Genetic Assessment of Connectivity in the Temperate Octocorals *Eunicella verrucosa* and *Alcyonium digitatum* in the North East Atlantic

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature.....

Abstract

Elucidating patterns of connectivity for species of conservation concern is crucial in the design of networks of ecologically coherent marine protected areas, and therefore is considered in the design of such a network recently proposed to the UK Government. However, data concerning connectivity are deficient for most invertebrate sessile taxa. Therefore, this study used microsatellite panels developed *de novo* to assess the population genetic structure and genetic connectivity of two temperate octocorals in the North East Atlantic. Microsatellite panels for both species show evidence of cross-species transferability, and therefore in future may prove to be useful monitoring tools for the target species but also for congenerics further afield in Europe.

Eunicella verrucosa (O. Alcyonacea: S.O.Holaxonia: F. Gorgoniidae), a threatened and IUCN red-listed sea fan, was sampled in the northerly extremes of its eastern Atlantic range in southern Portugal, Brittany, the South West UK and western Ireland. In this vicinity, connectivity appears to be defined at regional scales and localised cases of inbreeding and differentiation suggest that the population structure of this species is best described as a metapopulation. *Alcyonium digitatum* (O. Alcyonacea: S.O. Alcyoniina: F. Alcyoniidae), a soft coral, was sampled in the central portion of its range in Brittany, western Ireland, south west UK and the North Sea. This species exhibited very little population structure and apparent panmixia across the sampled range. However, high levels of heterozygote deficiencies and inbreeding in the majority of populations implies that the genetic structure of some populations of this species may be defined by self-seeding and rarer dispersal events that occur sufficiently often to offset divergence via genetic drift. Coalescent analyses indicate that in both species, migration between regions occurs asymmetrically. The presence of few duplicate genotypes in both datasets implies that sexual reproduction predominates in both species in the sampled area. *Eunicella verrucosa* is a charismatic species that is often used to promote marine conservation efforts in the UK and *A. digitatum* is a ubiquitous animal around western European coasts; the two species often occur together and both may suffer the damaging effects of mobile fishing gears. This research represents the first population genetic assessment of both species and the first time microsatellites have been used to assess population structure of octocorals in the North East Atlantic.

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Chapter 1. General Introduction

Part 1 – Why Assess Connectivity? Diversity, Connectivity and Population Models

1.1.1 The Importance of Genetic Diversity

Biodiversity is maintained by underlying genetic variation, of which high levels are critical to foster adaptive evolution and impart evolutionary resilience to changing selective regimes at population to landscape scales (Sgro *et al.* 2011). Reduced genetic diversity has been empirically proven to correlate with reduced population fitness, as evidenced by decreased survival, reduced population growth rates, an increased extinction risk, or other fitness declines associated with increased inbreeding (inbreeding depression; Bradshaw and Holzapfel 2008, Reed and Frankham 2002, Wright *et al.* 2008). This is of particular concern for endangered species where population sizes need to be maintained at levels that preserve current genetic diversity and reduce extinction risks (Reed and Frankham 2002). At large geographic scales, genetic variation present within a metapopulation (i.e., all populations of a species across its geographic range) may be reduced by the loss of a single population, which causes an overall genetic loss for the species in question and also for the region (Feral 2002). Furthermore, a loss of genetic diversity can be particularly detrimental to small or declining populations that characterize threatened species. Spielman *et al.* (2004) compared the extent of genetic diversity between 170 threatened taxa with their taxonomically related but non-threatened counterparts and found that 77% of them had lowered diversity. This result highlights that species can be detrimentally affected by diversity loss at relatively short timescales, as was also noted by Bradshaw and Holzapfel (2008) in their paper about genetic selection favouring adaptation to altered seasonality due to climate change.

High levels of genetic diversity are also essential for ecosystem function and resilience following disturbance (Underwood *et al.* 2009); decreased genetic diversity within populations, communities and ecosystems may have ecological consequences (reviewed in Hughes *et al.* 2008). Therefore understanding and measuring the extent of genetic variation within and between species and ecosystems is crucial to predict how they might respond to the many threats they are currently facing, and also to assist in developing protective measures that may enhance their persistence (e.g., Johannesson and Andre 06). Genetic diversity is usually quantified by several metrics, for example by comparing allelic and genotypic richness (the average number of alleles at a particular locus and the number of genotypes within a population respectively) and heterozygosity (the average proportion of loci that have two different alleles at a single locus within an individual, Hughes *et al.* 2008). Reduced

heterozygosity signifies reduced adaptive potential, increased extinction risk and compromised reproductive potential (Spielman *et al.* 2004).

1.1.2 Defining Connectivity

Assessing population divergence and inferring how this diversity is governed by import and export of individuals between them is achieved by assessing 'connectivity'. Connectivity has been broadly defined as "the extent to which populations in different parts of a species' range are linked by exchange of larvae, recruits, juveniles or adults" (Palumbi 2003). Understanding connectivity is a priority for the sustainable management of resources, the tracking of invasive species pathways, the determination of the impact of climate change and the design of protected areas (Levin 2006). Connectivity may also be defined as being either demographic or genetic (reviewed in Lowe and Allendorf 2010). Demographic connectivity refers to how population growth, or survival and birth rates, are affected by immigration, emigration, and recruitment (Thomas and Bell 2013), whereas genetic connectivity refers to how the extent of gene flow from migration mitigates divergence through genetic drift within subpopulations (Lowe and Allendorf 2010). Both types may occur at different temporal and spatial scales. For example, genetic connectivity may represent a historical event resulting in population homogenization, or it may be representative of large-scale biogeographic patterns, whereas demographic connectivity may occur on contemporary timescales between local populations linked by the exchange of individuals. As such, population connectivity is often also described as ecological (demographic) or evolutionary (genetic; e.g., Sale *et al.* 2010). In marine populations, the number of individuals maintaining each type of connectivity is often plotted as a function of dispersal distance from source populations in a so called 'dispersal kernel' (e.g., Steneck 2006, Steneck *et al.* 2009). In such diagrams, sufficient genetic connectivity to prevent local extinctions can be achieved by very few individuals relative to the large proportion of individuals that maintain demographic connectivity. Gene flow resulting from as little as one immigrant per generation may spread advantageous alleles, may mitigate local inbreeding effects or may act to maintain similar allele frequencies (termed 'adaptive', 'inbreeding' and 'drift' genetic connectivity respectively; Lowe and Allendorf 2010). Demographic connectivity is thought to stabilise populations, in cases where immigration compensates for low recruitment in the resident population, or at larger geographic scales where colonization of new unoccupied patches compensates for high extinction rates in occupied patches (Lowe and Allendorf 2010).

Marine populations are often described as 'open' or 'closed'; these concepts are also related to connectivity and describe the relative rates of recruitment from distant vs. local sources,

respectively. An open population implies that the extent of larval dispersal is great given the potentially large distance propagules may travel on oceanic currents, coupled with observed genetic homogeneity over large spatial scales (Cowen *et al.* 2000). Therefore, there is argument that the term 'open' can relate to both demographic and genetic connectivity, and distinguishing between them is important to prevent false assumptions that the extent in demographic openness will result in a corresponding change in genetic openness (Johnson 2005).

1.1.3 Using Population Genetics to Infer Connectivity

Inferring the extent of gene flow (and thus genetic connectivity) within and between populations based upon variations in allele frequencies (i.e., heterozygosity) is usually calculated using Wright's F-statistics, which describes the correlation between two randomly chosen alleles within subpopulations relative to two alleles randomly sampled from the total population. As such, the extent of inbreeding can also be determined due to the correlation between alleles if they occur in the same subpopulation (Wright 1951, Balloux and Lugon-Moulin 2002). Gene flow is summarized by the equation Fst = 1/(4Nm +1), where Nm represents the number of immigrants entering a subpopulation in each generation (N= local subpopulation size, m= the proportion of immigrants), and the expected divergence is a function of the number of immigrants Nm (Lowe and Allendorf 2010, Wright 1951). If a locus with two alleles is considered in two subpopulations, then Fst will attain a value of one if both subpopulations are completely homozygous (i.e., the same allele copy is bi-parentally inherited) and fixed for the alternative allele (i.e., monomorphic), and zero if the allele frequencies in each subpopulation are identical; in other words, hypothetically, Fst values vary between zero and one, with zero signifying no differentiation and one representing completely disparate populations (Balloux and Lugon-Moulin 2002). However, in reality Fst values between population pairs are typically low as total variation across all populations is averaged between them (Hellberg 2009) and problems remain given that divergence is ultimately inferred by population genetic theory. For example, violation of the assumption of F-statistics that subpopulations have attained mutation-drift equilibrium (i.e., a balance between genetic drift and gene flow) leads to an over-estimate of gene flow (Lowe and Allendorf 2010). This may be exemplified by a population that has been recently recolonized following local extinction, which will bear the same genetic signature as its source population despite an absence of ongoing gene flow (Hellberg *et al.* 2002). In marine species, large effective population sizes (i.e., Ne, numbers of individuals contributing genetic material between generations) resulting from pelagic larvae with extensive dispersal potential may result in reduced rates of genetic drift that suppress Fst values (Beger *et al.* 2014). The Fst concept is

also based upon an island model of gene flow, assumptions of which include equal likelihood of movement between subpopulations regardless of geographic distance, that subpopulations are equally sized and that migration and genetic drift are in equilibrium (Beebee and Rowe 2003). These assumptions are highly unlikely to be met biologically (Hellberg 2009). Another inherent problem in measuring genetic connectivity with Fst is that ongoing gene flow between populations and discontinued gene flow in recently separated diverging populations cannot be distinguished; the former represents a scenario of mutation-drift equilibrium whereas the latter does not, and both may generate similar Fst values (Beebee and Rowe 2003, Pearse and Crandall 2004). Therefore, patterns of population structure are often assessed in the context of alternative population models such as stepping stone models, in which adjacent subpopulations have a higher likelihood of exchanging migrants. This pattern can be tested by looking for evidence of isolation-by-distance in which genetic differentiation at neutral loci will theoretically correlate with geographic distance (Slatkin 1993). Basic population models, and how they may be identified from measures of genetic diversity, are illustrated in Figure 1.1.

1.1.4 The Importance of Assessing Marine Connectivity

An overall decline in oceanic ecosystems is well-documented and falling fish stocks have led to widespread implementation of marine reserves (Higgins *et al.* 2008). Understanding to what extent ocean health has been affected by anthropogenic influence and other disturbances is difficult, as oceanic and underwater surveys and monitoring began relatively recently compared to terrestrial ecosystems and we therefore have little baseline data to compare with new observations (Knowlton and Jackson 2008). However, it is recognised that benthic ecosystems and pelagic environments are severely impacted by natural and artificial disturbances and processes which has led to a critical need to protect marine environments and resources (e.g., Allison *et al.* 1998).

Topical anthropogenic impacts include the accelerated onset of ocean acidification, whereby oceanic pH decreases due to an increased absorption of atmospheric CO_2. This has a detrimental effect on calcifying organisms, as decreased saturation of carbonate ions caused by lowered pH makes precipitation of aragonite and calcite more difficult and renders calcium carbonate more susceptible to dissolution (reviewed by Raven *et al.* 2005). Increased acidification can also alter skeletal growth and survivorship, as seen in corals (Albright *et al.* 2008). Calcifying organisms are both planktonic and benthic (e.g., coccolithophores, corals, molluscs), therefore as increasing partial pressures of CO_2 lowers pH first in surface and then in deeper waters, many organisms and ecosystems will be negatively affected in tropical and

temperate waters, including coral reefs, deep-sea corals and seagrasses (e.g., Martin *et al.* 2008, Turley *et al.* 2007).

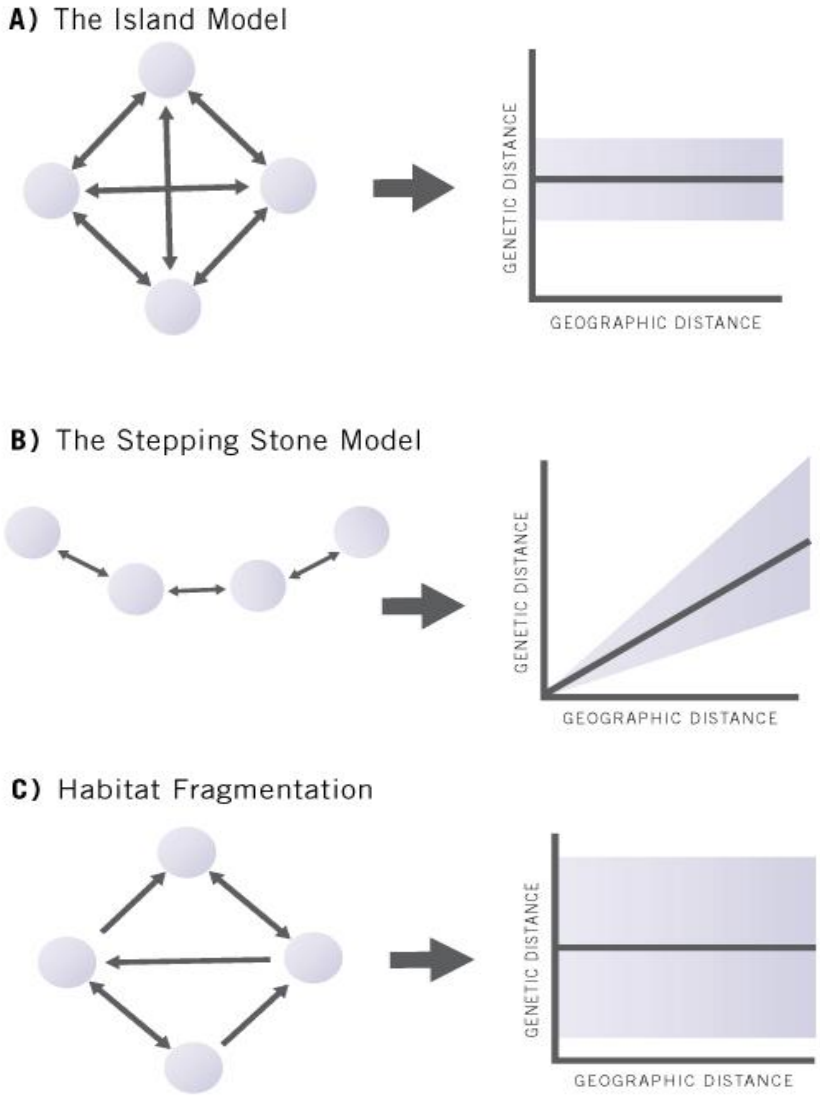


Figure 1.1 Conceptual illustrations of simple population models (left) and their theoretical effect on patterns of genetic diversity (right), illustrated by expected scatter plot distributions (shaded areas) surrounding approximate relationship between genetic and geographic distances (bold line). Modified from Beebee and Rowe (2008), Hutchison and Templeton (1999) and Whitlock and McCauley (1999). **1A)** The island model. Migrants disperse between all other populations, therefore no correlation between genetic and geographic distance is expected. Gene flow is dominant over drift and populations are not in equilibrium. Alternative explanations might be a recent recolonization event, in which case future patterns may be more similar to 1C. **1B)** The stepping stone model, when dispersal occurs between neighbouring populations and genetic similarity is expected to decline with the number of 'steps' between colonies.. Equilibrium occurs between mutation and genetic drift. **1C)** Habitat fragmentation. Gene flow has been lost between some populations due to, for example, vicariant events and as such gene flow is completely broken or asymmetrical. There is no equilibrium with genetic drift more influential than gene flow.

Climate change, primarily manifested in the ocean by an increase in sea surface temperatures, also has a detrimental effect on marine organisms. Elevated water temperatures induce the loss of symbiotic algae ('zooxanthellae') from anthozoan hosts as a so-called 'bleaching' process (a stress response also induced by ocean acidification, Baker 2001, Anthony *et al.* 2008). Temperature fluctuations may also cause range expansions in many marine animals, and has led to northward expansions in several temperate taxa (for example octocorals, barnacles and gastropods; Hiscock *et al.* 2004, Southward *et al.* 2004, Hellberg *et al.* 2001, respectively). Climate change can also influence connectivity by changing currents, for example the sea urchin *Centrostephanus rodgersii* is undergoing a pole-ward range extension due to the combined effects of elevated ocean temperatures and strengthening of the Eastern Australia Current (Ling *et al.* 2008). El Niño events, which some evidence suggests might be increasing in frequency due to climate change (Timmermann *et al.* 1999), have also contributed to range expansions. For example, the southerly latitudinal limit of the invasive mollusc *Cymatium keenae*, increased following a warming period of El Niño, and some specimens were found to persist 22° farther south than their original range limits (Ashton *et al.* 2008).

Anthropogenic disturbance also manifests as localised damage to marine ecosystems, including fishing activities such as bottom-trawling, which causes mechanical damage to benthic animals. This is well documented in some areas where reduced fin-fishery quotas have increased numbers of inshore fishing vessels, such as in the UK where scallop trawlers have damaged octocoral populations (e.g., *Eunicella verrucosa and Alcyonium digitatum,* Hinz *et al.* 2011), maerl coralline algae beds (Hall-Spencer and Moore 2000) and ancient deep-water scleractinian corals (e.g., *Lopehlia pertusa*; Hall-Spencer *et al.* 2002). Elsewhere, over-harvesting of selected populations has reduced overall genetic diversity and threatens populations of taxa with strong genetic structure, such as the Mediterranean red octocoral *Corallium rubrum* (Abbiati *et al.* 1993). Over-fishing of top-predators can negatively affect lower trophic levels and result in trophic cascades, for example, removal of sharks may increase ray population sizes which in turn significantly reduce scallop numbers (Heithaus *et al.* 2008). Local point-source pollution events also affect marine life directly or indirectly. Rocky shores and associated animals are acutely impacted by events such as oil spills or diffusion of toxic chemicals from shipping (e.g., Hawkins *et al.* 1999), and pollution from human or chemical effluent can introduce or increase the prevalence of disease in benthic organisms. This has been observed in a variety of taxa and habitats, such as *Zostera marina* seagrass beds (Hawkins *et al.* 1999), temperate octocorals (*Eunicella verrucosa*, Hall-Spencer *et al.* 2007), and tropical scleractinians, which are affected by at least eighteen described diseases of which many are attributed to anthropogenic activity (Sutherland *et al.* 2004). The presence of

pathogens that act opportunistically when combined with increased water temperatures can also rapidly decimate some ecosystems, as has been observed in Mediterranean octocorals (Cerrano *et al.* 2000).

These threats and disturbances could reduce genetic diversity and thus the efficacy of gene flow between populations of marine taxa. Elevated temperatures resulting from climate change may reduce post-settlement survivorship in some coral species (Randall and Szmant 2009), and may reduce larval dispersal potential in several phyla as colder temperatures are conducive to longer pelagic larval duration (O'Connor *et al.* 2007). Increased oceanic acidification may reduce growth rates in juvenile scleractinian corals, which has been linked to increased juvenile mortality and later onset of sexual maturity, which could ultimately result in smaller effective population sizes if adult populations are also impaired (Albright *et al.* 2008). Habitat disturbance such as trawling can alter the relative frequencies of asexual versus sexual reproduction and may induce clonality in some species such as hydroids (Henry and Kenchington 2004); clonal genets with reduced genetic diversity may predominate during times of disturbance and ultimately reduce the fitness of affected marine populations.

There is a large body of literature concerning marine larval dispersal in fish, which is understandable given the global decline of fish stocks (Bradbury *et al.* 2008). There is also considerable research concerning sessile invertebrates, although few marine reserves have been designed with the sole aim of conserving invertebrate species. This is quite surprising as they constitute a fundamental component of benthic biomass, have high commercial value as fisheries targets and high bioprospective value and, consequently, commercial harvesting of invertebrates has been responsible for substantial coastal degradation globally (e.g., Thorpe *et al.* 2000, Hawkins *et al.* 1999). A large proportion of research concerning connectivity of sessile invertebrates, in particular with regard to marine protected areas, has been conducted in tropical and sub-tropical regions (e.g., on corals, Atchinson *et al.* 2008). This is problematic as templates and guidelines established for designing MPAs nearer to the equator may be inapplicable at higher latitudes; larvae of both fish and invertebrates develop more slowly at lower temperatures which could lengthen larval dispersal distances (O'Connor *et al.* 2007) and alter inferences of connectivity. It has been suggested that larval dispersal of fish may increase by as much as 8% per degree of latitude from the equator, which ultimately results in higher levels of gene flow in temperate regions (Laurel and Bradbury 2006). Some habitats in temperate regions may also harbour elevated levels of species richness for some taxa, exhibit endemism or act as refugia resulting from environmental stressors at lower latitudes or from historical events (e.g., Barnes and Griffiths 2008, Hewitt 2004, Johannesson and André 2006). Therefore, temperature has a direct and important influence on population connectivity and

obtaining more data on gene flow among temperate species and habitats is critical for the development of MPAs and MPA networks at higher latitudes.

Part 2: Processes Driving and Ways to Measure Connectivity

1.2.1 Correlating Larval Dispersal with Connectivity

Adult mobility is limited for most benthic marine organisms, therefore dispersal and subsequent recruitment of gametes and larvae are the primary mechanisms through which demographic and genetic connectivity occurs (Cowen *et al.* 2006). Initially, connectivity between marine populations was assumed to be relatively high compared to terrestrial populations as many marine species have long-lived pelagic larval phases, which led to the assumption that marine populations were highly connected and that larvae could readily disperse between them (Becker *et al.* 2007). However, this assertion is untrue of many sessile marine species and there is now considerable evidence that many marine populations exhibit strong population structure, even if currents and pelagic larval durations are theoretically sufficient to transport propagules considerable distances (Hellberg 2009). Furthermore, several studies have highlighted the prevalence of self-seeding in benthic marine populations (Sammarco and Andrews 1998, Swearer *et al.* 2002). These observations have important implications for marine conservation, as isolated or unconnected populations threatened by ecosystem decline or habitat fragmentation are highly vulnerable to a loss of diversity and localized extirpation if they are not sufficiently protected.

Inherent in the concept of marine connectivity is the migration of gametes or larvae between 'source' (originating) populations and 'sink' (receiving) populations and subsequent subsidy (i.e., recruitment from non-local sources) and self-recruitment (i.e., recruitment from the same population; Cowen and Sponaugle 2009). Measuring connectivity directly (e.g., by observing and tracking larvae) is very difficult logistically given their miniscule size compared to their environment and potential range (e.g., Barber *et al.* 2002b). However, as gametic and larval import ultimately determines to what extent new genetic material enters a population, comparing genetic diversity between putative source and sink populations is a way to indirectly infer connectivity between them, as the extent of genetic divergence resulting from mutation, genetic drift and localised directional selection and homogenisation resulting from gene flow and balancing selection can be measured and compared using molecular methods (Hedgecock *et al.* 2007). In the marine environment, genetic breaks have been recorded between populations of species with potentially large dispersal distances, suggesting that

oceans are not always open systems whereby propagules are carried passively on currents (e.g., Barber *et al.* 2000). In some cases, gene exchange occurs only between adjacent populations in a stepping stone-like scenario and in this case, genetic distance is thought to correlate positively with geographic distance from a source population ('isolation by distance', e.g., Bradbury and Bentzen 2007; Figure 1.1). However, such a simplistic correlation is not always observed and other factors including organismal life-history, local current regimes, geographical barriers, historical / geological events (e.g., plate tectonics, glacial maxima) and combinations thereof are more appropriate indicators for explaining genetic structure across a species' range (e.g., Sherman *et al.* 2008, Waters 2008).

Propagule dispersal between marine communities promotes genetic continuity and maintains a supply of new recruits; for sessile taxa this is usually the only means of maintaining connectivity (Shanks *et al.* 2003). Consequently, considerable effort has been put into understanding how larval biology and life history traits, primarily reproductive strategies and pelagic larval duration, affect dispersal potential and therefore patterns of population structure (Levin 2006). Descriptions of these traits, such as how and where larvae develop and how these traits theoretically affect connectivity patterns are summarised in Table 1.1. Greater dispersal ability is generally expected to correlate with increased gene flow (Bohonak 1999); life history traits conducive to reduced or increased dispersal capacity are often used as a proxy to infer the extent of connectivity between populations.

Most benthic marine animals produce planktonic propagules (typically gametes or larvae) that settle onto suitable habitat following a planktonic developmental phase and there is considerable variation between taxa as to how long this phase lasts, ranging from minutes to months (Shanks *et al.* 2003). As propagule dispersal underpins movement of genetic material, which in turn determines adaptive potential and species resilience, assessing pelagic larval duration (PLD) and understanding how variation in life history strategies influences dispersal has become a crucial component in the study of connectivity (Bradbury *et al.* 2008b), and by extension in the design of marine protected area networks. Actual larval dispersal distance determines connectivity patterns, so hypothetically dispersal distance will be farther for organisms with a longer PLD, ultimately leading to a positive correlation between PLD and genetic homogeneity (e.g., Watts and Thorpe 2006). There is an additional expectation that direct developing or brooding species with limited dispersal will exhibit lower genetic connectivity than species with far-dispersing larvae (e.g., Richards *et al.* 2007, but see Miller and Ayre 2008b for evidence to the contrary). Therefore, understanding larval movement dynamics has become a critical focus in the design of marine reserves and their placement (Palumbi 2003).

Dispersal potential is usually inferred from several means, such as variation in life history traits, including adult reproductive mode, larval biology (e.g., nutritional and developmental modes), inference from similar data known for congeners, by tracing the expansion of invasive species or by predicting movement according to local currents (e.g., Dupont *et al.* 2009**,** Jones and Carpenter 2009). These data, whether generated theoretically or empirically, allow some prediction of how far larvae may be expected to travel, and hence how connected marine populations might be. However, the correlation between genetic connectivity and larval dispersal capacity is increasingly shown to be unpredictable. Many studies have shown that maximum dispersal potential is achieved much more infrequently than previously expected and that even species with long-lived larvae may exhibit philopatric behaviour (i.e., settle near to their natal origins) and rely on local larval retention as a source for new recruits. This has been observed when unexpectedly low rates of gene flow occur given expected pelagic larval duration and has been recorded in tropical and temperate areas, at a variety of depths, and across many taxa (e.g., coral reef fish: Almany *et al.* 2007, Jones *et al.* 2005, Taylor and Hellberg 2003; seamount invertebrate taxa: Mullineaux and Mills 1997, Samadi *et al.* 2006; molluscs: Piggott *et al.* 2008; corals: Hellberg *et al.* 2002; for reviews see Bradbury *et al.* 2008b, Jones and Carpenter 2009, Kinlan and Gaines 2003, Swearer *et al.* 2002). Distinct populations have also been noted among species with a high expected dispersal potential when prevailing local currents are considered (Barber *et al.* 2002a), although modelling efforts have demonstrated that larval dispersal inferred from water flow may also over-estimate dispersal potential (Cowen *et al.* 2000). However, many cases do exist where dispersal and connectivity have correlated closely with predicted patterns based upon life history strategies and hydrodynamics in an isolation-by-distance / stepping stone manner, for example in sponges (Duran *et al.* 2004a), bryozoans (Goldson *et al.* 2001), and molluscs (Sotka *et al.* 2004).

Table 1.1: Primary marine larval development patterns and their theoretical effect on the extent of connectivity. Descriptions and expected connectivity patterns complied from Jablonski and Lutz 1983, Jones and Carpenter 2009, Kinlan and Gaines 2003, Pechenik 1999. Superscript numbers denote examples of supporting references as follows: 1= Goffredo *et al.* 2004, 2= Hellberg 1994, 3= Hellberg 1996, 4= Underwood *et al.* 2009, 5= Golson et al. 2001, 6= Duran *et al*. 2004b, 7= Kelly and Palumbi 2010, 8= Janson 1987, 9= Watts and Thorpe 2006, 10= Mokhtar-Jamai *et al.* 2011.

	Description	**Expected extent of connectivity**
Developmental Mode		
Direct development	No intermediate developmental stage; juveniles typically emerge from parent with limited dispersal	Low [1,2,8,9]
Indirect development	Development includes free-living larval stages, gametes or spores	High [5]
'brooders'	e.g., the eggs are retained in the maternal polyp	Low [3,4,10]
'broadcast spawner'	e.g., both the eggs and sperm and released into the water column	High [3,4]
Mixed development	Where early developmental stages are encapsulated, but later stages emerge as free-swimming, pre-metamorphic larvae	
Nutritional Mode		
Lecithotrophic	Pertaining to larvae that derive nutrition from the yolk reserve of the egg from which they develop - i.e., non-feeding	Low [5,6]
Planktotrophic	Pertaining to larvae that subsist upon planktonic food sources - i.e., feeding	High [5]
Non-planktotrophic	Larvae that may be planktonic or planktotrophic, generally with a lower dispersal capability than planktotrophs	
Developmental site		
Pelagic	Larvae that develop in the open sea	High [7]
Planktonic	Where a significant portion of larval development time is spent freely swimming in surface waters	High
Aplanktonic	Lacking a dispersive phase; development to juvenile stages occurs entirely, for example, within egg masses or brood chambers	Low
Neritic	Larval development occurs in the near-shore water column	Medium
Benthic/demersal	Larval development occurs in or on the substratum	Low [7]

A variety of known and unknown biological and hydrodynamic processes are responsible for larval retention. 'Closed' populations that receive a negligible influx of external recruits may incur elevated rates of inbreeding and reduced fitness, which could render them vulnerable to genetic bottlenecks (e.g., Bell and Okamura 2005). On the other hand, a lengthy larval phase might ensure increased genetic continuity, but at the risk of being carried beyond habitat suitable for juvenile development or of being exposed to increased predation (e.g., White *et al.* 1998, Cowen and Sponaugle 2009). There is some suggestion that most dispersal distances being either low (e.g., <1km) or high (e.g., >20km) (with few in-between) is due to evolutionary strategies (Shanks *et al.* 2003). This could, theoretically, represent the aforementioned trade-off and the apparent lack of mid-range-dispersing species may have relevant implications for the design of marine reserve networks and is therefore a concept worthy of further review (Shanks *et al.* 2003). However, a bimodal distribution of dispersal distance as reported by Shanks *et al*. (2003) is unlikely to be a pandemic pattern and local hydrodynamic regimes, and reproductive mode of the species in question would also need to be considered (see below).

1.2.2. Correlating Reproductive Strategies with Connectivity

Sessile marine invertebrates have evolved a diverse array of reproductive strategies to deal with physical properties of aquatic environments, such as production of feeding larvae that profit from abundant food, or release of planktonic propagules that overcome reduced diffusion rates of oxygen and other molecules (reviewed in Strathmann 1990). Some modes of reproduction are more conducive to farther larval dispersal than others and extent of gene flow between populations is often expected to relate directly to the reproductive strategy employed. Although a full review of these strategies and the cues that trigger them is beyond the scope of this introduction, the principal reproductive modes utilised by sessile marine invertebrates and how they relate to connectivity will be considered. Direct-developing animals are those that lack a developmental stage in the plankton, sometimes also completely lacking a larval stage and sometimes producing fully developed larvae competent to settle shortly after release. There are many variations on this theme, and larvae may be brooded internally (viviparity), or brooded on the colony surface, but directly-developed larvae are usually the result of internal fertilisation from either hermaphroditic or gonochoric (= dioecious) parents and may be produced sexually or asexually (e.g., Fautin 2002, Goffredo *et al.* 2004). Theoretically, directly-developing animals would exhibit little genetic connectivity between populations due to limited dispersal which has actually been observed in several taxa including the dogwhelk (*Nucella lapillus*, Bell and Okamura 2005), several brooding hard and soft corals (e.g., *Corallium rubrum,* Abbiati *et al.* 1993, *Madracis decactis,* Atchison *et al.* 2008), ascidians (e.g., *Styela clava*, Dupont *et al.* 2009; *Pycnoclavella communis*, Perez-Portela and

Turon 2008) and cushion stars (*Parvulastra exigua*, Sherman *et al.* 2008). There are, however, exceptions to this trend and unexpectedly high gene flow has been measured in animals that develop directly, such as amphipods (although in this case, rafting on fragments of host sponge was thought to be the reason; Richards *et al.* 2007).

An alternative strategy to direct development is spawning, where gametes are synchronously released from adults, fertilisation occurs in the water column, and the gametes and subsequent larvae are thought to be able to travel relatively large distances. Some research supports this hypothesis, where spawning taxa show little structure in relation to direct developers. For example, the solitary coral *Paracyathus stearnsii* broadcast spawns planktotrophic larvae, whereas the co-occuring solitary coral *Balanophyllia elegans* broods large, non-feeding larvae; as expected, more genetic subdivision was observed in the latter (Hellberg 1996). On the contrary, Hunter & Halanych (2008) found that the brittle stars *Astrotoma agassizii* maintained high connectivity over large spatial scales (>500km) despite a brooding strategy.

As the dispersal phase of most benthic marine invertebrates occurs during early life history stages that are usually planktonic, it is reasonable to assume that local- and regional-scale water movement plays the lead role in population connectivity. However, biological processes also play a significant role in larval dispersal and connectivity does not always correlate with dispersal distances inferred from currents alone (Cowen and Sponaugle 2009). Biotic factors and their impact upon dispersal varies spatially and temporally, and can be taxon dependent; for example predation rates may vary between nearshore and offshore sites, timing of reproduction varies from regular gamete release for several months a year (e.g., the coral *Madracis decactis*, Atchison *et al.* 2008) to annual adult spawning aggregations (e.g., several fish, lobsters), and sessile invertebrates employ a diverse array of reproductive strategies as outlined above (Cowen and Sponaugle 2009). In addition, high fecundity in marine organisms is usually accompanied by high rates of larval and post-settlement mortality that may reach 100% due to, for example, desiccation and predation (reviewed in Gosselin and Qian 1997, Cowen *et al.* 2000), or even cannibalism by adults in the new environment (which can result in up to 80% mortality of potential recruits in the mussels *Perna perna* and *Mytilus galloprovincialis*, Porri *et al.* 2008).

Biological traits inherent in the larvae may also affect dispersal distances and hence connectivity. Planktotrophic larvae (which feed on plankton whilst in the water column) are thought to travel further than lecithotrophic larvae (which derive a source of nutrients from

the parent, such as yolk), which in turn disperse farther than directly developing larvae (e.g., Shanks 2003). Some propagules such as eggs may be positively buoyant which may expose them to wind-driven surface currents with a large potential for dispersal (Roberts *et al.* 2009). Behavioural mechanisms also affect spatial distribution and dispersal capacity of larvae. For example, some octocorals exhibit negative geotaxis (*Corallium rubrum*, Abbiati *et al.* 1993) and strong negative geotaxis followed by positive phototaxis has been observed in several mussel species (*Mytilus* spp.**,** Gilg and Hilbish 2003). Swimming behaviour of bivalve larvae significantly affects dispersal potential (*Crassostrea* spp., North *et al.* 2008), as can larval vertical migration in the water column (Roberts *et al*. 2009). Connectivity also depends upon the availability of suitable substrate for settlement and differential recruitment and genetic heterogeneity may be partly determined by spatial variation in settlement cues (released from biofilms, food sources or host organisms, for example) (Barber *et al.* 2002a).

1.2.3. Asexual vs. Sexual Reproduction, Recruitment and Connectivity

Genetic diversity in benthic marine populations is ultimately determined by recruitment of new individuals which are produced from source adults either asexually via fragmentation, through self-fertilisation, fission, budding or production of parthenogenetic larvae (producing 'ramets', or clonal modules), or sexually via release of gametes or larvae (resulting in 'genets', or genetically distinct individuals; Jackson and Coates 1986). Rates of asexual versus sexual reproduction vary among and within taxa at spatial and temporal scales; in some species one reproductive mode is exclusive or dominant over the other, but in other species both modes contribute in varying proportions to population sustainability (Foster *et al.* 2007). Spatial variation in reproductive mode has been noted in several species across their range. For example, clonal reproduction was found to be more prevalent in western than eastern populations of the Caribbean elkhorn coral *Acropora palmata* (Baums *et al.* 2006). The contribution of asexual reproduction to populations of the deep water scleractinian *Lophelia pertusa* was also found to be highly variable across the North East Atlantic (Le Goff-Vitry *et al.* 2004). In addition, marginal populations in a species range may exhibit differentiation in asexual and sexual reproductive mode. Johannesson & Andre (2006) suggest that asexual reproduction is more common in marginal populations of several seaweed species in the Baltic Sea and Ayre and Hughes (2004) observed elevated rates of clonality in a peripheral coral population on Lord Howe Island, Australia.

The reproductive output of asexual versus sexual reproduction can vary temporally on relatively short to longer-term demographic timescales. In some anthozoans taxa there is evidence that sexual reproduction occurs on a seasonal basis but asexual fission may occur

between cycles (Bronsdon *et al.* 1997). Asexual reproduction may start to predominate over several generations and there is considerable evidence that an increase in asexual propagation is directly attributable to an environmental disturbance. For example, Henry and Kenchington (2004) observed increased clonality and reduced fertility in the hydroid *Sertularia cupressina* following the simulation of injury due to bottom trawling. Some populations of *Lophelia pertusa* subject to intense bottom trawling are now predominantly asexual due to the mechanical reduction of colonies to sexually immature sizes (Le Goff-Vitry *et al.* 2004). The same disturbance has also been suggested as a potential cause for reduced numbers of sexually mature colonies in overharvested populations of the red octocoral *Corallium rubrum* (Santangelo *et al.* 2003). Asexual reproduction may also be more apparent when gonochoric species experience skewed sex ratios and there is a lack of males. This has been recorded in several species of coral where brooded larvae may be produced parthenogenetically (see Fautin 2002)

Asexual propagation may be considered an adaptation to ensure survival during stressful periods. However, it would be misleading to assume that this mode of reproduction is always less beneficial to an organism than sexual reproduction and that it only predominates during periods of disturbance. The prevalence of asexual reproduction has important evolutionary implications for maintaining genetic diversity in sessile invertebrates, especially for clonal species where larvae are thought to disperse shorter distances and are more philopatric than aclonal larvae (Jackson and Coates 1986). In addition, small effective population sizes (i.e., individuals that contribute genetically to the next generation) within large census population sizes (i.e., total number of individuals, not all of whom will reproduce) are common in clonal aggregates and may promote inbreeding, particularly where dispersal distances are short (e.g., Hellberg 1994). In terms of connectivity, there is a body of evidence implying that frequent asexual reproduction maintains locally adapted clonal populations and that sexual reproduction is a relatively rare event employed as a means to produce farther-dispersing colonists. Therefore, asexual reproduction could be more relevant on an ecological timescale (i.e., contemporary / ongoing geneflow) and sexual reproduction on an evolutionary timescale (reviewed in Cowen and Sponaugle 2009). Limited sexual exchange has been observed repeatedly in predominantly clonal animals with large geographic ranges. For example, populations of the coral *Seriatopora hystrix* exhibit strong genetic differentiation across their range on the Great Barrier Reef, thought to result from rare long distance founder events from sexually produced genets (van Oppen *et al.* 2008, Ayre and Hughes 2004), and several founder events may explain genetic structure in the Pacific stomatopod *Haptosquilla pulchella* (Barber *et al.* 2002b) and introduced European ascidians (Lopez-Legentil *et al.* 2006).

Loss of genetic diversity in a species will impede adaptation to novel environments resulting from disturbance such as climate change (Bell and Okamura 2005), although genetic consequences of frequent asexual propagation do not necessarily reduce fitness or even reduce genetic diversity. This has been noted in the USA in north-western populations of the clonal temperate octocoral *Alcyonium rudyi,* where inbreeding is insignificant and diversity is comparable to that expected for sexually reproducing philopatric species with similar dispersal characteristics (McFadden 1997). It is thought that long generation times of many clonal species might compensate for fewer recombination events from reduced sexual reproduction, as genet longevity preserves existing genotypic diversity (McFadden 1997).

1.2.4 Ways to Measure Connectivity

1.2.4a) Non-Molecular Methods

Despite the importance of assessing connectivity, measuring it is inherently difficult due to the logistical constraints of isolating and tracking minute propagules (larvae or gametes) *in situ* and as such empirical connectivity data remain elusive. Nonetheless, several studies have attempted to determine demographic connectivity directly, for example by rearing spawned larvae in laboratory conditions (e.g., Albright *et al*. 2008), and examining planktonic samples at various stages of development and combining the observations with hydrodynamic models (van der Molen *et al*. 2007, Tian *et al*. 2009). Another direct approach is the measurement of geochemical tags in calcified structures that can be used as signatures to determine the natal origins of an animal; for example, variable elemental or isotopic composition in calcified morphological structures such as otoliths, shells and statoliths over environmental gradients can be measured using mass spectrometry and compared to a reference site (reviewed in Thorrold *et al*. 2007). This approach is limited to certain taxa and has been used on various fish and bivalve taxa. For example, the oyster *Ostrea lurida* (Carson 2010) was assigned to natal stocks based upon trace element chemistry of larval aragonitic shells and connectivity in the temperate wrasse *Coris julis* (Fontes *et al*. 2009) in the Azores was tested the same way. The benefit of this technique is that natal signatures can be determined with relatively high success (up to 82% in *O. lurida*; Carson 2010) and at small spatial scales (tens of kilometres; Fontes *et al*. 2009), but with the caveat that connectivity patterns cannot be assigned if samples from several sites exhibit the same chemical signature (Fontes *et al*. 2009). Biomarkers such as tetracycline have also been used to tag otoliths to examine dispersal patterns of fish from known parents, which could then be compared to genetic parentage identity (e.g., in the clown fish *Amphiprion polymnus*; Jones *et al*. 2005).

Logistical constraints on empirical measurement of larval dispersal can be overcome by the use of high resolution biophysical models that can determine likely connectivity pathways based upon local and regional hydrography (typically lagrangian particle simulations; e.g., Siegel *et al*. 2003) that in some cases is coupled with biological traits such as larval behaviour and expected mortality. This type of analysis is probably the easiest way to infer demographic connectivity, and larval dispersal estimates based upon models are the most commonly used method to inform size and spacing criteria for networks of marine protected areas (e.g., Berglund *et al*. 2012, Kaplan *et al*. 2009, Roberts 1997, Sale *et al*. 2010, Shanks *et al*. 2003,). Biophysical models have also been used extensively to estimate connectivity in a range of threatened, commercial or other marine invertebrates, such as brittle stars (Lefebvre *et al*. 2003), scallops (Tian *et al*. 2009) and oysters (North *et al*. 2008). Unfortunately, there are significant caveats to connectivity modelling. For example, dispersal kernels, (i.e., larval settlement probability distributions typically based upon pelagic larval duration plus mortality plotted with distance from the natal site) are governed by many complex abiotic and biotic factors which may disrupt a uniform dispersal kernel pattern (see above, Cowen and Sponaugle 2006). Biological traits such as larval behaviour, longevity, survivorship and post-settlement processes are usually unknown, and flow models that are combined with linear mortality estimates usually over estimate connectivity as they do not account for high initial mortality and diffusion (Cowen *et al*. 2000).

1.2.4b) Molecular Methods

Molecular techniques are currently the only means through which genetic connectivity and recruitment can be measured and they can determine gene flow indirectly by estimating the extent and pattern of genetic divergence between populations, or more directly by assigning individuals to a subpopulation or parents (Lowe and Allendorf 2010). A wide selection of molecular techniques is available for detecting allelic variants with genetic markers (=genotyping). They vary in cost and resolution, but all are based upon statistically validating differences between either DNA sequences or the frequency and composition of alleles (copies of a gene of interest) within and between individuals from the populations of interest (reviewed in Ryman *et al.* 2006). As genes are recombined and exhibit inter-generational variation (leading to genetic drift), assignment of marine larvae to a source adult population or individual is usually inferred from population genetic models derived from evolutionary theory (Hellberg *et al.* 2002).

Techniques employed have taken either a multi-locus or single-locus approach, i.e., genetic information is derived from alleles found in several genomic positions or in just one. The

former identifies variation in anonymous genes from unidentified regions within the genome simultaneously, the plus side being that the methodology is relatively affordable and rapid and high levels of variation between individuals is obtained. The negative repercussions, however, are that some of this variation may be attributed to non-target organisms, may be non-Mendelian in inheritance (which impedes assignment to a population), or only allow presence and/or absence scoring (in which dominance alone is detectable and can be non-comparable between studies, reviewed in Sunnucks 2000). Single-locus approaches are more precise and target specific genes or regions, and both allele variants from co-dominant inheritance can be scored and therefore evolutionary models based upon Mendelian inheritance can be applied (Hellberg *et al.* 2002). Some of the most popular techniques that have been applied to detect genetic variation in marine organisms will be briefly described.

Allozyme electrophoresis is a multi-locus technique that differentiates between individuals by analysing allele frequency variation in expressed enzymes, which are separated and visualized by gel electrophoresis (Page and Holmes 1998). Electrophoretic mobility is determined by variation in weight and ionic charge due to variation in amino acid sequences (and hence underlying DNA sequences) of the proteins, and gel banding patterns are compared (as opposed to actual changes in a gene sequence, therefore it does not provide as much information as direct DNA sequencing methods). Nonetheless, it was a very popular means to determine population structure as it is relatively cheap, and there is some indication that allozymes are more sensitive for detecting low levels of gene flow (Hellberg 1994). Allozymes have been used to elucidate population structure between marine invertebrates with limited larval dispersal (Abbiati *et al.* 1993, Hellberg 1994), to examine levels of inbreeding in different environments (Sherman *et al.* 2008) and in different parts of a species range (Goffredo *et al.* 2004), to elucidate taxonomic affinities between congeneric species (McFadden 1999), and to assess the effectiveness of marine protected areas (Miller and Ayre 2008a). A limitation of allozymes is that they provide limited genealogical information (Sunnucks 2000) and allozyme methods have now largely been superseded by more modern DNA-based techniques, such as AFLPs, microsatellites and SNPs.

Amplified Fragment Length Polymorphism (AFLP) is a multi-locus method based on variation in the length of DNA fragments generated from digestion with restriction enzymes. Following digestion, short DNA 'adapters' are attached to each end of the fragment, which can then be selectively amplified using PCR (Vos *et al.* 1995). Size patterns from resulting fragments can be visualised and compared using gel electrophoresis. Advantages for AFLPs include a relatively low cost, no prerequisite for DNA sequence data and high overall variability; however, a major drawback is that alleles are only detected as present or absent and therefore the method

cannot detect recessive alleles (Henry and Kenchington 2004). In addition, AFLP fragment patterns exhibit limited reproducibility and therefore there is limited comparative capacity between studies (Sunnucks 2000). AFLPs have been used to assess marine connectivity, for example in scleractinian coral populations on oil platforms in the Gulf of Mexico (Atchison *et al.* 2008).

Microsatellites, or simple sequence repeats (SSRs), are short tandem repeats of DNA motifs consisting of 2-10 base pairs (Hellberg *et al*. 2002). Microsatellites are thought to have functional roles in chromatin organization, regulation of DNA metabolic processes and gene regulation, although as they constitute a high proportion of non-coding DNA relative to protein-coding regions, they are generally considered to be evolutionary neutral markers (a contestable concept, reviewed in Li 2002). As genetic markers, microsatellites have several favourable properties for inferring population structure. For example, they have relatively high rates of mutation and are highly polymorphic, multi-allelic and co-dominant (Andreakis *et al.* 2009). As neutral markers, the extent of polymorphism is proportional to their underlying rate of mutation, and therefore evolutionary models can be applied to microsatellite size and frequency data to infer population divergence (Ellegren 2004). As such, microsatellites are extremely popular in assessing connectivity in marine populations. This is particularly evident, for example, from both finfish and invertebrate fisheries research, where microsatellites are used to infer source (stock) populations, to examine migratory routes and to examine effects of over-fishing on genetic diversity (e.g., Miller *et al.* 2009).

When reference genomes are unavailable, microsatellites must be directly characterised through cloning and detection and sequencing of candidate loci to identify microsatellite flanking regions from which specific primers can be identified (Jarne and Lagoda 1996). Although the development of microsatellite markers is laborious and time consuming (Chapter 2), next generation sequencing technologies combined with the availability of more genomic resources for taxa of interest are beginning to improve yield and efficiency of their development, as seen for the scleractinian *Acropora millepora* (Wang *et al*. 2008). Microsatellites have been developed for a number of invertebrate taxa, from both tropical and temperate regions and have been applied to several studies examining connectivity in the context of conservation or marine reserves (e.g., Costantini *et al*. 2011, Le Goff-Vitry *et al*. 2004, Yasuda *et al*. 2008,).

Since 2000, high-throughput methods have led to the possibility of amassing vast amounts of genomic data rapidly; microchip-based single nucleotide polymorphisms (SNPs) are replacing microsatellites and becoming the marker of choice for population genetics (Sunnucks 2000).

SNPs are a 1bp polymorphism in a gene. Reduced resolving power conferred by bi-allelism and slower evolution compared to multi-allelic microsatellites can be overcome using larger marker sets typically an order of magnitude higher than a microsatellite panel (although a relatively small array of 80 SNPs was sufficient to determine parentage as successfully as 11 microsatellites in a wild sockeye salmon population, Hauser *et al.* 2011). Relative to microsatellites, SNPs are disadvantageous in cost, the development of high-throughput assays is arduous and they may have limited cross-species transferability (Hauser *et al.* 2011, Seeb *et al.* 2011). Nonetheless, once available, SNPs offer higher accuracy, more power, higher reproducibility and faster data collection than microsatellites (Seeb *et al.* 2011). They have therefore proved highly valuable in diverse applications, including comparative and ecological genomics (e.g., Wang *et al*. 2008) and in clinical diagnostics, where they are used extensively (e.g., to detect susceptibility to breast cancer; Easton *et al.* 2007).

In summary, molecular techniques have been employed with increasing frequency for conservation purposes, for example in fisheries management and in marine reserve design (reviewed in Hellberg 2002 and Hedgecock *et al*. 2007). Although the use of molecular markers can give an empirical measure of realized gene flow (i.e., a measure of inter-generational genetic input mediated by migration) as opposed to theoretical values (e.g., inferred from but not measured directly from ocean currents), they are also problematic. For example, microsatellites are limited in that high locus polymorphism can lessen sensitivity in detecting population differentiation, which may confound interpretations of connectivity. The occurrence of insignificant low Fst values between populations may conclude in inferences of high connectivity, whereas the result may be a consequence of low power from the use of several highly variable markers, as suggested by Hellberg (2009). A limitation in resolving weak genetic differentiation is particularly relevant to populations characterised by high effective population sizes and reliance on dispersive phases for connectivity, a likely scenario in many marine populations. Negative correlations between Fst estimates and locus polymorphism have been demonstrated empirically (e.g., in walleye Pollock; O'Reilly *et al.* 2005) and therefore interpreting low estimates must be done with caution.

Part 3: Octocorals

1.3.1 Taxonomy, Ecology, and Importance

The class Anthozoa forms one of five cnidarian classes and typically consists of colonial coelenterates lacking a medusoid stage, which are divided between the subclasses

Hexacorallia (including the scleractinian corals and anemones, generally animals with tentacular arrangements in multiples of six) and Octocorallia (i.e., gorgonians, soft corals, sea pens, with eight pinnate tentacles, Daly *et al.* 2007). Octocorallia are prominent members of benthic communities from circum-polar regions through temperate zones to the tropics, with an intertidal to abyssal depth range (Breedy 2009). Shallow tropical species and some shallow temperate octocorals contain endosymbionts of the ubiquitous dinoflagellate *Symbiodinium* spp., although the ecology and diversity of octocoral symbioses are less well characterized than for scleractinians (Holland 2006). The Octocorallia is represented by more than 3000 species, of which 75% are thought to occur in depths below 50 metres (Yesson *et al*. 2012). In fact, the extent of the biodiversity of deep-sea octocorals is probably unknown, given that these deeper species are still being described and that recent conservation efforts to mitigate the effects of deep-sea trawling have been based upon the prevalence of deep-sea octocorals in bycatch (e.g., Lopez-Gonzalez *et al*. 2001, Watling and Auster 2005).

The evolutionary affinity of octocorals has recently been questioned, and mitochondrial phylogenomics suggests that the Anthozoa are not monophyletic and that octocorals, itself still a monophyletic group, may form the sister group to the Medusozoa and not the Hexacorallia as was previously thought (see Kayal *et al*. 2013). Octocorals do not usually produce a massive supportive calcareous matrix like scleractinian corals but instead contain microscopic calcite elements, termed sclerites (or 'spicules'), that provide both a supportive and defensive role (e.g., Breedy 2009). Sclerites are made of calcite but also contain magnesium and strontium (Weinbauer and Velimirov 1995a). As the morphology of octocoral sclerites varies enormously in size, shape, colour, distribution and abundance between coenenchymal layers, they have traditionally been used as a character to delineate species and infer taxonomy (e.g., Bayer 1961). Octocorals are also colloquially termed gorgonians (or sea rods or sea fans) and soft corals. This refers to members to the paraphyletic order Alcyonacea, which represents most octocorals but excludes blue corals and sea pens (the orders Helioporacea and Pennatulacea respectively; Fabricius and Alderslade 2001). Gorgonians are typically arborescent or rod-like animals of the suborders Holaxonia, Scleraxonia or Calcaxonia, and contain a solid axial skeleton that varies taxonomically in composition and may be composed of protein ('gorgonin'), calcite or aragonite, whereas soft corals are lobate, fleshy members of the suborders Alcyoniina and (again) Scleraxonia and lack this consolidated skeleton and rely instead upon hydrostatic pressure and their sclerite mass for support (Fabricius and Alderslade 2001). As octocorals do not secrete massive calcium carbonate structures, they are not considered to be reef-building, in contrast with the scleractinian hexacorals also characteristic of- and prevalent in tropical coral reefs. However, spicules may form a substantial component

of calcium carbonate sediments in some areas (e.g., the Ligurian sea; Weinbauer and Velimirov 1995b). Furthermore, spicules may be consolidated at the base of colonies in some species to form 'spiculite', as seen in the fossil record and present-day colonies of the fleshy genus *Sinularia* (Jeng *et al*. 2011); therefore the role of octocorals in reef-building may need reconsideration. Octocorals also differ from scleractinians in that they produce secondary metabolites with allelopathic capabilities which have important ecological functions including antipredator defense and space competition (Sammarco and Coll 1992). For example, soft corals contain chemicals either toxic or distasteful to predatory fish and therefore act as a feeding deterrent (Mackie 1987) and these chemicals may also inhibit growth or cause tissue necrosis in adjacent species competing for space (Sammarco and Coll 1992). Interest in the chemical ecology of octocorals is prevalent following the discovery that they often prove cytotoxic to human pathogens or exhibit antiproliferative activity to cancer cell lines; for example to human breast and prostate adenocarcinoma (by *Eunicella cavolini*; Ioannou *et al*. 2009). Secondary metabolites produced by octocorals may also be anti-inflammatory, such as the pseudopterosin class of compounds produced by the Caribbean gorgoniid *Pseudopterogorgia elisabethae* which are harvested commercially in the Bahamas for use in the cosmetics industry (Goffredo and Lasker 2008). Toxicity of secondary compounds is also not restricted to human pathogens; significant antifungal activity has been demonstrated in *Pseudopterogorgia* spp. and *Pseudoplexaura* spp. to both coral and human pathogens (*Aspergillosis sydowii* and *A. flavus*, respectively; Kim *et al.* 2000) and Caribbean *Briareum asbestinum* shows toxicity against the malarial parasite *Plasmodium falciparum* (Ospina *et al*. 2003). Understandably, octocoral fisheries are thus of great interest to the pharmaceutical industry.

Octocorals are an extremely important component of benthic biomass in shallow and deep waters and constitute microhabitats and substrate for other marine invertebrates, including ophiuroids, barnacles, shrimp, anemones, hydroids, molluscs (e.g., Cupido *et al*. 2012, Herrera *et al*. 2012). Octocorals provide structural complexity in many ecosystems where they may form the dominant epifaunal group, such as on seamounts (Baco and Shank 2005) or in the Mediterranean where they form dense gorgonian forests and represent an emblematic member of Mediterranean coralligenous communities (Coma *et al.* 2004).

1.3.2 Threats to Octocorals

Globally, octocorals are threatened by anthropogenic activity and the impacts of alternative climate scenarios. Members of the genus *Corallium* include the so-called 'precious corals' that have been harvested for thousands of years, primarily for use in jewellery, such as the species

C. rubrum that has undergone considerable depletion in the Mediterranean (Abbiati *et al*. 1993). *Corallium lauuense* and *C. secundum*, also harvested for jewellery, are abundant on seamounts in the Hawaiian Islands and the former, known as 'red-coral' is extremely valuable (Baco and Shank 2005). In these species, the effects of over-harvesting are likely compounded by high rates of inbreeding (Baco and Shank 2005). Commercial fishing activity is also a threat to many octocoral species as incidental mechanical damage by fishing gear targeting other species can decimate populations. The temperate pink sea fan, *Eunicella verrucosa*, is one such species and is threatened by the increased efforts of inshore shellfish fishing (e.g., for king scallops *Pecten maximus*) resulting from the collapse of continental shelf finfish fisheries in Europe (Lumbis 2008). Damage by mobile towed benthic fishing gears has thus resulted in localised conservation efforts to protect sea fan habitat by closing certain areas to trawling (Atrill *et al*. 2011). Another temperate octocoral, the ubiquitous *Alcyonium digitatum,* is affected detrimentally by benthic trawling and its abundance is greatly reduced in fished vs. non-fished areas (Hinz *et al*. 2011). Octocorals are also sensitive to long-line fishing gear and deeper species appear to be particularly vulnerable as they are often caught as bycatch (Watling and Auster 2005).

The effects of climate change on octocorals are less studied than for scleractinians and remain poorly understood. The phenomenon of coral bleaching, whereby tropical anthozoans lose their endosymbiotic dinoflagellate symbionts (*Symbiodinium* spp) in response to short-term environmental variability, has also been recorded in octocorals (Prada *et al*. 2009). This implies that octocorals are vulnerable to increasing sea surface temperatures (SSTs), especially as the diversity of their symbiotic complement (and therefore adaptive potential) may be reduced in relation to other reef taxa (Baker and Romanski 2007, Holland 2006). Likewise, the negative effects of decreasing oceanic pH have been reported for marine invertebrates and calcifying plants such as the temperate urchin *Psammechinus miliaris* (Miles *et al*. 2006), seagrasses (Martin *et al*. 2008) and the sand dollar *Dendraster excentricus* (Chan 2012) but are poorly understood in octocorals. Surprisingly, initial research implies that octocorals may be more resilient to the effects of ocean acidification than other tested marine invertebrates. Gabay *et al*. (2013) subjected the three zooxanthellate octocoral species *Heteroxenia fuscescens*, *Ovabunda macrospiculata* and *Sarcophyton* sp. to reduced pH regimes and did not record significant differences in polyp behaviour or cellular biochemistry, sclerites, polyp weights and zooxanthellae density and chlorophyll content. These data imply that acidification had negligible effects on the species of octocorals studied and the authors suggest that, subject to further research, octocoral tissues may buffer against the effects of decreased pH. However, a contrary finding of impaired spicule formation was reported in *Corallium rubrum* colonies

subjected to acidified conditions (Bramanti *et al*. 2013), suggesting that other species may be adversely affected by reduced seawater pH. Thus the potential resilience of octocorals to acidification requires further study.

Octocorals are prone to diseases and this has been best documented in the Mediterranean, where in 1999 the Ligurian Sea saw the mass mortality of potentially millions of sea fans including *Paramuricea clavata*, *Eunicella singularis* and *E. cavolini* (Cerrano *et al*. 2000), and where a second mass die-off occurring in 2003 caused even greater mortality in *P. clavata* (Cupido *et al*. 2012). In both cases, temperature anomalies leading to a rise in seawater temperature likely led to infection by opportunistic bacterial or fungal pathogens. A disease outbreak has also been recorded on *Eunicella verrucosa* in southwest England, between 2003 and 2006 (Hall-Spencer *et al*. 2007). In the latter study, a consortium of bacterial *Vibrio* species were likely to be the causative agent in the infection, inducing necrosis and the sloughing off of coenenchymal tissue exposing underlying gorgonin. This contrasts with a well documented fungal pathogen from the Caribbean region, *Aspergillosis sydowii,* which affects sea fans in the genus *Gorgonia* and causes necrosis but additionally tumours, galls and a darkening of pigmentation (Mullen *et al*. 2006). Diseases caused by bacterial and fungal pathogens in the absence of temperature anomalies indicate that gorgonians may be sensitive to localised pollution or run-off; *A. sydowii* is a terrestrial fungal pathogen but there is also evidence that it can be transported on substrates in African soil dust that transverses the Atlantic (Shinn *et al*. 2000). Regardless of the trigger, disease outbreaks can have important consequences for octocoral population dynamics. For example, Cupido *et al*. (2012) observed reduced gross reproductive output in *Paramuricea clavata* populations after the mass die-off and octocoral longevity and slow growth rates are likely to exacerbate the detrimental effects of diseases (Cerrano *et al*. 2000).

Part 4: Connectivity and Marine Conservation Policy

In response to unprecedented rates of global biodiversity loss and following international commitment to sustainable development, the United Nations Environment Program (UNEP) Convention on Biological Diversity (CBD) gained 163 signatures at the 'Earth Summit' in Rio de Janiero in 1992 (http://www.cbd.int). As of 2005, there were over 100,000 protected areas representing over 12% of the terrestrial surface of the Earth (Chape 2005). The rationale behind protected areas is that by reducing the loss of habitat and exploitation of species, the growth of populations will be enhanced enabling them to produce more offspring and enjoy enhanced survivorship (Mora and Sale 2011). In some ecosystems, maximizing protected areas

is balanced against the potential loss of ecosystem services and therefore since the CBD, great effort globally has been put into the design, size and spacing of protected areas to maximize conservation benefits while maintaining their economic value (e.g., Roncin *et al*. 2008). The International Union for Conservation of Nature (IUCN) therefore defines a protected area as "a clearly defined geographical space, recognised, dedicated and managed, through legal or other effective means, to achieve the long-term conservation of nature with associated ecosystem services and cultural values" (Laffoley 2008).

Increasingly, the impacts of anthropogenic activity together with rapidly changing climatic conditions are acting to damage marine resources and to reduce marine biodiversity, leading to a critical need to protect marine ecosystems in addition to terrestrial ecosystems (Allison *et al.* 1998). Following the collapse of many fisheries of commercial importance, initial measures to protect the marine environment focused primarily on maintaining such stocks (e.g., Higgins *et al.* 2008). Gradually, however, the extensive damaging effects of human activities and the contemporary accelerated rate of decline of other marine species and ecosystems was realised, including the unprecedented rate of coral reef mortality (Mora *et al*. 2006), the disturbance of sessile benthic fauna and habitats by towed fishing gear (Hinz *et al*. 2011), and the threats posed by ocean acidification (e.g., Turley *et al*. 2007). Therefore, evidence of declines in marine harvests, loss of biodiversity, and degradation of habitats has prompted calls for the establishment of a global system of Marine Protected Areas (MPAs) (Laffoley *et al.* 2008) and MPAs are fast becoming the mainstream management tool for conserving biodiversity in oceans and seas worldwide (Agardy *et al.* 2003), though evidence to date suggests that MPA designation and design is frequently based more on political considerations than scientific data (Agardy *et al.* 2003, Jones and Carpenter 2009, Laurel and Bradbury 2006). The IUCN (1988) definition of a MPA is "any area of intertidal or subtidal terrain, together with its overlying water and associated flora, fauna, historical and cultural features, which has been reserved by law or other effective means to protect part or all of the enclosed environment" (Johnson *et al*. 2008 page 10). More recently, MPA design has shifted towards developing networks of marine protected areas, defined as a "collection of individual MPAs operating cooperatively and synergistically, at various spatial scales, and with a range of protection levels, in order to fulfil ecological aims more effectively and comprehensively than individual sites could alone" (in McLeod *et al*. 2009). Several networks have now been established globally, for example in central California (Kaplan *et al*. 2009).

In Europe, commitments to marine environmental protection are being implemented under several policies, including the OSPAR convention (1992) - an amalgamation of the 1972 Oslo

and 1974 Paris conventions that were developed initially to address pollution risk in the NE Atlantic, which now has fifteen signatories (http://www.ospar.org). More recently, the EU Marine Strategy Framework Directive (MSFD 2008) has been developed which requires that each member state establish 'good environmental status' and 'coherent and representative networks' of MPAs by 2020 (e.g., http://www.defra.gov.uk).

In the UK, the development of a network of MPAs is highly topical, and as such two key requirements of the Directive, an assessment of the status of UK seas and the development of characteristics detailing what good environmental status means for UK seas, have recently been published (Defra Marine Strategy Part One report, www.jncc.defra.gov.org). Commitments to develop an MPA network in the UK are being met by the wider OSPAR network (Evidence Review MCZ Final Report, Defra 2013a) as well as the Marine (Scotland) Act, the Northern Ireland Bill and the Marine and Coastal Access Bill of 2009, which implements initiatives in marine planning, licensing, marine conservation and coastal access. The new MPA network will include and develop upon areas previously protected under European legislation, including European Marine Sites: Special Areas of Conservation (SAC) and Special Protection Areas (SPA), marine areas of Ramsar sites (for protection of wetlands), Sites of Scientific Special Interest (SSSIs), Marine Conservation Zones (MCZs) in English and Welsh seas, Nature Conservation MPAs which include Scottish inshore and offshore waters and MCZs developed for Northern Ireland (Evidence Review MCZ Final Report: Defra 2013a).

In England (the primary region under consideration in this study) development of proposals for UK MPA designation was conducted between 2008-2010 by four regional groups, in line with national guidance from the UK government statutory conservation bodies, Natural England and the Joint Nature Conservation Committee (JNCC) and Defra (2008). The four regional groups were Finding Sanctuary (southwest England), Balanced Seas (southeast England) Net Gain (the North Sea) and Irish Sea Conservation Zones (the Irish Sea excluding Welsh and Irish territorial waters) (Defra 2013a). In addition, an independent scientific advisory panel of primarily academic marine experts was appointed to support the work of the four regional groups, and each regional group worked with the scientists and stakeholders to decide upon potential areas to be designated as MCZs. Their final recommendations were submitted to government in September 2011 and were assessed by independent consultation (Defra 2013a). Ultimately, in December 2012 only 30 of the proposed 127 sites were accepted by the UK Government. At the time of writing this thesis, they were undergoing final consultation until March 31st, 2013 (defra.gov.uk). There appears to be relatively few cases where genetic assessment of connectivity has been incorporated into MPA network design; genetic data was

not included in the MPA network design guidelines in the UK, even though connectivity is one of the seven principles that were incorporated in to the network design following OSPAR and IUCN guidelines (Defra 2008). However, the need for incorporating molecular population data into MPA Network design has been widely recognised (e.g., von der Heyden 2009).

Part 5: Study Species

1.5.1 *Eunicella verrucosa* (Pallas, 1766) and *Alcyonium digitatum* Linnaeus, 1758

This study will focus upon two important octocoral species in the UK and North East Atlantic, the 'pink sea fan' *Eunicella verrucosa* (Octocorallia: Alcyonacea: Holaxonia: Gorgoniidae) and 'dead man's fingers' *Alcyonium digitatum* (Octocorallia: Alcyonacea: Alcyoniina: Alcyoniidae).

Eunicella verrucosa is an IUCN red-listed species, subject to damage from bottom trawling activity, and is protected in the UK under the Wildlife and Countryside Act of 1981 and is also a UK Biodiversity Action Plan (BAP) species (Hall-Spencer *et al.* 2007). Despite an extensive range in the North East Atlantic, from Angola to Western Ireland, its range in the British Isles is limited to the South West, Pembrokeshire and southern and western Ireland (e.g., Grasshoff 1992). *Alcyonium digitatum* also has an extensive trans-Atlantic range which spans Portugal to Norway, Iceland and the North Sea in the NE Atlantic, and eastern Canada to Cape Hatteras in the USA in the NW Atlantic (Hartnoll 1975, Watling and Auster 2005). *Alcyonium digitatum* is not protected, but it is locally depleted in some areas by benthic trawling (Hinz *et al.* 2011) Both species often co-exist in the same habitat and they are often presented in publicity materials to promote UK-based marine conservation efforts. Colonies representative of specimens found in UK waters are shown in Figure 1.2.

Figure 1.2 Exemplary octocorals of the UK and NE Atlantic. Left: *Eunicella verrucosa* pink morph (copyright Paul Kay, from naturalengland.org.uk). Right: *Alcyonium digitatum* white morph (copyright Keith Hiscock, from marlin.ac.uk).

Research Objectives

The aim of this research is to provide the first genetic assessment of *Eunicella verrucosa* and *Alcyonium digitatum* in the south west portion of their range in the UK and beyond, and to determine the extent of population structure and the extent of connectivity between populations in this area at a variety of spatial scales. This research was commissioned by Natural England (Project no. SAE 03-02-146) to coincide with the design and developmental period of the UK's first Marine Protected Area Network between 2008-2012. Few molecular markers are available for temperate Anthozoa, and none are available to determine population-level relationships within our species of interest. Therefore, the study was split into two parts:

1) Development of microsatellite markers *de novo* for each species. The objective of this part of the research was to develop and publish markers that would be available for my study and also for future assessments of population structure in each species. These microsatellites would represent (to my knowledge) the first developed from octocorals in the North East Atlantic including the UK and will be the first developed from each species. Therefore this study relied exclusively on these microsatellites and assessment of genetic connectivity was entirely based on microsatellites.

2) Assessment of genetic connectivity of *Eunicella verrucosa* and *Alcyonium digitatum* in the North East Atlantic. Using the microsatellites developed in Part 1), population structure and genetic connectivity was assessed in each species in the UK (with particular focus on South West England and Wales), Brittany, western Ireland, and Portugal (in the case of *E. verrucosa*) and the North Sea (in the case of *A. digitatum*). The objective of this research was to gain an overview into the genetic diversity within and between populations of each species, to make inferences about their degree of genetic connectivity at various spatial scales, (from adjacent sites to regionally disparate populations) and to determine if the vulnerability of each species could be determined genetically. Although the reproductive biology of each species is relatively poorly understood (Chapters 3 and 4), I hypothesized that genetic structure would be less pronounced in *Alcyonium digitatum.* Anecdotal evidence suggests that this species is likely to produce lecithotrophic larvae, as does *Eunicella verrucosa,* although *A. digitatum* is unusual in that it spawns hibernally and as such may be more exposed to wind-driven surface currents.

In this thesis, Chapter 2 describes part one of the study and outlines the development, screening and results from microsatellite isolation from each species. Chapter 3 and Chapter 4

describe patterns of connectivity of *Eunicella verrucosa* and *Alcyonium digitatum* respectively. Finally, Chapter 5 discusses the results from both parts of the study and highlights the implications of these data in terms of conservation efforts.

Chapter 2. Development of Microsatellite Markers in the Temperate Octocorals *Eunicella Verrucosa* and *Alcyonium Digitatum*

Abstract

Two panels of microsatellite loci were developed *de novo* for the temperate octocorals *Eunicella verrucosa*, an IUCN red-listed species with a limited distribution in the British Isles, and *Alcyonium digitatum*, an abundant species found sublittorally on many coastlines around Britain and Ireland. These panels represent the first microsatellites to be isolated from any British octocorals and from any octocorals in the NE Atlantic. Microsatellites were isolated from three separate enriched genomic libraries, two of which were combined for *E. verrucosa*. In total, 76 unique microsatellite sequences were screened for *E. verrucosa*, and 54 for *A. digitatum*, ultimately resulting in two panels of fourteen and eleven loci, respectively. The panel from *Eunicella verrucosa* was tested in 44 individuals from Mewstone Ledges, Plymouth Sound, Devon, UK and alleles per locus ranged between two and ten. Expected heterozygosities in this population were between 0.05 and 0.82, and significant deviation from expectations of Hardy-Weinberg equilibrium was detected at three loci following correction for multiple tests; these loci also had evidence for null alleles. The *Alcyonium digitatum* panel was tested in a population of 42 individuals from Trenemene Reef in the Isles of Scilly, UK. Alleles per locus were more numerous in this species and ranged between three and twenty-seven and expected heterozygosities were between 0.07–0.96. Two loci in this panel deviated from Hardy-Weinberg equilibrium possibly due to null alleles. Evidence of linkage disequilibrium was not detected in either panel. Cross-amplification attempts were made using both panels; for *A. digitatum*, this included three congeneric species from the French Mediterranean, and for *E. verrucosa*, three gorgoniid species collected from the Mediterranean and southern Portugal. The *E. verrucosa* panel amplified particularly well in *E. singularis*, and also successfully at most loci in *E. cavolini* and *Leptogorgia sarmentosa*. The *A. digitatum* panel amplified relatively well in *A. coralloides* and *A. acaule*, but with limited (but some) success in *A. palmatum*. These markers should provide an excellent means for making a long-overdue assessment of connectivity and population structure in these important and vulnerable animals, and provide a useful resource for future monitoring and conservation efforts of particular octocorals whose ranges often surpass national boundaries.

2.1 Introduction

2.1.1 What is a Microsatellite?

Microsatellites, or simple sequence repeats (SSRs), are short tandem repeats of DNA motifs of 2-10 base pair nucleotides in length (Hellberg *et al.* 2002). Minisatellites are also short tandemly repeated sequences but are more than ten base pairs in length and will not be considered further. Microsatellites are named according to the number of bases in one repeat, for example, a two base pair repeat (e.g., GC) is a dinucleotide, a three base pair repeat is a trinucleotide, a four is a tetra- and so on. As genetic markers, microsatellites have several favourable properties for inferring intrapopulation structure; they are highly variable, codominant (i.e., inheritance from both parents can be inferred), neutral (theoretically), are easily scored and as they are short, may be isolated from poor quality DNA such as hair, fossils or faeces, making them ideal for conservation genetics applications where intrusive sampling may be difficult (Luikart and England 1999). The high polymorphism of microsatellites is thought to be due to mutations arising through DNA slippage (i.e., imperfect replication due to misalignment of nascent and template DNA) which increases or decreases motif copy number and therefore changes the length of the microsatellite (reviewed in Schlotterer 2000). Polymorphism can be observed within an individual, known as a heterozygote, which is (in a diploid taxon) when a different allele is inherited from each parent and therefore two alleles of different length can be detected, as opposed to homozygotes, when the same allele has been inherited from each parent and therefore both genes carry a microsatellite of identical length. As variation between alleles can be deduced between individuals, measuring allelic variation of microsatellites and assessing their frequency within and between populations of the same species can give a statistically- inferred measure of divergence and explains their widespread popularity in population genetics. These inferences are usually based upon expected vs. observed frequencies in heterozygotes and homozygotes in accordance with the Hardy-Weinberg equilibrium model, the assumptions of which include sexual reproduction, non-overlapping generations, random mating, identical allele frequencies in each sex, diploid individuals, bi-allelic inheritance and minimal migration and mutation (Hartl and Clark 2007). As genotypes of individuals can be detected, microsatellites are also used for assignment of individuals to a population or cohort, and can be used to examine intrapopulation characteristics such as the extent of inbreeding or the effective population size (a.k.a. Ne, i.e., the number of breeding individuals contributing genetic input to subsequent generations).

2.1.2 Evolution of Microsatellites

Microsatellite mutation rates are much higher than at point mutations on coding loci gene and generally range from 10^{-2} to 10^{-6} (Li *et al.* 2002), with variation in this rate observed between different taxa; in yeast estimates are between 10^{-4} to 10^{-5} per locus per generation, whereas in humans rates are thought to be at 10^{-3}, compared to 10^{-9} to 10^{-10} at point mutations (Hancock 1999). These mutations are thought to stem from unequal crossing over (when imperfectly aligned homologous chromosomes recombine) or by slip-strand mis-pairing errors during replication and recombination, which results in the loss or gain of motifs, with more evidence supporting the latter as the most common mechanism (Eisen 1999). Understanding microsatellite evolution is important in population genetics, where it is inferred statistically upon the basis of theoretical mutation models and used to explain differences in allele frequencies. Although microsatellite evolution is still relatively poorly understood and is dependent upon many factors (e.g., taxon, motif, location in the genome, neutrality), mutation rates are usually inferred by one of two models. These are the infinite allele model (IAM, Kimura and Crow 1964), where each mutation creates a novel allele, mutations can include several tandem repeats and the resulting allele state is novel to the population, and the stepwise mutation model (SMM, Kimura and Otha 1978), whereby one repeat motif is lost or gained per mutation (with equal probability) and thus an allele state may already have occurred in the population (Estoup and Cornuet 1999, Jarne and Lagoda 1996). In both models, the number of repeat motifs in an allele has no upper or lower boundary, although in reality there appears to be an upper size limit of microsatellite repeat numbers as very long microsatellites are uncommon and likely unstable and constrained by selective pressure (Amos 1999). Under the IAM, different size alleles differ equally from each other in terms of mutational steps independently of their size, whereas under the SMM, alleles of similar size are thought to be more similar in terms of mutational steps than alleles of very different size (Jarne and Lagoda 1996). These models are somewhat simplistic and contradictory research exists where empirical data do or do not fit allele frequencies predicted by the models; further complications may arise when motifs are imperfect or compound (i.e., interrupted by non-repeat base-pairs or motifs). It is recognised that these two models represent extreme mutation models and that microsatellites are likely to evolve between the two, as such there are several variations on these models including two phase models (e.g., Rienzo *et al.* 1994) when occasional multistep mutations are allowed in addition to one step mutations (Balloux and Lugon-Moulin 2002). In some cases, asymmetrical mutations have been observed, where the likelihood of microsatellite expansions or contractions are unequal (Estoup and Cornuet 1999). Furthermore, inter-specific differences between closely related species have also been noted by the

presence of longer repeats being repeatedly found in homologous microsatellites in one but not the other species (e.g., human microsatellite markers are longer when typed in humans than in chimpanzees). Reasons explaining this are unclear but this indicates that mutation rates of microsatellites can vary highly even among closely related lineages (Amos 1999). In summary, microsatellite mutation rates are unlikely to be uniform across either closely-related or distantly-related lineages.

Determining the most likely model of microsatellite evolution is possible either directly by observing variation over several generations in a pedigree, or indirectly using population genetic theory, where the expected number of alleles in a population can be inferred from observed heterozygosity based upon the IAM or SMM and the probability of rejecting either model is calculated upon the difference in the observed and expected data (Jarne and Lagoda 1996, Valdes *et al.* 1993). The paucity of empirical data being an exact fit for either model suggests a need for caution when using either model in population genetic analysis. However, R-statistics have been developed which assume the SMM (Slatkin 1995) and take into account the actual size of alleles, as opposed to the consideration of frequency rather than size in F-statistics.

2.1.3 Genomic Distribution of Microsatellites

In eukaryotes, microsatellites are located throughout the genome, in both non-coding and protein coding regions, but there is variation in their distribution and motif identity between coding, non-coding regions and intergenic spacer regions, with the lowest abundance thought to be in coding regions (i.e., exons, Toth *et al.* 2000). An increased availability of genomic data generated by next-generation technologies has allowed for comparisons of microsatellite abundance, distribution and motif type between certain taxa, although for others data remain scarce. For example, Toth *et al.* (2000) compared microsatellites in several vertebrate, arthropod, fungi and plant genomes. They found that in primate introns and intergenic regions, mononucleotides are very common and tetranucleotides are much more frequently encountered than trinucleotides. Furthermore, they noted that tetranucleotides were more abundant in introns and intergenic regions of all vertebrate taxa studied. Rodents had a higher abundance of microsatellites than all other taxa studied, with the highest frequency of dinucleotides; in comparison, this motif was scarce in fungi. They also described a higher frequency of (G+C) rich motifs in exons compared to other regions, and a higher exon proportion of trinucleotides and hexanucleotides, and they also suggest that pentanucleotides are more frequent than tetranucelotides in invertebrates. This latter assertion may be taxon-specific, as Meglecz *et al.* (2012) described higher proportions of tetranucleotides as opposed to

pentanucleotides in all of the metazoan non-chordate taxa screened during their research. In some taxa, microsatellites are notoriously difficult to isolate (e.g., arthropods, molluscs, cnidarians); reasons for this remain unclear but may be linked to similarity in flanking regions adjacent to microsatellites (potential primer sites), recombination events or associations with transposable elements (McInerney *et al.* 2011). There is also evidence that success may be species dependent even between closely related animals due to variation in inter-specific genomic complexity between species (McInerney *et al.* 2011). Across all eukaryotes, Poly (A) and poly (T) repeats are thought to be the most abundant microsatellites in all genomes and it is the most common motif in human genomes (Hancock 1999).

2.1.4 Functionality of Microsatellites

The function of microsatellites is largely uncertain. There is evidence that in some cases they may have a regulatory and functional coding role in transcription in eukaryotes as they are often found upstream of coding genes in promoter regions. Here they may serve as enhancer elements, may bind to proteins, or may even be found as coding sequences within an actual protein (Kashi and Soller 1999). An increased number of tandem repeats in a promoter region may correlate positively with transcription activity; motifs characterized in promoter regions are varied and may be di-, tri-, or tetranuceleotide, for example TCCC, TC and TG are known from several taxa (Kashi and Soller 1999). However, variation in numbers of trinucleotide repeats within genes are more likely to result in phenotypic effects, as a transcribed trinucleotide found within a gene (i.e., a codon) may encode an amino acid that alters protein function (and may also be subject to selection). For example, irregular numbers of glutamine residues are known to alter the mouse Sry gene to cause complete and partial sex reversal, whereas in humans it alters androgen receptor function to increase overall risk of early onset and risk of prostate cancer (shorter repeats), or impairs spermatogenesis (longer repeats, Kashi and Soller 1999). Phenotoypic effects of trinucleotide expansions are also evident in human diseases, where instability in them is associated with inherited diseases including myotonic dystrophy, spino-bulbo muscular dystrophy, Huntingdon's disease, Friedreich's ataxia, autosomal dominant pure spastic paraplegia and synpolydactyly (Valdes *et al.* 1993; Kashi and Soller 1999). Several of these diseases are unusual in that age of onset decreases and severity increases in successive generations, or in that they are linked to paternally or maternally inherited chromosomes and, thus, one sex may carry but not manifest the phenotype (reviewed in Rubinsztein *et al.* 1999). In these examples, substantial increases in trinucleotide copy number are the causal agent, this is typified in X-linked mental retardation (fragile X syndrome). This condition is thought to be linked to extensive increases in a CGG repeat in exon 1 in the gene FMR1, which is typically between six and

fifty repeat units, whereas affected individuals have over 200 to more than 1000 repeats (Page and Holmes 1998). A significant association between microsatellites and genes known to cause certain diseases (such as narcolepsy, multiple sclerosis and insulin-dependent diabetes mellitus) in the human major histocompatibility complex (HLA) has also been demonstrated by calculating linkage disequilibrium between them (the non-random association of alleles that are not necessarily located in close physical proximity to each other). In this case, the microsatellites themselves are not thought to affect susceptibility to disease, but can be useful for disease mapping and genetic screening (Carrington *et al.* 1999).

Although some microsatellites may therefore be associated with altered phenotypic states, the majority of them are thought to evolve neutrally though genetic drift over time (Hellberg 2007). In classical population genetics, statistical inferences are usually based upon the assumption that loss of alleles though genetic drift is balanced with new alleles arising from migration and mutation; neutral markers with no selective pressure are therefore most useful and best fit these assumptions (Marko and Hart 2012).

2.1.5 The Application and Utility of Microsatellites

Due to their high rates of intraspecific polymorphism, microsatellites have been used extensively in population genetics to infer demographic history and migration, and in conservation genetics to determine rates of hybridization and inbreeding, to detect bottlenecks, and to measure dispersal and connectivity patterns. In population genetics, prominent examples include elucidating diversity, evolutionary history and migration patterns of human populations. For example Zhivotovsky *et al.* (2003) used 377 loci to confirm that microsatellite diversity was highest in Africa, that migration of modern humans arose from a common ancestor in sub-Saharan Africa and that, at a coarse scale, three clusters of modern humans could be defined (Africa / Eurasia, East Asia/Oceania, and America). Interestingly, at finer resolution within these groups, they also noted unexpected patterns of relatedness such as the Basques, Sardinians and Orcadians being more closely related to Middle-Eastern populations than to other Europeans.

In conservation genetics, microsatellites are used for a vast array of purposes and are used extensively (see below). They have been employed to examine the spread of invasive species (e.g., sea squirts, DuPont *et al.* 2006, 2007), to determine rates of hybridization (e.g., between the endangered Ethiopian wolf and domestic dogs, Beaumont and Bruford 1999), to determine the extent of inbreeding (e.g., in threatened precious corals, Costantini *et al.* 2007a), to examine evidence for a bottleneck (e.g., in threatened European tree frogs, Broquet *et al.* 2010), to

determine parentage (e.g., in wild and captive gorillas and orang-utans, Field *et al.* 1998), to assess relatedness (e.g., within burrow clusters of endangered wombats, Taylor *et al.* 1997), and to assess the long-term effects of conservation management on genetic diversity of fragmented populations (e.g., the Mauritius parakeet, Raisin *et al.* 2012). In some cases, microsatellites may be linked to a sex chromosome, in which case they may also be used to determine patterns on the basis of gender (e.g., in sticklebacks**,** Shikano *et al.* 2011).

2.1.6 Microsatellites in Marine Conservation

In recent years, conservation genetics approaches are increasingly being applied to threatened marine ecosystems and species, knowledge about which is often scant compared to terrestrial systems. This is particularly evident from finfish and invertebrate fisheries, many of which are under intense fishing pressure globally and some of which have suffered huge declines in stocks. As such, molecular markers have been used to determine the source and fitness of stock and replenished populations, to examine migratory routes and to examine the effects of over-fishing on genetic diversity. Microsatellite panels have been used in many commercial species of fish such as Atlantic salmon (*Salmo salar*, e.g., Griffiths *et al.* 2010, 2011), Atlantic cod (*Gadus morhua,* Glover *et al.* 2010) and Atlantic bluefin tuna (*Thunnus thynnus thynnus*, Carlsson *et al.* 2004), and in invertebrates including cuttlefish (*Sepia officinalis*, Wolfram *et al.* 2006), abalone (*Haliotis rubra,* Miller *et al.* 2009) and spider crabs (*Maja brachydactyl,* Sotelo *et al.* 2008). Microsatellites have also been used to assess population structure in other ecologically important species, such as those landed as bycatch or bioindicator species (e.g., dab *Limanda limanda,* Tysklind *et al.* 2009), and from species indirectly threatened by fishing activity (e.g., the great white shark, *Carcharodon carcharias* Blower *et al.* 2012). Using molecular techniques for marine invertebrate fisheries poses many more challenges than for finfish and in many cases these systems may be particularly vulnerable to overfishing due to their intrinsic biological traits (e.g., low mobility, or poorly understood dispersal and life histories, reviewed in Thorpe *et al.* 2000).

As global efforts to mitigate the effects of overfishing, habitat destruction, anthropogenic impacts and climate change are increasing, there is a growing need for the design and implementation of marine reserves and marine protected areas (MPAs). In most cases, MPAs and MPA networks are implemented to preserve fish stocks (e.g., Blyth-Skyrme *et al.* 2006); increased fish abundance and biomass within reserves and often in neighbouring areas (i.e., spillover effects) have been repeatedly demonstrated (e.g., Goni *et al.* 2008, Garia-Charton *et al.* 2008). Threatened sessile benthic taxa are also now garnering interest, especially tropical coral reefs that have undergone rapid degradation in

the latter half of the 20th century (Knowlton and Jackson 2008). Attention is also shifting towards sessile benthic temperate taxa, many of which have poorly understood biology and are suffering threats such as habitat degradation and fragmentation, environmentally-induced disease outbreaks, overfishing and the effects of climate change and ocean acidification (e.g., Thorpe *et al.* 2000).

In the UK, commitment to international conservation treaties is being met by the implementation of a network of MPAs, which was proposed to government in December 2012 and is undergoing consultation until March 2013. Guidelines for this network highlight several sessile invertebrate taxa, including the IUCN red-listed gorgoniid *Eunicella verrucosa*, a charismatic sea fan with a limited UK distribution confined to South West England, the south west of Wales and western and southern Ireland. Basic biology concerning life history traits of this species lacks empirical data, and a genetic assessment of its population structure has never been undertaken. Therefore, to address this deficiency and address the UK MPA network guideline criterion of connectivity, we developed a panel of novel microsatellites for this species and used them to assess patterns of genetic divergence around the coasts of south west England and Wales and the North East Atlantic. We developed a second panel to assess population structure and connectivity of a second octocoral species, the alcyoniid *Alcyonium digitatum*, an animal with extremely high abundance around the British Isles, but also lacking empirical data concerning biology or population structure.

This chapter outlines the development and testing of both microsatellite panels, undertaken primarily at the NERC Bimolecular Analysis Facility at The University of Sheffield. Connectivity patterns and population structure of *E. verrucosa* and *A. digitatum* determined by the microsatellites are described in Chapters 3 and 4 respectively. The development of the microsatellite panels resulted in two primer notes, which were published in Conservation Genetics Resources in April 2013 (Appendix 1 and 2).

2.2 Materials and Methods

This section describes the development of both microsatellite panels, and laboratory-based data acquisition, sampling, and genotyping methods for the entire project. Methods concerning statistical and population genetics analyses from the resulting data will be outlined in the respective chapters for each species.

2.2.1 Site Selection

For the entire project, samples of *Eunicella verrucosa* were collected at thirty sites ranging from southern Portugal to western Ireland, including northwest France and south west England and

Wales. Samples of *Alcyonium digitatum* were collected from twenty-one sites across the same region, together with additional samples from two sites in the North Sea (Tables 3.1 and 4.1 and Figures 3.1 and 4.1).

2.2.2 Sample Collection

All octocoral samples used in this study were collected between September 2008 and May 2012, with the exception of several *Eunicella verrucosa* colonies that were collected from Skomer Marine Reserve in Pembrokeshire, Wales in 2007. All samples of *Alcyonium digitatum* were collected specifically for this research. Similarly, the majority of *Eunicella verrucosa* samples were collected specifically for the project, though some additional samples were acquired from previously collected populations in the Marseilles area of France, southern Portugal ('EvARM', Table 3.1), and several aquaria-maintained colonies originating from near Padstow on the north coast of Cornwall were provided by London Zoo ('nr Padstow', Table 3.1). Nearly all samples were collected by SCUBA diving by myself, Dr Jamie Stevens, and many volunteer divers (please see Acknowledgements); additionally, two sets of *A. digitatum* were collected from the east coast of England during bottom trawls conducted during CEFAS research cruises ('CEFAS MIX' and 'T342 CEFAS', Table 4.1). Due to depth and time restrictions imposed by SCUBA, typical depths of populations sampled were forty metres or shallower. At each site, *E. verrucosa* colonies were sampled by removing a 3 cm branch (usually terminal) using sea-snips; as this species is protected in the UK, all specimens were collected according to UK wildlife licensing laws as per the terms and conditions of Natural England licences granted to Jamie Stevens (Natural England licences 20080861 and 20090943) and subsequently by the Marine Management Organisation (license number 001). Wherever possible in sites with high sea fan abundance, we avoided small (juvenile) colonies and tried to sample from larger, apparently healthy individuals, although as we attempted to collect a minimum of forty individuals per site (in order to maximise statistical validity of genotypic data), this was not always feasible. Although in this study necrotic colonies were avoided, some sites sampled have previously reported incidences of disease in the vicinity, these are Lundy Island in the Bristol Channel, Mewstone Ledges and Breakwater Fort in Plymouth Sound and Sawtooth in Lyme Bay, Dorset (Hall-Spencer *et al.* 2007). No colonies were tagged or tracked *in situ* during this study, although most individuals collected at Skomer Marine Reserve are well mapped and known individually to park staff of the Countryside Council for Wales; therefore, genotypes of these individual colonies could theoretically be assigned to a live individual. *Alcyonium digitatum* colonies were also sampled by removing a thumb-sized branch with scissors. For both species, we attempted to sample colonies at least a metre apart, to avoid sampling ramets (duplicate genotypes) or clonal individuals; other studies have sampled

potentially clonal individuals at spatial scales from 5m apart (e.g., Goffredo *et al.* 2009; Foster *et al.* 2012) but this was not always possible, depending upon local distribution of the sea fans. For *A. digitatum*, this strategy was less obvious given that clusters or 'patches' of individuals could be found in close proximity to each other (whereas *E. verrucosa* colonies are often more sparsely aggregated). Distribution of samples was also highly site-specific and dive time limitations meant that collecting populations over a wide bottom area was not always possible. Furthermore, given ambiguity over reproductive strategies employed by the two species, and their unknown pelagic larval durations and potential distances of larval dispersal (see 'Introduction' sections in Chapter 3 and Chapter 4), we appreciate that this method was a somewhat crude means to avoid collecting duplicates. Therefore, we subsequently relied on the detection and removal of duplicate haplotypes prior to statistical analysis following genotyping (see below). During sample collection, individuals were placed into mesh dive bags and immediately after collection, either on the boat, on the dock or in the laboratory, were divided into batches of up to ten individuals (depending upon the size of the specimen taken) and placed into 50ml falcon tubes containing 100% ethanol to approximate a 1:5 – 1:10 volume of sample tissue to alcohol. In the case of some larger *A. digitatum* fragments, incisions were made into the tissue (taking care not to cut through the sample), to maximize entry of ethanol into the internal tissues. Several sets of donated samples appeared to be degraded and thus when conducting my own sampling this step was carried out to avoid endonuclease activity prior to DNA extraction. Wherever possible, ethanol was changed within twenty-four hours of collection, and was usually replaced twice in order to reduce levels of degraded and 'mucousy' material. Samples were then catalogued, recorded in the project database and placed individually into glass vials containing 100% ethanol, which were then stored in cold room facilities at The University of Exeter at 4^0 C pending analysis. In some instances, a small amount of tissue was removed straight after processing and placed in 1.5ml microcentrifuge tubes containing ethanol during cataloguing of samples for immediate DNA extraction. Following initial processing, timing of subsequent analysis did not appear to have affected success of molecular analysis, suggesting that our preservation and storage protocol is robust.

2.2.3 DNA Extraction

Wherever possible, DNA was extracted from approximately 10-20 whole octocoral polyps that were plucked manually from each colony using forceps. If polyps had retracted following collection and their removal from calyces was impossible, scalpels were used to shave a slice of either $1cm^2$ (approximately) of surface tissue from *Alcyonium digitatum*, or coenenchymal tissue (excluding the gorgonin axis) of 1-2cm in length from *Eunicella verrucosa*. Shavings were inspected visually to verify

presence of at least 10-20 polyps per cross section or calyces per slice to ensure animal tissue was present for digestion in addition to the proteinaceous skeletal matter.

Genomic DNA was extracted from both octocoral species using the Wizard® SV Genomic DNA Purification System (Promega Corporation, Madison, Wisconsin) according to the manufacturer's protocol for animal tissues using a microcentrifuge. Briefly, 275 µl of a prepared 'digestion solution master mix', containing 200µl nuclei lysis solution, 50µl 0.5M ethylenediaminetetraacetic acid (EDTA, ph 8.0), 20µl proteinase K (20mg/ml) and 5µl RNase A solution (4mg/µl), was added to each sample in a 1.5ml microcentrifuge tube and samples were incubated overnight in a 55^{0}C water bath. The next morning, samples were centrifuged at 2,000g for 2 minutes to pellet undigested coenenchymal tissue and the supernatant transferred to a fresh microcentrifuge tube containing 250µl of Wizard® SV Lysis Buffer. Following a 15-second vortex, the lysate was transferred to a Wizard® SV Minicolumn assembly and centrifuged at 13,000g for 3 minutes. *Alcyonium digitatum* extractions usually had residual lysate on the column at this stage, so were spun again at 13,000g for a further minute following the manufacturer's suggestion. The minicolumn was then transferred to a fresh microcentrifuge tube and washed four times by centrifuging the sample with 650µl Wizard® SV Wash Solution at each step. Finally, the minicolumn was dried by centrifuging at 13,000g for 2 minutes and DNA was eluted twice into 50µl of nuclease-free water warmed to 65^{0}C, resulting in an elution of total volume 100µl. This elution volume is significantly less than the recommended total amount of 500µl. However, I found these volumes to be too weak for genotyping, so reduced it significantly to increase DNA concentration (at the expense of overall yield) and added the step of heating the elution medium (nuclease-free water) to 65^{0}C, following the manufacturer's suggestion that this would help to maximise DNA yield. The elution was left at room temperature for approximately ten minutes, and divided into two aliquots of 50µl. DNA was stored in -20^{0}C in freezers in the Biosciences department at The University of Exeter, or in the Department of Plant and Animal Sciences at The University of Sheffield.

2.2.4. DNA Quantification

Following extractions, 1.5µl of genomic DNA was quantified using a FLUOstar OPTIMA plate-reading fluorometer, using either Quant-iT™ PicoGreen® dsDNA (Invitrogen) fluorescent nucleic acid strain (diluted 39µl into 20ml low TE buffer (10 mM Tris, pH 8, 0.1 mM EDTA), of which 150µl was added to per sample, or 200µl of Hoescht dye (1µg/ml in 1xTNE buffer, which is 50 mm Tris–HCl [pH 7.4] 100 mm NaCl, 0.1 mm EDTA). Each assay was standardised against calf thymus standards and a blank (the elution buffer, Promega nuclease-free water); standards were typically 1.5µl each of 0, 1.0625,

3,125, 6.25, 12.5, 25, 50, and 100ng/µl. Standard curves were calculated and a best-fit linear regression was plotted through standards. DNA concentrations varied widely; poor DNA yields were more common for *Alcyonium digitatum* (less than 10ng/µl) than for *Eunicella verrucosa,* although for both species, concentrations above 50ng/µl were considered to be high. Ranges were typically between 20-40ng/µl (data not shown). Following DNA quantification, aliquots were taken from stock solutions, diluted to working concentrations of 10ng/µl and transferred by population into deep-well plates ready for downstream PCR pipetting.

2.2.5 Isolation of Microsatellites

Microsatellites were developed and tested for both species at the NERC Biomolecular Analysis Facility (NBAF), Department of Animal and Plant Sciences, University of Sheffield, where all genotyping and laboratory work (with the exception of DNA extractions) was also undertaken over several visits between 2009 and 2012. Additionally, a further microsatellite library was developed for *Eunicella verrucosa* at the Evolutionary Genetics Core Facility of Cornell University between October 2008 and March 2009; loci from this library were also tested in Sheffield and added to microsatellites developed at NBAF to complete the final multiplexes selected. All libraries were constructed from DNA obtained from one individual. For *E. verrucosa*, the individual was collected from East Tenants Reef, Lyme Bay, Devon in September 2008 (50 39.143 N, 02 52.728 W). For *A. digitatum*, the individual in question was collected from the Volnay Wreck in the Manacles area off the Lizard Peninsula, Cornwall, in March 2008 (50 04.372 N, 04 59.802 W). A small (2cm) branch clipping was used to extract DNA from *E. verrucosa* with a Qiagen Plant Mini kit according to manufacturer's instructions (Qiagen), whereas a Wizard Kit (Promega) was used to extract DNA from *A. digitatum* following the manufacturer's animal tissue protocol (Promega). Presence of high-molecular weight genomic DNA was verified on 2% agarose gels (in TBE [tris base, boric acid and EDTA) and quantified with a Nanodrop 2000 (ThermoScientific) and subsequently shipped on ice to Cornell and Sheffield facilities for library construction (*Eunicella verrucosa*: 16.5ng/ul, A260:A280 = 1.77, *Alcyonium digitatum* 14.5ng/ul, A260:A280 = 1.71)

The respective protocols used for microsatellite development by NBAF and Cornell are described briefly here. At Cornell, microsatellites were developed for *E. verrucosa* only, following a modified protocol of Hamilton *et al*. (1999). Using the restriction enzymes *BsaA I* and *Hinc II*, DNA was digested and an SNX linker was attached to the resulting blunt-ended fragments. The DNA mixture was then enriched for repeats by hybridization to the following 3'-biotinylated oligonucleotides: (GT)8, (TC)9.5, (TA)15, (TTA)11, (GTT)6.33, (TTC)7, (GCT)4.33, (GAT)7, (GTA)8.33, (GTG)4.67, (TCC)5,

(TTTA)8.5, (TTTG)5.25, (TTTC)6, (GATA)7, (GTAT)6.25, (GAAT)5.5, (GATT)5.5, (GTTA)6.25, (TTAC)6.75, (GATG)4.25, (GGTT)4, (GCTT)3.75, (GTAG)4.5, (GTCA)4.25, (GTTC)4, (TCAC)4.25, (TTCC)4.25, after which fragments were captured on streptavidin-coated magnetic beads. Polymerase chain reaction (PCR) was used to make the fragments double-stranded using the SNX forward primer, purified, and digested with *Nhe*I prior to cloning into *Xba*I-digested, dephosphorylated pUC19. Colonies were grown on ampicillin-containing Luria-Bertani agar plates, and replicated onto nylon membranes prior to probing with radiolabelled oligonuceleotides used in enrichment. Positive clones were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with a universal M13 forward primer flanking the cloning site. Reactions were cycled at 94°C for 50 sec, 56°C for 20 sec, 60°C for 4 min (x35) and were analysed using an ABI 3100 or ABI 3730xl automated sequencer. Sequences were trimmed of vector and linker sequences and checked for contigs (CodonCode Aligner v 2.0.3); unique singletons, or one sequence from an assemblage of contigs, were exported as FASTA files. These were then sent to Exeter and I continued from this step with primer design (see below). At NBAF, libraries were developed for both species using a modified protocol of Armour *et al.* (1994), which digests DNA with MboI (Promega) before ligation with the linkers (Sau-L-A and Sau-L-B). DNA fragments with linkers were then size selected from agarose gels (250-750bp) and enrichment for the microsatellite motifs $(GT)_n$, $(CT)_n$, $(GTAA)_n$, $(CTAA)_n$, $(TTTC)_n$ and $(GATA)_n$ and their complements was carried out. These probes are bound to magnetic beads following Glenn and Schable (2005), and enriched DNA is recovered via PCR using Sau-L-A as a primer. Fragments were cloned into a plasmid using a TOPO vector, grown on agar plates and sequenced bi-directionally using Sanger sequencing ABI BigDye v3.1 and ABI 3730 at the NBAF Facility Edinburgh at the University of Edinburgh.

All primers were designed using the program Primer3 (Rozen and Skaletsky 2000), and in some cases OligoCalc v3.25 (Kibbe 2007) was also used to check the suggested primers for self-complementarity. Ideal primer properties including incorporation of a GC clamp of 3 base pairs at the 3' end, length of around 18-24bp, a high CG content, similar melting temperatures for the forward and reverse primer, and avoidance of hairpin bends, self annealing or self complementarity were taken into consideration to maximize efficient specificity (e.g., Dieffenbach *et al.* 1993). However, short flanks or GC content of singletons meant that this was not always possible; for example (the forward primers of 'tet_67/EverEXE_41 is directly adjacent to the repeat motif and 'tet_69/EverEXE_42' has a potential hairpin bend). In this case, primers were designed as optimally as feasible. All of the unique FASTA sequences tested from both libraries were submitted to the European Molecular Biology Laboratory repository on completion of the screening process and are numbered with the

accession numbers HF677589–HF677704 (*Alcyonium digitatum,* 116 sequences) and numbers HF677589–HF677704 and HF913257–HF913263, HF913265–HF913269, HF913383–HF913422 (*Eunicella verrucosa* library one, from Cornell, 52 sequences) and HF913256, HF913264, HF913270–HF913382 (*E. verrucosa* library 2, from NBAF Sheffield, 115 sequences).

2.2.6 Testing and Selecting Microsatellite Loci

For *E. verrucosa*, 74 singletons in total were obtained from the Cornell microsatellite library and 115 from the NBAF library. Thirty primer pairs were designed from the NBAF library and 33 from the Cornell library. After initial screens and testing, an additional set of 19 was designed from the Cornell library – resulting in 82 primer pairs designed, of which 76 were eventually tagged with FAM/HEX fluorescent dyes (Applied Biosystems) and tested. For *Alcyonium digitatum*, 116 unique FASTA files were obtained from NBAF. Initially, 30 primer pairs were designed from these, and subsequently, an additional batch of 24 pairs was designed, all of which were FAM/HEX labelled and tested; therefore 54 primer pairs in total were screened in *A. digitatum*. All primers were arranged into several batches of duplexes with one FAM and one HEX in each which were tested sequentially in four individuals from the same population for *E. verrucosa* (e.g., 'Isles of Scilly nnw Flat Ledge' individuals 17, 18, 19 and 20 for initial duplexes and various individuals from West Tennents reef for subsequent batches, DNA stocks permitting), or four individuals from the same population for *A. digitatum* (e.g., 'Manacles Cairn Du' individuals 37, 38, 39 and 40 for initial duplexes followed by 8 individuals from the same population for subsequent duplexes).

Duplexes were designed manually (in Excel) so that wherever possible, FAM and HEX primers with similar melting temperatures but different sizes (based upon expected fragment sizes) were placed together, in order to avoid potential issues with bleedthrough and flashover, and to avoid mis-interpretation of allelic diversity from primers dyed with the same fluorescent tag. Ultimate multiplexes and duplexes are shown in Tables 2.3 and 2.4 for *E. verrucosa* and *A. digitatum* respectively.

Template DNA was diluted into a minimum of 10ng/µl concentrations, 1µl of which was dried on PCR plates and amplified in a 2µl PCR reaction containing 0.2 µM of each primer and 1µl QIAGEN multiplex PCR mix (QIAGEN Inc.; Kenta *et al*. 2008). PCR reactions were conducted using a DNA Engine Tetrad 2 thermal cycler (MJ Research, Bio-Rad, Hemel Hempstead, Herts., UK) and for all primer pairs designed at NBAF, the following profile was used ("QMix58"): incubate at 95°C for 15 minutes, followed by 44 cycles (94°C for 30 seconds denature, 58°C for 90 seconds anneal, 72°C for

90 seconds extension), and a final extension of 60°C for 30 minutes. This profile was used due to uniformity of primer melting temperatures in these sets. Duplexes designed from the Cornell library had a more variable range of melting temperatures, for this reason a 'touch-down' profile was used when testing them ("JenTD"): incubation at 95°C for 15 minutes, then 15 cycles of 94°C for 30 seconds denature, 65°C for 90 seconds anneal, 72°C for 60 seconds extension decreasing by 1°C each cycle, followed by 27 cycles of 94°C for 30 seconds, 50°C for 90 seconds, 72°C for 90 seconds, ending with a final extension of 72°C for 10 minutes. PCR products were diluted prior to genotyping using an automated ABI 3730 48-well capillary DNA Analyser (Applied Biosystems, California, USA). Amplification success and allele sizes were measured using GENEMAPPER v3.7 software (Applied Biosystems, California, USA).

At the four-individual testing stage using the first 15 duplexes, amplification success rate was particularly poor for *A. digitatum*, so I then tested all duplexes again on 24 individuals from the same population (Manacles, Carn Du) to get a better idea of how successful and variable primers would be and if they might be more successful if repeated in more individuals. Selected duplexes were repeated for the same reason for *E. verrucosa*, in 24 individuals from the same population (Isles of Scilly nnw Flat Ledge). Usual protocol at NBAF suggests that at this stage, a 24 individuals in a population likely to be in Hardy-Weinberg equilibrium is used to screen initial microsatellites; this allows deviations from these expectations to be used as a measure of how useful the microsatellite itself may potentially be (as opposed to an 'established' available marker that would itself be trusted to infer that the population as opposed to the marker deviates from HWE). However, given the poorly understood biology of both species with respect to the reproductive and life history properties of each, a complete lack of information about migration between populations in my sampling region and, at this stage, limited availability of samples, I selected a 'typical' *A. digitatum* population from the Manacles, Cornwall (typical in terms of knowing it was not an isolated site and I had samples from nearby) and the *E. verrucosa* population West Tennents Reef that theoretically could be both a source and a sink in the UK (in terms of known ocean currents), and which was not at the limits of the known westerly or easterly range of the species in British waters.

2.2.7 Data Preparation: *Eunicella verrucosa*

All genotyping for *Eunicella verrucosa* was done using three multiplexes comprising 18 loci (Table 2.3). From these, four loci were impossible to score reliably due to practical problems (incompatible bleedthrough / flashover signal or consistently bad amplification), resulting in a final data set of 14 loci which were scorable and suitable for further analysis. To avoid spurious results from missing

data, initially individuals for which more than 6 out of 14 loci had failed to amplify (5 failures allowed, 36%) were removed from the final data matrix. This resulted in 1055 individuals from 30 different sites around South West England and Wales, western Ireland, North West France, Portugal and the Mediterranean (Figure 3.1, Table 3.1). Some analyses were subsequently conducted with more stringent screening, in which an individual with a failure rate of 4 or more loci out of 14 was excluded from the data matrix, i.e., 3 failed (no amplification) loci or less (21%) were kept in the data matrix – this resulted in a revised data set comprising 979 individuals.

After removal of individuals with 4 or 6 missing loci, the occurrence of duplicate genotypes was tested for using the 'Identity Analysis' option in Cervus v3.0.3 (Kalinowski *et al.* 2007), allowing for no mismatching loci. This would give a measure of clonality and occurrence of individuals potentially resulting from asexual reproduction within each population. In the most stringent data set (4 or more failed amplifications), from 478,731 pairwise comparisons of 979 individuals, 28 exact-match genotypes were detected – in some cases this included unequal amplification success in the two individuals (e.g., data from 12 loci from one individual that exactly matched data from 10 loci that amplified successfully in the second individual would count as a 100% match). If duplicate genotypes occurred within the same population, all but one were removed, resulting in a final data matrix comprising 955 individuals of which two were an exact match ('EvArm_22' and 'Faro2_10', from separate but adjacent sites in southern Portugal approximately 4km distant).

Using this data matrix, evidence for the presence of null alleles, stuttering and large allele dropout was checked for in all populations using Microchecker v2.2.3 (van Oosterhout *et al.* 2004), with a maximum allele size set at 400bp, a 95% confidence interval and 1000 iterations. Input GENEPOP files suitable for import into Microchecker were generated in Cervus v3.0.3 (Kalinowski *et al.* 2007) and were Bonferroni corrected in that program (Rice 1989); therefore, this correction was not re-applied in Microchecker. This program omits missing data from analyses and, as allele sizes in this dataset did not conform to expected sizes for perfect motif repeats, analyses were conducted including suspect data. Results from this analysis are presented in Table 3.2.

2.2.8 Data Preparation: *Alcyonium digitatum*

Using a stringent dataset, where the failure allowance threshold was set at two out of eleven loci, the occurrence of duplicate genotypes was tested using the 'Identity Analysis' option in Cervus v3.0.3 (Kalinowski *et al.* 2007), allowing for no mismatching loci or fuzzy matching. Out of 666 genotypes, eleven were identical to another haplotype; in this case all identical pairs happened to be from the same populations as each other and therefore one of the pairs was removed from the data, resulting in a final data matrix of 655 individuals. Using this data matrix, evidence for the presence of null alleles, stuttering and large allele dropout was checked using Microchecker v2.2.3 (van Oosterhout *et al.* 2004) as above.

2.3 Results

2.3.1 Numbers of Usable Loci

2.3.1.1 *Eunicella verrucosa*

For *Eunicella verrucosa*, 18 polymorphic microsatellite loci were identified and tested in all populations in three mutliplexes with a mixture of 6FAM / HEX fluorescent dyes or labelled with a 4dye kit (Applied Biosystems). However, similar allele sizes and repeated problems with bleedthrough (despite template dilutions and adjusted injection times of the ABI 3730) for some of the loci meant that the data was incomprehensible and they were dropped from all further analyses; therefore, a panel of fourteen usable loci was the final result from microsatellite development in this species (resulting in three PCR reactions required per individual). Final selection is shown in Table 2.1.

2.3.1.2 *Alcyonium digitatum*

For *Alcyonium digitatum*, eleven loci were identified. These were also multiplexed with a 3/4/dye kit (ABI), but significant failures in amplification with primers labelled this way, large allele sizes or potential problematic primers (e.g., potential anneals between them) resulted in poor returns and unreliable reads; therefore, all *A. digitatum* loci were run as 6FAM/HEX duplexes in all populations (i.e., 6 PCRs per individual). Eleven loci were selected for final analysis; these are shown in Table 2.2.

Table 2.1 following page: Characterisation of fourteen *Eunicella verrucosa* microsatellite loci (for details please refer to Appendix 1). T_m, primer melting temperature, [§], a touchdown PCR program was used (see above for details); Size range, Observed allele size range (bp) within the amplified individuals; *N*, number of individuals amplified and successfully genotyped (of 44 tested); *K*, number of alleles; H_O, Observed heterozygosity; H_E, Expected heterozygosity; Estimated null allele frequency (calculated using CERVUS v3.0.3, Kalinowski *et al.* 2007); P_{HW}, probability for Hardy–Weinberg equilibrium calculated using GENEPOP v.4.2 software (Raymond and Rousset 1995; Rousset 2008), asterisk indicates significant values ($p<0.05$) after correcting for multiple tests using false discovery rate (Benjamini and Hochberg 1995).

Locus	Clone / EMBL Accession No.	Repeat motif	Fluoro-label	Primer sequences (5' – 3')	T_m (°C)[§]	Size range (bp)	*N*	*K*	H_O	H_E	Est. null Allele fq	P_{HW}
Ever001	PSFShef01C02	$(AATG)_{17}$	HEX	F:ACTGCAACTGTTCATCGTCAG	59.0	237–269	43	9	0.51	0.66	0.13	0.01*
	HF913256			R:AAACTAGCCGGTCTATAACTCTCG	59.4							
Ever002	PSFExe15_tet1	$(TTAC)_6$	HEX	F:ATGTTGAGCTGCGTCCTTCGC	67.1	105–117	43	4	0.33	0.36	0.02	0.10
	HF913257			R: GTACAATCGAGTGGGTGTGC	59.0							
Ever003	PSFExe21_tet16	$(TTGA)_8$	HEX	F:TCTCGCAGAACTATCGCCG	62.6	170–182	44	4	0.16	0.19	0.17	0.15
	HF913258			R: AGTTATCAGTGTTCATGACTCG	54.5							
Ever004	PSFExe33_tri22	$(CAA)_{12}$	6FAM	F:CAACAATGAAAGCGGCAACAGC	67.0	148–183	44	9	0.68	0.66	-0.05	0.78
	HF913259			R: CATCTTCGACACCTTCATCC	58.1							
Ever005	PSFExe41_tet67	$(GATT)_6$	6FAM	F:GCAACTTGGTTTTAATAAACG	54.4	230–235	44	2	0.05	0.05	-0.00	1.00
	HF913260			R: GACGATGATGTTAAGAGCGGG	61.9							
Ever006	PSFExe49_tri38	$(GTT)_{22}$	6FAM	F:GCCGTTGGTGGTATCTATGG	60.2	353–394	44	9	0.66	0.82	0.11	0.02
	HF913261			R: GTTGTTTTAGAGCGACAGCAGC	61.5							
Ever007	PSFExe10_di_17	$(GA)_{10}$	6FAM	F:GGTAACAAACTTAGCACAGC	52.8	226–242	42	5	0.55	0.58	0.03	0.51
	HF913262			R: GCTAATAATGAGCCAATCACCC	59.8							
Ever008	PSFExe48_tri33	$(GAT)_6$	6FAM	F:CATTGTCCCTGTATCGATGG	58.8	151–154	38	2	0.61	0.50	-0.10	0.95
	HF913263			R: ATTTTCGTTTTTCGGGATCCC	63.3							
Ever009	PSFShef06E10	$(AATC)_8$	6FAM	F:ATACAAGTTCTGGTGGCATGG	59.9	92–131	43	9	0.44	0.66	0.20	0.00*
	HF913264			R:CCCTCCTGTAAATCAGCATATTG	59.9							
Ever010	PSFExe17_tet3	$(TATC)_{13}$	6FAM	F:GCATAATGACTCTGTCAATGTC	54.8	240–253	44	4	0.14	0.13	-0.03	1.00
	HF913265			R: CTTCTCATAGACGGTTTTATACAC	54.2							
Ever011	PSFExe34_tet28	$(TCAA)_7$	VIC	F:GGGCCACAAATTTATCAGCAGC	64.9	144–153	44	3	0.46	0.42	-0.05	0.82
	HF913266			R: CTTGGAATAAAGCCAAAAATGC	60.0							
Ever012	PSFExe47_tri32	$(GAT)_6$	6FAM	F:AAACGTAGGCACCAAGATGG	60.0	204–210	35	3	0	0.11	0.77	0.00*
	HF913267			R: TGGCTGCGAGGTATTATCTG	59.0							
Ever013	PSFExe50_tri45	$(CAA)_{14}$	PET	F:CAAAAACGACAACAGCAACGG	63.8	127–164	44	10	0.75	0.79	0.02	0.37
	HF913268			R: CATCGTCCTAATTGTTGGTGG	60.2							
Ever014	PSFExe24_tri1	$(GTT)_4$	6FAM	F:GCTTGTAGTGGTTGCCGTCG	64.0	317–357	44	5	0.73	0.70	-0.02	0.64
	HF913269			R: CGAACATTCTCACAGTTGATTGGC	65.1							

2.3.2 Final Panels In Comparison With Other Octocoral Microsatellites

In order to compare microsatellites generated in this study with other research, properties of all microsatellites isolated from octocorals in studies published to date were summarized; in total, this comprises twelve panels in ten species including those from this study (Table 2.5). Panels have been described from three Mediterranean species, two from the Caribbean and three from the Pacific; *Eunicella verrucosa* and *Alcyonium digitatum* represent the first temperate NE Atlantic octocorals and the only anthozoans in the British Isles to have microsatellite markers developed for them (besides the anemone *Nematostella vectensis,* an introduced species only found in estuarine environments in the south and south east of England, Darling *et al.* 2006). At the time of writing, no polar octocorals appear to have microsatellites or other genetic markers published yet.

Numbers of loci isolated from octocorals vary between two and fourteen per species with an average of eight usable loci retained; from my experience, this is a low number compared to the initial numbers of positive clones isolated or potential sequences identified during the development and screening process. For example, where data are available, from the number of primers tested as few as 5% may be usable (Liu *et al.* 2005) to a maximum of 54% (Mokhtar-Jamai *et al.* 2010). In several cases, octocoral microsatellites are initially so few in numbers that several libraries may be combined to obtain sufficient numbers of polymorphic loci (LeDoux *et al.* 2010, Liu *et al.* 2005b, Mokhtar-Jamai *et al.* 2010, this study for *Eunicella verrucosa*). Furthermore, where papers proceed the publication of a primer note, some loci prove unusable when tested in a larger dataset, therefore numbers of loci in population genetic assessments of some species are even lower than in the corresponding primer note (e.g., Andras *et al.* 2013, Costantini *et al.* 2007a and 2007b, Yasuda *et al.* 2008). Overall, there does not appear to be a clear relationship between numbers of primers tested and numbers of final usable loci; in this study, the return rate for *E. verrucosa* was approximately 18% (between both libraries) and was 20% for *A. digitatum*, whereas in the blue coral *Heliopora coerulea*, a 191 primer set yielded a 6% return of eleven loci. There is not enough data at this stage to determine if success at isolating microsatellites in octocorals is taxonomically correlated; a similar return (26%) was obtained for *Eunicella singularis* (Cataneo *et al.* 2010) to *E. verrucosa*, although reasons for this may be coincidental and with different laboratories using different enrichment approaches or probes, it is difficult to determine if similarity is based upon methodology or genomic characteristics.

In terms of motifs, it is clear that octocorals may contain some complicated, long, and imperfect repeats, for example, *Corallium lauuense* (Baco *et al.* 2006) and *Heliopora coerulea* (Yasuda *et al.* 2008). The *Alcyonium digitatum* panel includes three penta-nucleotide repeats, whereas *Eunicella verrucosa* did not contain any and had a high proportion of tetra-nucleotide repeats (7/14). A penta-nucleotide was also reported in *Gorgonia ventalina* (Andras and Rypien 2009). Numbers of alleles per locus also varied between species (although as the data in Table 2.5 comes from primer notes, it is worth pointing out that in some cases only a few individuals were tested and therefore the entire range of allelic diversity may be under-represented). In the Plymouth Mewstone Ledges population presented in the *E. verrucosa* primer note, between 2-10 alleles were found per locus (N=44), a similar number to *E. singularis* (2-9, Cataneo *et al.* 2010; in the current study between 1-8 alleles/locus in *E. singlularis* were found (N=12); see Table 2 in the primer note for *E. verrucosa,* Appendix 1). For *A. digitatum*, 3-27 alleles / locus were found among the Isles of Scilly Trenemene population (N= 42). It appears that *A. digitatum* has a higher allelic richness than *E. verrucosa*, and that (from limited data), *Eunicella spp.* are on the lower end of allelic richness in octocorals genotyped with microsatellites so far.

Table 2.2 (following page) *Tm* primer melting temperature (calculated using Primer3), "ManV1**"*** Expected / Observed: allele sizes expected based on the individual cloned and sequenced and genotyping of the individual colony from which the library was created (id: ManV1), *k* number of alleles, *N* number of individuals successfully amplified and genotyped (of 42 tested), H_O Observed heterozygosity, H_E Expected heterozygosity, estimated null allele frequency (calculated using CERVUS v3.0.3, Kalinowski *et al.* 2007), $_PHW$* *p*-value for HWE test calculated using GENEPOP v.4.2 software (Raymond and Rousset 1995; Rousset 2008). Asterisk indicates significant values ($p<0.05$) after correcting for multiple tests using the false discovery rate correction (Benjamini and Hochberg 1995).

Locus	Clone name	Repeat motif	Fluoro label	Primer sequences (5'-3')	Tm°C	"ManV1"* Exp/Obs size	Obs allele size (bp)	K	N	H_O	H_E	Est.null allele fq.	$_PHW$*
Adig001	Adig85D10	$(TG)_8$	HEX	F: ACATACTCGGCTCATACTCGTG	59.3	124/	114–129	7	42	0.36	0.41	+0.0901	0.096
				R: CTCGTGCTCACAGACAAACAC	59.5	118, 122							
Adig002	Adig86B02	$(TG)_{12}$	HEX	F: GACTGGAGATTATGTTTTCATCG	57.8	219/	216–237	9	42	0.69	0.68	-0.0012	0.4
				R: ACGCATTCAGTTTTCTCTAACC	57.6	212, 220							
Adig003	Adig87B11	$(GT)_9$	HEX	F: TTTCAATTGTGCTACTGTTTGG	57.9	146/	136–152	6	41	0.34	0.67	+0.2990	0*
				R: TTGACGTTTCTAATTGCAATACC	58.3	145							
Adig004	Adig87E02	$(ATCT)_8$	6FAM	F: GACCTATGACGCATGCTCTG	59.4	129/	126–205	13	32	0.28	0.85	+0.5049	0*
				R: CGGATGCGAATTCTTTATCTTAC	59.2	126							
Adig005	Adig87H01	$(ACACA)_9$	6FAM	F: ATGACACGATAAACCCAAACG	59.7	116/	74–175	18	42	0.88	0.92	+0.0173	0.522
				R: ATTTGTTGTGTTGTGCTGTGC	59.7	89, 114							
Adig006	Adig87H03	$(TAT)_8$	HEX	F: CTTATTTGTAAAGCGCCTTGAAC	59.4	142/	130–142	3	42	0.21	0.20	-0.0488	1
				R: AAGCACGCTTCAAGACAACTC	59.4	142							
Adig007	Adig88A04	$(ATGGT)_5$	6FAM	F: GTGGTGTGGTAGTGGTGTGG	59.8	176/	166–202	4	41	0.07	0.07	-0.0096	1
				R: GCGTGTTCGGACTACATAGC	58.4	177, 182							
Adig008	Adig88C09	$(TACA)_{22}$	HEX	F: TTGTGGAAATCAACTATTTCTGTTG	59.5	179/	95–220	24	42	0.98	0.95	-0.0176	0.652
				R: TCTGTGACTGGTTCATATTGTGG	59.9	161, 177							
Adig009	Adig88E08	$(TGTTG)_{17}$	6FAM	F: GTGTTTCATCACCCTTGCAG	59.1	249/	196–258	13	39	0.82	0.90	+0.0424	0.326
				R: CCGACGATTGTTATAAAGATTAAATG	59.3	242, 253							
Adig010	Adig88E09	$(ATCT)_{16}$	6FAM	F: CAGTTTCTACTGCAATGGTTATTC	57.1	122/	111–264	27	30	0.87	0.96	+0.0434	0.196
				R: ATTAGGGAAACAAGCTTCGAC	57.5	120, 167							
Adig011	Adig89C09	$(TGT)_{11}$	HEX	F: GGTCGGGTTAGACGTGGAG	60.5	118/	98–169	16	41	0.76	0.70	-0.0606	0.644
				R: GTCACTCGAACTCGGCATTC	60.8	101, 120							

Table 2.3 Eighteen loci multiplexed and genotyped in all *Eunicella verrucosa* samples (clone names given). Multiplex names 10, 11 and 12 correspond to the multiplex combination being tested and have no other significance; therefore multiplex 1, 2 and 3 are replacement names. Multiplex 1 is 6FAM™ (blue) / HEX™ (green) only and therefore genotyping was done with reference to the Genescan™ 500 ROX ® size standard as follows: (-35, -50), 75, 100, 139, 150, 160, 200 (-250), 300 (-340), 350, 400, 450, 490, 500bp, Applied Biosystems). Multiplexes 2 and 3 included the 4-dye kit 6FAM™ (blue), VIC® (green), NED™(yellow/black) and PET® (red) dyes, Applied Biosystems) and were genotyped with reference to the Genescan™ 500 LIZ ®size standard (as above, Applied Biosystems). * Exe24 was attempted in two multiplexes, as a 6FAM™ and a HEX™ – best results were obtained from Multiplex 2 and therefore data from Multiplex 1 were discarded. Unreliable reads, problems with bleed-though and flashover resulted in the loss of data from the loci 06F03, Exe29, Exe44 and Exe35; these loci were selected from initial screens and are likely to be informative markers. Size ranges and allele numbers are from the entire *E. verrucosa* dataset (N=955), this data for the above discarded loci stems from initial screens and probably unlikely represents the full extent of allelic polymorphism.

***Eunicella verrucosa* Multiplexes**

Multiplex 10*				
Multiplex 1 / 10*				
Locus	**Dye**	**MOTIF**	**Size Range**	**No. Alleles**
01C02	HEX	(AATG)17	233-274	15
Exe15	HEX	(TTAC) 6	105-121	5
Exe21	HEX	(TTGA) 8	166-182	5
Exe33	6FAM	(CAA)12	148-183	13
Exe41	6FAM	(GATT)6	230-239	3
Exe49	6FAM	(GTT) 22, imperfect	337-394	18
Exe24*	HEX	(GTT) imperfect	317-364	9
Multiplex 2/ 11				
Locus	**Dye**	**MOTIF**	**Size Range**	**No. Alleles**
06E10	6FAM	(AATC)8	92-176	18
Exe17	6FAM	(TATC) 13, imperfect	237-266	9
Exe34	VIC	(TCAA) 7	140-157	5
Exe47	6FAM	(GAT) 6	204-217	5
Exe50	PET	(CAA) 14	127-170	14
06F03	NED	(GA)8	147-153	2
Exe24*	6FAM	(GTT) imperfect	317-364	9
Multiplex 3 / 12				
Locus	**Dye**	**MOTIF**	**Size Range**	**No. Alleles**
Exe10	6FAM	(GA) 10	223-260	11
Exe48	6FAM	(GAT)6	151-154	2
Exe29	NED	(GAT) 26, imperfect	125-167	9
Exe44	VIC	(TGA), 6 imperfect	139-146	2
Exe35	PET	(GTAT) 7	141-151	2

Table 2.4 Twelve loci duplexed and genotyped in all *Alcyonium digitatum* samples. Locus names here correspond to clone names, for 'new' names please refer to Table 2.2. All duplexes (or the two single-plexes) were tagged using 6FAM™ (blue) or HEX™ (green) and therefore genotyping was done with reference to the Genescan™ 500 ROX ® size standard as follows: (-35, -50), 75, 100, 139, 150, 160, 200 (-250), 300 (-340), 350, 400, 450, 490, 500bp, Applied Biosystems). Please note some loci were tested in several duplexes indicacted by *. Adi88G01 was impossible to score and amplified unreliably and was therefore discarded, and final genotyping was done using eleven loci (please see the *Alcyonium digitatum* primer note, Appendix 2).

***Alcyonium digitatum* Duplexes**

Duplex	Duplex Name	Locus	Dye	MOTIF	Size Range	No. Alelles
Splex,1	JanMmix1	Adi88E08*	6FAM	$(TGTTG)_{17}$	181-278	25
Splex, 1	JanMmix1	Adi87B11*	HEX	(GT)9	136-157	9
2	JanMmix2	Adi88A04	6FAM	$(ATGGT)_5$	166-202	5
	JanMmix2	Adi89C09	HEX	$(TGT)_{11}$	81-169	25
3	JanMmix4	Adi85D10	HEX	$(TG)_8$	114-140	12
	JanMmix4	Adi88G01	HEX	(GCGTGTGC)	157-216	19
4	JanMmix7	Adi87E02	6FAM	$(ATCT)_8$	118-318	39
	JanMmix7	Adi88C09	HEX	$(TACA)_{22}$	87-258	36
5	JanMmix9	Adi87H01	6FAM	$(ACACA)_9$	74-180	23
	JanMmix9	Adi87H03	HEX	$(TAT)_8$	130-152	7
6	Mplex26	ADI86B02	HEX	$(TG)_{12}$	210-241	14
	Mplex26	Adi87B11*	HEX	$(GT)_9$	136-157	9
	Mplex26	Adi88E09	6FAM	$(ATCT)_{16}$	99-264	43

Table 2.5 Summary of all microsatellites isolated from octocorals to date. # loci per paper refers to those used in subsequent publications, * is when several authors use the same loci (refer to Reference column for details).

Area	Taxonomy	Species	# Loci (Primer Note)	# Loci (Paper)	Motifs (as published)	Allele Size Range / Largest Motif Range*	Alleles per Locus	# Positive Clones, # of Loci Sequenced, # Retained	Success (%) of primers tested	Reference
NE Atlantic	S.O. Holaxonia F. Gorgoniidae	*Eunicella verrucosa*	14	n/a	(GA) (GTT: 2 loci, one interrupted) (CAA: 2 loci) (GAT: 2 loci) (AATG) (AATC) (TTAC) (TTGA) (GATT) (TATC) (TCAA)	92-394 / 353-395 (tri)	2-10	Attenpt 1: 115 +ve clones sequenced, 30 primer sets designed and tested, 2 loci retained. Attempt 2: 74 unique microsatellites sequenced, 52 primer sets designed, 46 of which labelled and tested, 12 loci retained	7 attempt 1 26 attempt 2	Holland *et al.* 2013b, this study
	S.O. Alcyoniina F. Alcyoniidae	*Alcyonium digitatum*	11	n/a	(GT) (TG: 2 loci) (TAT) (TGT) (TACA) (ATCT) (ATCT) (ATGGT) (TGTTG) (ACACA)	74 - 264 / 111-264 (tetra)	3-27	115 +ve clones sequenced, 54 primer sets designed and tested, 11 retained	20	Holland *et al.* 2013a, this study
Mediterranean	S.O. Scleraxonia F. Coralliidae	*Corallium rubrum*	7	4 (2007a) 5 (2007b)	(CA), (AC) ,(GT) (GTT) ,(TTG) (CAGA) ,(CAAA)	175-536 / 256-536 (tetra)	5-26	302 +ve clones, 227 sequenced, 24 potential loci / primers designed, 7 loci 'perfect'	29	Costantini and Abbiati 2006, Costantini *et al.* 2007a, 2007b
	" "	*Corallium rubrum*	8	8	(GT:4 loci), (CA: 2 loci), (GTTT3GT16), (AC2,A,AC,G,AC3TA,AC7)	93-356 / 140-536 (di)	7-67	Attempt 1: 150 +ve clones, 115 sequenced, primers designed for 22, 12 tested, 6 retained. Attempt 2: 40 +ve clones sequenced, primers desgined for 12, 2 retained	50 attempt 1 17 attempt 2	LeDoux *et al.* 2010 (plus 2 from above), supp mat.
	S.O. Holaxonia F. Plexauriidae	*Paramuricea clavata*	8	n/a 1*	(CT), (GT: 2 loci), (GTT: 2 loci), (CAA: 2 loci), (GTT6,GTGTC,GTT3, AT,GTT2)	147-356 / 172-337 (tri)	2-12	45 +ve clones, 36 loci identified (mention of short flanks),		Agell *et al.* 2009, 1 of these loci used in Mokhtar-Jamai *et al.* 2010
	" "	*Paramuricea clavata*	11	5*	n/a	n/a	3-24	Attempt 1: 3 libraries made, 32 clones from each sequenced, primers for 11 potential microsatellites, 6 retained, Attempt 2: 124 +ve clones, 18 primer sets tested, 5 retained	54 attempt 1 28 attempt 2	Mokhtar-Jamai *et al.* 2010, Mokhtar-Jamai *et al.* 2011. *plus 1 locus from Agell *et al.*2009
	S.O. Holaxonia F. Gorgoniidae	*Eunicella singularis*	7	n/a	(GT), (ATT, AAT, AAC: int), (ATGT), (GTT/GCT: int), (TTTAGGG:imp)	180-285 / n/a	2-9	252 sequenced (animal and dinoflagellate), 27 primer pais tested for octocoral. 4 from E. singularis, 3 from E. cavolinii	26	Cataneo *et al.* 2010

Area	Taxonomy	Species	# Loci (Primer Note)	# Loci (Paper)	Motifs (as published)	Allele Size Range / Largest Motif Range*	Alleles per Locus	# Positive Clones, # of Loci Sequenced, # Retained	Success (%) of primers tested	Reference
Pacific	S.O. Scleraxonia F. Coralliidae	*Corallium lauuense*	6	3 (2005)	(CA: 2 loci) (CA5,TA,CA,25) (CAT18CGT,CAT7) (CA20,TA,CA3,TA,CA1)(CAGA 3,CA5,CG,CA19,CG,CA5,CG2, CA,CG,CA8)	75-284 / 75-179 (di)	16-45	50 +ve clones containing microsatellite sequenced, 15 labelled and six polymorphic and free from extraneous PCR bands	40	Baco *et al.* 2006, Baco *et al.* 2005
	S.O. Calcaxonia F. Ellisellidae	*Junceella juncea*	4	4 (2005b)	(AC) (GT) (GA) (A6,GT8,G15)	114-340 / 114-213 (di)	4-17	Attempt 1: 235 clones containing insert more than 400bp sequenced, 175 hybridized to microsatellitesequence, 51 had flank longer than 50bp. 21 primer pairs tested, of which 3 amplified and 1 was polymorphic. Attempt 2: 9 sequences with a repeat motif, primers designed for 6 of them, 3 retained (amplified well and polymorphic)	5 attempt 1 50 attempt 2	Liu *et al*. 2005a, 2005b
	O. Helioporacea F. Helioporidae	*Heliopora coerulea*	11	9	(TC6, AC5: 2 loci) (AC5,TC7) (AC6,AG5) (TC5,TG9) (AC6, AG6, AC6) (AC6, TC5, T3, CA4) (AC6, TC5, AC15, TC5) (AC6, AG7, AC, AG4) (AC5, TC7, TT, TC2, TT, AC7) (AC5, TC7, T3, AACTT, AC7)	81-274 / 208-274 (compound di)	3-20	191 primer pairs designed, of which 11 were polymorphic and retained	6	Yasuda *et al*. 2008
Caribbean	S.O. Holaxonia F. Gorgoniidae	*Pseudopterogorgia elisabethae*	2	3*	(CA: 2 loci), 3rd locus (Pel1) n/a	134-224 / 208-224	1-27	80 +ve clones, 28 sequences, msats in 18, primers for 10 designed, 5 usable (2 unique loci after 2005 corrigendum)	20	Gutierrez-Rodriguez & Lasker 2004a, Gutierrez-Rodriguez *et al*. 2005, *addition of 'pel1' from ITS region, Gutierrez-Rodriguez and Lasker 2004b
	S.O. Holaxonia F. Gorgoniidae	*Gorgonia ventalina*	10	7 (2012)	(CT: 2 loci) (GT) (TG) (CAT) (GTT) (TTGTG) (TTC4, 6bp, TTC7), (AAC25, 35bp, AAAC16), (TTTG10, 8bp, TTTG13)	117-589 / 317-589 (penta)	4-52	400 +ve clones sequenced, primers designed for 85 unique microsatellite sequences, all primers tested on 16 colonies (8 locations), 10 retained	8.5	Andras & Rypien 2009, Andras *et al.* 2013

2.4 Discussion

2.4.1 Microsatellites in Anthozoa and Comparisons with *E. verrucosa* and *A. digitatum*

Comparisons between the two sub-classes Hexacorallia and Octocorallia may not be directly appropriate, as taxonomic relationships within the Cnidaria remain uncertain and it has recently been suggested that the class Anthozoa is polyphyletic, as opposed to monophyletic as previously thought (based upon mitochondrial phylogenomics, Kayal *et al.* 2013). Likewise, relatively little is known about the nuclear or organellar genomes of each sub-class, although there are some known differences between them. For example, scleractinians have a slow rate of mitochondrial evolution and nuclear genes are thought to evolve at a faster rate than mitochondrial genes in Anthozoa (the opposite of most other metazoans, Shearer *et al.* 2002), although mitochondrial evolution is thought to be even slower in octocorals, possibly due to the presence of a mtDNA mutation repair mechanism (Bilewitch and Degnan 2011). However, a general lack of molecular data ensures that comparisons between octocorals and hexacorals and other cnidarians is inevitable; in terms of published numbers, there are more panels currently available for hexacorals than octocorals, which probably reflects increased research effort in this group due to interest in preservation of globally threatened coral reefs, as opposed to genomic differences between them. From these, it appears that numbers of usable loci also rarely exceed ten per study; for example, in the threatened corals *Lophelia pertusa* (10 loci, LeGoff-Vitry *et al.* 2004), *Acropora palmata* (8 loci, Baums *et al.* 2005), *Montastraea annularis* (4 loci, Foster *et al.* 2007, 6 loci Foster *et al.* 2012), *Porites lobata* (9 loci, Polato *et al.* 2010), *Seriatopora hystrix* (5 loci, Maier *et al.* 2009), *Cladocora caespitosa* (13 loci, Casado-Amueza *et al.* 2011), and *Montastrea faveolata* (9 loci, Davies *et al.* 2012). It is interesting to note that the latter panel (Davies *et al.* 2012) resulted from screening of expressed sequence tag (EST) data; from 33,206 sequences, 544 were found to contain repeats, of which 173 were unique, 59 were non-redundant, and from 53 primer pairs only 9 loci were retained. Therefore, it appears that increased availability of next-generation sequencing (NGS) data is unlikely to significantly ameliorate discovery of more loci in corals, and that low returns compared to the number of unique microsatellites screened are highly likely to reflect genomic paucity of microsatellites in these animals as opposed to limits imposed by technological, time or funding constraints.

The distribution, characteristics and abundance of microsatellites in anthozoan genomes are poorly understood, although difficulty in obtaining them has been recognized both in scleractinians and octocorals (Marquez *et al.* 2000, Liu *et al.* 2005 respectively). Difficulty in obtaining microsatellites, however, is not restricted to Anthozoa and has also been reported for a range of other invertebrate

taxa including molluscs, (e.g., McInerney *et al.* 2011), mites (Evans *et al.* 2003) and flies (Griffiths *et al.* 2009). Additional research indicates that coverage in gastropods may vary 6-fold even between species (Meglecz *et al.* 2012). In the scleractinian *Acropora palmata*, the trinucleotide repeats AAC and AAG were found to be in low abundance when compared to several vertebrate taxa, whereas AAT was abundant (Baums *et al.* 2005). This research also highlighted the difficulty of obtaining polymorphic AC repeats, and the authors isolated AC motifs that aligned to coding regions of congeneric corals. More recently, NGS-based genome screens of non-model eukaryotes have confirmed that microsatellite coverage per megabase of DNA is very low in some phyla compared to others, including Cnidaria (and Bryozoa, Echinodermata and Onychophora, compared to, for example, Chordata and Arthropoda, Meglecz *et al.* 2012). Although Meglecz *et al.* only included one species of anthozoan (the bubble-tip anemone, *Entacmaea quadricolor*), the data also revealed a paucity of dinucleotide repeats in certain phyla including Cnidaria, whereas typically dinucleotide motifs are the most abundant and often constitute more coverage than all other motifs combined. Meglecz *et al.*'s genome screens revealed that AAT was shown to be the most frequent trinucleotide repeat across all phyla, as was found in *A. palmata* by Baums *et al.* (2005).

There is some thought that low abundance of microsatellites in corals may correspond to relatively small genome size and, indeed, variation in abundance of microsatellites has been shown between classes of higher chordates such as birds and mammals (Marquez *et al.* 2000). However, other research suggests that a paucity of microsatellite isolation may stem from genomic 'complexities' such as mutations and cryptic repetitive regions within flanking regions, as opposed to a lack of microsatellites within the genome (e.g., plants, insects, crustaceans and molluscs; Tero *et al.* 2006, Meglecz *et al.* 2007, Bailie *et al.* 2010, McInerney *et al.* 2011, respectively). As there is a general lack of available genomic data across all anthozoan taxa, the theory that genome size plays a factor in microsatellite abundance remains unproven in this group. Physical location of microsatellite repeats within the genome is also largely unknown in Anthozoa. Baums *et al.*(2005) reported an AC rich motif within an intergenic spacer region between two repetitive histone genes in *A. palmata* and Gutierrez-Rodriguez and Lasker (2004b) located an undisclosed motif within the ITS2 region of 18s rDNA of the octocoral *Pseudopterogorgia elisabethae*. In the current study, penta-nucleotide repeats were isolated in *A. digitatum* (3/11 motifs), and they have also been reported in *Gorgonia verntalinia* (Andras and Rypien 2009). Meglecz *et al.* (2012) suggest that penta-and hexa-nucleotides are rare, and in their genome scans of non-model eukaryotes, observed that penta-nucleotides were only detected infrequently in seven out of thirty-eight metazoan non-chordate taxa. Although only one anthozoan species was examined in their study (*E. quadricolor*, see above), it is interesting to note

that tri- and tetra-nucleotide repeats are the most abundant in its' genome; *Eunicella verrucosa* had a high abundance of tetra-nucleotides (7/14 motifs) and in both species in the current study, di-nucleotides were the least common motif, whereas tri-, tetra- (and in *A. digitatum*, also penta-) nucleotides were the most numerous.

Imperfect repeats are likely to occur in several of the microsatellites developed for this study. (e.g., – locus Exe33/Ever004 in *Eunicella verrucosa* has microsatellite-like repeats elsewhere in the FASTA sequence from which primers were developed (data not shown). Although these alleles were not sequenced to confirm this, complex mutations including point mutations and indels both within and in the flanking regions of microsatellites have been demonstrated in other anthozoan taxa (the anemone *Nematostella vectensis*, Darling *et al.* 2006). There are also some reports of potential triploidy, or evidence for duplicate microsatellite regions in anthozoans; larvae showing three peaks at more than one locus were considered triploid in *Acropora palmata* (although no adults shared this pattern, Baums *et al.* 2005). In the current study, although three peaks were not consistently present at any one locus in all individuals, some incidences of potential polyploidy were recognised during the screening process in the two species studied and in the cross-species amplifications; of course, this could also result from contaminated template DNA, genotyping misreads (flashover / bleedthrough), stutter etc. In these cases, these loci were rejected from final selection.

2.4.2 Cross-Species Amplification of Microsatellites

Successful cross-species amplification for both panels developed in this study highlights their utility in genetic assessments of other octocoral species, in particular the 13/14 loci from the *Eunicella verrucosa* panel that amplified in *E. singluaris*, and to a lesser extent, the 6/11 *Alcyonium digitatum* loci that amplified with most success in *A. acaule* (Table 2 in the respective primer notes, Appendices 1 and 2). In hexacorals, Ridgeway and Gates (2006) suggest that microsatellites are not transferable across genera. In this study, 10/14 *E. verrucosa* microsatellites successfully amplified in *Leptogorgia sarmentosa*, a different genus but within the same family as *E. verrucosa* (F. Gorgoniidae). Furthermore, accidental addition of *A. digitatum* positive controls to an *E. verrucosa* plate during this research resulted in their amplification at certain loci, indicating potential trans-generic, trans-familial and even trans-subordinal amplification (data not shown). Therefore, microsatellites in octocorals may potentially be more conserved than in scleractinians, and, although based upon somewhat anecdotal evidence, would appear to warrant further investigation, including sequencing the loci of interest in all of the relevant species. Cataneo *et al.* (2010) report cross-species amplification between *E. singularis* and *E. cavolini*, but other authors appear to have not tested their

primers in other species (Table 2.5) – therefore, there is at this time a lack of data concerning transferability of octocoral microsatellites between species and/or at higher levels.

In conclusion, isolation and screening of microsatellites in the octocorals *Eunicella verrucosa* and *Alcyonium digitatum* resulted in the development of two new sets of molecular makers which should prove useful for elucidating genetic patterns governing the distribution of these charismatic animals. Please refer to Appendices 1 and 2 for the final panels used during throughout this research.

Chapter 3. Genetic Assessment of Connectivity and Population Structure in the Threatened Octocoral *Eunicella verrucosa* in the UK and NE Atlantic

Abstract

In the UK, efforts are underway to develop an ecologically coherent marine reserve network in order to fulfil commitments to international conservation legislation. One criterion outlined in design guidelines for this network is connectivity. Currently, inadequate genetic data is available to assess connectivity of sessile, benthic organisms targeted by the network, including the octocoral *Eunicella verrucosa*. This species is subject to damage by inshore fishing effort and as such is listed as 'vulnerable' on the IUCN red list, is protected by UK law and is one of seven cnidarian species of conservation interest targeted specifically by the network. The range of *E. verrucosa* in the eastern Atlantic is vast, from Angola to Ireland with a Mediterranean presence, yet in the British Isles, its distribution is limited to South West England and Wales, and Ireland. Therefore, my study area constitutes the northerly part of its range. *Eunicella verrucosa* is believed to be a gonochoric summer broadcaster that produces lecithotrophic larvae with limited pelagic duration. Although these data are anecdotal, the latter life history traits are conducive to philopatric settlement and strong population structure. I therefore hypothesized that population structure in *E. verrucosa* would be relatively pronounced.

To test this assertion, I developed a panel of DNA microsatellites and used them to assess population structure and connectivity of *E. verrucosa* at local to regional spatial scales between south west England and Wales, western Ireland, Portugal and Brittany. In total, 955 individuals collected from 30 sites were genotyped. Contrary to my expectations, my data suggest limited population structure at small to large spatial scales across most of the sampled range coinciding with local inbreeding, conforming to a metapopulation dynamic. However, strong regional differentiation was identified with asymmetrical migration between regions, with both Irish and Portuguese clusters being the most divergent. As my data stem from northerly limits to the range of *E. verrucosa*, the Irish populations might be considered marginal and as such exhibit isolation evidenced by inbreeding and lowered allelic richness. These data imply that life history traits are unreliable proxies for connectivity and that genetic data should be incorporated into l marine reserve design. Furthermore, this study confirms that source and sink dynamics extend beyond national boundaries and inter-governmental collaboration is needed to effectively conserve regional biodiversity.

3.1 Introduction

3.1.1 Connectivity of Sessile Invertebrates in the NE Atlantic

Genetic connectivity between populations drives patterns of genetic structure and is a critical means to maintain population fitness, resilience and diversity. In fact, the preservation of genetic diversity is recognised by The International Union for Conservation of Nature (IUCN) in its own right as being worthy of conservation efforts (Reed and Frankham 2003). For sessile marine animals, connectivity between populations usually relies on a meroplanktonic (i.e., temporarily planktonic) larval dispersive phase as the exclusive opportunity to migrate. Reproductive life history traits, pelagic larval duration, larval behaviour, survivorship, availability of suitable settlement substrata and cues, post-settlement metamorphosis and recruitment, hydrodynamic regimes and meteorological conditions are just some of the biotic and abiotic variables that determine migration between geographically disparate populations. Due to the inherent difficulties of measuring most of these parameters *in situ* (see Chapter 1), comparing genetic signatures and using them to infer similarity or divergence between populations is a valuable and accurate means to assess connectivity. In marine ecosystems, 'open' (i.e., open to external recruits) populations were historically assumed for sessile taxa given the passive dispersal of larval stages and the extent of ocean currents, coupled with observed genetic homogeneity of some marine species over large spatial scales (Cowen *et al.* 2000). However, findings of unexpected genetic structure over small spatial scales has proven that this assumption is not always valid and that conservation strategies, including placement and sizing of marine reserves, needs to take empirical measures of connectivity into account (Barber *et al.* 2000). Recognition of the importance of demographic (i.e., ongoing) migration of individuals means that connectivity is indeed often a target criterion in the design of marine reserves globally, and numerous studies and guideline reports emphasise this; connectivity is recognised as a key criterion in the design of marine reserves (e.g., Lubchenco 2003). The focus of connectivity research in marine ecosystems spans diverse taxa, from fish (Galarza *et al.* 2009), to diverse invertebrates (Almany *et al.* 2009, Le Goff-Vitry *et al.* 2004, McCook *et al.* 2009, Piggott *et al.* 2008), marine algae (Coleman and Kelaher 2009), and seagrass (Ferber *et al.* 2008). Most of this research is motivated to gain a better understanding of population structure for conservation purposes. There is also growing recognition of the need not only to conserve connectivity of populations based upon movement of individuals, but also to maintain connectivity between habitats or to protect ecosystems so that connectivity may be facilitated between them. For example, different life cycle stages such as breeding and feeding may occur in adjacent yet different ecosystems, a prominent example being movement of juvenile fish from mangroves, their nursery habitat, to adjacent coral reefs (Mumby and

Hastings 2008). The importance of maintaining habitat connectivity may even be extrapolated to a larger scale by considering between realm connectivity, i.e., between terrestrial, freshwater and marine environments, which is relevant for animals such as anadromous salmonids that migrate to freshwater to spawn, birds that migrate seasonally from inland wetlands to coastal areas, and terrestrial crabs that have a pelagic marine larval stage (reviewed in Beger *et al.* 2010). The importance of connectivity along bathyal gradients has also recently been recognised, for example between shallow and deeper coral reef environments (Olsen and Kellog 2010).

3.1.2 *Eunicella verrucosa* (Pallas, 1766)

3.1.2.1 *Eunicella verrucosa* – Taxonomy

Eunicella Verrill, 1869, is an octocorallian genus within the suborder Holaxonia (an unspiculated, proteinaceous axis with a hollow core, Fabricius and Alderslade 2001) comprising approximately 36 species. At least nine of these are found in the eastern Atlantic, primarily in western Africa, the Atlantic coasts of western Europe and the Mediterranean - *E. cavolini* Koch, 1887, *E. ctenocelloides* Stiasny, 1936, *E. filiformis* Studer, 1879, *E. filum* Grasshoff, 1992, *E. gazella* Studer, 1878, *E. granulata* Grasshoff, 1992, *E. labiata* Thomson, 1927, *E. singularis* Esper, 1791, *E. verrucosa* (Pallas, 1766), in Stiasny 1936, Grasshoff 1992 and Watling and Auster 2005. Based on sclerite analysis, the genus has been ascribed to the family Gorgoniidae (e.g., Grasshof 1992, although it is sometimes listed in the Plexauriidae, e.g. Stiasny 1936, iucnredlist.org, pinkseafan.wildlifetrusts.org). The Gorgoniidae is characterised by small sclerites (Fabricius and Alderslade 2001), often reticulating flabellate forms and includes the charismatic sea fans and sea plumes well known from tropical coral reefs. In the North East Atlantic and Mediterranean, three species of *Eunicella* are well represented, *E. verrucosa* , *E. cavolini* and *E. singularis*- the latter two being among the most prevalent octocorals (along with *Paramuricea clavata* and *Corallium rubrum*) in rocky sublittoral communities of the western Mediterranean (Gori *et al.* 2007, Sartoretto and Francour 2012). *Eunicella singularis* is the only known species of the genus to harbour symbiotic dinoflagellates of the genus *Symbiodinium* more commonly associated with tropical Anthozoa, although mostly at shallow depths as deeper colonies are typically azooxanthellate (Gori *et al.* 2011). *Eunicella cavolini* and *E. verrucosa* are obligate heterotrophs and the latter has been shown to switch its diet seasonally from zooplankton in the winter months to sedimentary organic matter in the summer (Cocito *et al.* 2013). *Eunicella verrucosa* is also the only species of the three to occur in the UK, where it has a limited distribution and is threatened by anthropogenic activity and therefore is a protected species (see below). As for many anthozoans, morphological variants

are recognised in *E. verrucosa* which has two colour morphs - colonies are either pink or white, although they can also be pale orange or in some cases exhibit patches of several colours from pink to pale brown on the same colony (personal observations). *Eunicella singularis* is also known to display two distinct morphotypes where colonies vary in branching pattern with depth (Gori *et al.* 2012).

3.1.2.2 *Eunicella verrucosa* – Range and Distribution

Besides its limited UK distribution, *Eunicella verrucosa* has an extensive range in the eastern Atlantic, which extends from Angola to western Ireland and includes Cape Verde, the Canary Islands and Madeira (Grasshoff 1992, Stiasny 1936). It is also prevalent in the western Mediterranean basin with representation in the Alboran Sea and along the Algerian and Moroccan coastlines, but a more sporadic distribution in Spain, France and the Tyrrhenian Sea, including Corsica (Sartoretto and Francour 2012). Its distribution therefore spans the marine provinces of the Gulf of Guinea, West African Transition, Lusitanian, Mediterranean and the southern part of the Northern European Seas (or Boreal) (as defined by Spalding *et al.* 2007). *Eunicella verrucosa* typically inhabits rocky substrates in areas of high turbidity and moderate to high water flow at upper and lower circalittoral depths between 10-155m, although colonies occasionally extend deeper (e.g., to 200m in Corsica and >500m in Fuerteventura, Grasshoff 1992). In the UK, the distribution of *E. verrucosa* is restricted to the south west of England, southern Wales and the south and west coasts of Ireland where despite high abundance and density in some areas, it is considered nationally rare but locally common (Hiscock *et al.* 2010). Furthermore, some colonies are found as shallow as four metres, therefore the UK likely constitutes the most northerly and shallowest portion of *E. verrucosa*'s range and large peripheral populations are located in northern Donegal Bay in western Ireland, north Pembrokeshire in Wales and Worbarrow Bay in Dorset, England (Tinsley 2005, marlin.co.uk). Interestingly, there appears to be a north / south gradient in the distribution of *E. verrucosa* colour morphs – in Dorset, Devon, Cornwall, Wales and Ireland, colonies are predominantly pink and white morphs are rarely encountered or unknown from these areas. However, the proportion of white colonies increases south of the UK and they are more abundant in the Isles of Scilly, Brittany, and the Mediterranean (Jamie Stevens pers. comm.).

Biotic and abiotic factors limiting the range of *E. verrucosa* remain unclear and there is some suggestion that its range may be extending north and/or into shallower waters in some areas. In the UK, Hiscock *et al.* (2004) suggest a northerly range extension may occur in response to climate-change induced temperature increases, and at its easterly limits, scattered occurrences of *E. verrucosa* colonies have recently been documented beyond Portland Bill, a

geographic feature thought to delineate the range boundary for Lusitanian species due to temperature or habitat availability constraints beyond it (Herbert *et al.* 2007). However, the noted presence of *E. verrucosa* here appears to be due to a previous lack of data as opposed to a recent introduction in this area (Tinsley 2005). Sartoretto and Francour (2012) documented an abundance of colonies at unusually shallow depths in the Marseilles area, most likely due to local fluctuations in turbidity and sediment load. However, how exactly environmental parameters and local hydrodynamic regimes interact to restrict the distribution of *E. verrucosa* remains unclear; it is possible that larval import is inhibited by local hydrodynamic regimes and biogeographic barriers, in addition to locally unfavourable environmental correlates.

3.1.2.3 *Eunicella verrucosa* – Biology, Ecology and Importance

Despite its extensive range and prevalence in the NE Atlantic and Mediterranean, the biology of *E. verrucosa* remains poorly understood, although a larger body of research is emerging describing growth patterns, secondary biomass production, population dynamics, and biometry of the Mediterranean congenerics *E. singularis* and (to a lesser extent) *E. cavolini* (e.g., *E. singularis*: Skoufas 2006, Linares *et al.* 2008, Gori *et al.* 2011 and 2012, Munari *et al.* 2013, *E. cavolini*: Weinbauer and Velimirov 1995a, 1995b, 1996). All three species have been screened for cytotoxic activity and antifungal compounds have been described from *E. cavolini* (Cimino *et al.* 1984), compounds prohibitive to growth of human prostate and breast cancer cell lines from *E. singularis* (Ioannou *et al.* 2009) and compounds with some (albeit low) activity against mice lymphoma and human melanoma cell lines from *E. verrucosa* (Ortega *et al.* 1994). Ongoing monitoring schemes have examined population dynamics and growth rates of *E. verrucosa* in the UK and the Mediterranean. From this, it has emerged that *E. verrucosa* is a slow growing and long-lived octocoral species. For example, Sartoretto and Francour (2012) measured growth rates of colonies at two depths over a ten-year period and found average growth rates between 3.33 and 0.62 cm per year, depending on the age of the colony (fastest growth observed in juveniles). From this study, a lifespan of at least 35 years was inferred for the largest colonies in the Marseilles area, whereas in the Brittany area, a similar assessment of growth rates suggests colonies may attain ages up to 54-60 years old (Coz *et al.* 2012).

The reproductive ecology of *E. verrucosa* is also poorly understood in comparison with its Mediterranean congenerics, although research funded by the English statutory conservation body Natural England and the Countryside Council for Wales have attempted to ameliorate this (Jones *et al.* 2008 and Munro 2004 respectively, Munro and Munro 2003). Some information is available, although in an unpublished format (www.marlin.ac.uk/biotic). This website suggests that a lecithotrophic mode of larval feeding and corresponding short life is

likely, with an estimated settlement distance of less than 1km from the parent colony. However, this assertion is based upon observed time taken for new recruits to colonise shipwrecks more than 1km distant of purported spawning adults and field observations of size classes at particular reefs, rather than connectivity data. The majority of octocorals studied to date are gonochoric, unlike scleractinian corals (reviewed in Kahng *et al.* 2011). Based on field observations from Skomer in Wales and Lyme Bay, Devon, *E. verrucosa* is thought to be an iteroparous gonochoric broadcast spawner, with a spawning period in late summer between August and September (Munro 2004). This likely coincides with water temperature maxima, and temperature cues for gamete release are also known in *E. singularis* (Gori *et al.* 2007). The role of lunar periodicity, a fundamental trigger for gamete release in many tropical and temperate corals and octocorals (e.g., Coma *et al.* 1995), remains unclear in *E. verrucosa* as spawning occurred at different phases of the lunar cycle in subsequent years in colonies sampled at Lyme Bay (Munro 2004). This suggests that temperature may be a more significant cue for spawning in *E. verrucosa*. Sex ratios at the two sampled sites indicate a female bias of two females per male, although in both cases sample sizes were small (N=6 or 15 colonies). Female biased sex ratios have also been noted in some *E. singularis* populations (Gori *et al.* 2007). However, as sea fans are found in large numbers in many areas, including East Tennants reef in Lyme Bay, more extensive sampling needs to be done to determine if this ratio is representative of a bigger population (Munro 2004). In aquaria established at London Zoo and The Deep Aquarium in Hull*, E. verrucosa* colonies spawned in April and June, corresponding to tank temperature increases, although fertilisation and settlement of larvae proved elusive in this study (Jones *et al.* 2008). Therefore, to date, no published data is readily available on larval dispersal, behaviour, pelagic larval duration, settlement, survivorship or recruitment in *E. verrucosa* in any part of its range. Furthermore, the likely age at which colonies become sexually mature remains unknown, although longevity of the species and estimated age at maturity in other octocorals suggest it may be as late as 13 years in some populations (Coz *et al.* 2012).

Eunicella verrucosa is an extremely important component of benthic biomass and provides structural complexity in habitats and is a substrate for various macrofauna and epiphytes. For example, the obligate epiphytic sea anemone *Amphianthus dohrnii* lives solely upon octocorals and has a restricted UK distribution to *E. verrucosa* and *Swiftia pallida* colonies (the 'northern sea fan',marlin.ac.uk). Occurrences of this anemone are thought to be in decline, as such it is also considered to be nationally 'rare' and is a Biodiversity Action Plan (BAP) priority species (jncc.defra.gov.uk). Other associated macrofauna include the barnacle *Solidobalanus fallax* and the nudibranch *Tritonia nislodhneri*, which is commonly found on *E. verrucosa* colonies on

which it feeds and is its only UK host (Hiscock 2005). Occasionally, dogfish and cuttlefish egg cases (Mermaid's purses) are embedded in *E. verrucosa*'s branches (Tinsley 2005). *Eunicella verrucosa* therefore plays a considerable ecological role in maintaining ecosystem biodiversity and species richness and could be considered an ecosystem engineer (Coz *et al.* 2012).

3.1.2.4 *Eunicella verrucosa* - Threats and Vulnerability

The likely slow growth rate, longevity and, in some areas, sparse distribution of *E. verrucosa* renders it susceptible to the numerous anthropogenic and environmentally-driven fluctuations it currently faces. Historically, colonies were harvested for ornamental value and for jewellery (Hall-Spencer *et al.* 2007) but more recently, mobile fishing gears and the detrimental effects of benthic trawling on *E. verrucosa* are well documented, especially in the south west UK where restrictions on scallop trawling have been implemented to protect rocky reefs on which sea fans are abundant (Atrill *et al.* 2011, Lumbis 2008). Trawling damage and stock exploitation of *E. verrucosa* have also been documented in Morocco (Franchimont *et al.* 2001). As such, *E. verrucosa* is protected in the UK under the 1981 Wildlife and Countryside Act (schedule 5), is a listed BAP species, and is listed under the IUCN red list as 'vulnerable A1d', which suggests population reductions of at least 20% based upon current or projected exploitation levels (as opposed to, for example, habitat loss, iucnredlist.org). Consequently, its conservation status in the UK has resulted in its listing as one of 7 cnidarian species of concern in the draft design guidelines for the UK MPA network (Jackson *et al.* 2008). The effects of environmentally- or climatic driven fluctuations on *E. verrucosa* are poorly understood and are currently under-studied. Research into a disease outbreak (likely caused by *Vibrio* bacteria) indicates that colonies may be more susceptible to disease at increased temperatures (Hall-Spencer *et al.* 2007). Long-term temperature increases have also been shown to be detrimental (in vitro) to calcification and photosynthesis in Mediterranean *E. singularis* (Ferrier-Pages *et al.* 2009). The effects of ocean acidification on *E. verrucosa* as a potential threat are unclear and data is also deficient in this regard.

3.2 Methodology

3.2.1 Sample Collection

Samples of *Eunicella verrucosa* were collected for this study between March 2009 and May 2012 from twenty seven sites in south west England, western Ireland, Brittany and Portugal. In addition, samples were donated from tank specimens held at London Zoo (collected in June 2005), from Skomer Marine Reserve in Wales (collected in June 2006 and 2007) and from the Marseilles area of France (EvMai, collected during unspecified dates between 2009-2010).

Details of each site and maps showing their location are given in Table 3.1 and Figure 3.1. Samples were collected at a variety of spatial scales to test connectivity at a) fine spatial scales (sites separated by 0-10km), b) local spatial scales (sites 10-25 km apart), and c) regionally (sites more than 25 km apart). All samples were collected by SCUBA diving; protocols for sample collection are outlined in Chapter 2. As outlined in section 3.2.3 above, connectivity patterns, sex ratios, dispersal and biological traits of *E. verrucosa* are poorly understood, and therefore I appreciate that each sampling site may not represent a discrete population. Nonetheless, for simplicity, each sampled site is hereafter referred to as 'population' and the names of each population correspond to the sampling site at which it was collected. The average number of individuals included in my final dataset after removal of duplicate genotypes and with a failure threshold of three loci per population is thirty-two.

3.2.2 Allelic Patterns in *Eunicella verrucosa* Data

Protocols for microsatellite development and for lab-based genotyping methods are detailed in Chapter 2; this section includes methods used for population genetic analyses after genotyping had been completed from all individuals used in this study. Genotyping data was compiled into a matrix, with an inclusion threshold of three or less failures out of the fourteen final selected loci. Duplicate genotypes were identified in Cervus (Kalinowski 2007) using the 'Identity Analysis' option; those occurring in the same population were subsequently removed, resulting in a final data set comprising 955 individuals from 30 sites (Table 3.1 shows numbers of individuals included in the final data matrix from each site).

Before summary statistics were calculated, allele frequency patterns for each population were visually compared by locus using 'bubble' plots, which allowed an overview of obvious differences in allelic distribution across the dataset (N=955, Figure 3.2). Allele frequency distributions were subsequently summed and graphed by locus (across all populations and individuals), which provided an overview of frequencies and size classes of alleles in the dataset, of the extent of imperfect repeat distributions and an idea of the likelihood that loci conformed to a stepwise mutation-like or infinite allele-like pattern of evolution (Figure 3.3).

The potential presence of null alleles, occurrence of stutter peaks, evidence for large allelic dropout and incidences where more than 50% of alleles occurred in one size class were tested by population-by-population using Microchecker v2.2.3 (van Oosterhout *et al.* 2004), with the maximum allele size set at 400bp, a 95% confidence interval and 1000 iterations (Table 3.2). Finally, a list of private alleles for each population was generated in GenAlEx v6.5b.3 (Peakall and Smouse 2012, Table 3.3).

3.2.3 Exploring Locus Diversity Indices and Testing for Outliers

In order to determine if genetic differentiation resulted from one or a few loci, or was due to several or all of them (i.e., a genomewide effect), average Fst (theta) and heterozygosity metrics were calculated for each locus across all data using FSTAT v2.9.3.2 (Goudet 2002) following Weir and Cockerham's (1984) estimation, using a 5% nominal level for multiple tests. Allelic richness (Ar) was calculated per locus by rarefaction algorithms implemented in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003) to examine how many alleles were present at each locus independently of the sample size. Allelic richness values are presented by locus in Table 3.4 and by population in Table 3.6.

To test for outlier loci and to identify the extent of selective neutrality of loci, the program LOSITAN was used (Antao *et al.* 2008). This program compares the expected distribution of Wright's inbreeding coefficient (Fst, Wright 1922) with expected heterozygosity (He) under an island model of migration (with neutral markers) to detect outlier loci, identified as those with aberrantly high or low Fst values compared to neutral expectations. LOSITAN was run following default and/or the author's recommended settings; 50,000 simulations, "Neutral mean Fst" and "Force mean Fst" options selected, a 95% confidence interval and false discovery rate correction set at 0.1 for both infinite alleles (IAM) and stepwise mutation (SMM) models. Graphical outputs from LOSITAN simulations and loci lists highlighting candidate loci under selection are shown in Appendix 4.

Table 3.1 List of sampling sites for *Eunicella verrucosa*. Bold type indicates sites or areas in which *Alcyonium digitatum* was also sampled. *=colonies with the white colour morph were sampled at some sites as indicated, otherwise all colonies were pink. Some samples were kindly donated to me; however, when preserved in ethanol, pigment leaches out of the colonies and they appear to be white. Therefore there may be some question over the original colour morph of some of these colonies (indicated by n/a). N represents samples included in the final dataset and not the total numbers collected or extracted; these individuals amplified in at least 11/14 loci. Samples from EvMai were a donation and I do not have exact coordinates for this sample. Samples are colour coded here and in subsequent results into the following groups; BLUE = UK, PINK = Ireland, YELLOW = Portugal, RED = Mediterranean (one site) and GREEN = Brittany.

Country	Code	N	Colour Morph*	Date Collected	GPS	Site
U.K.	DevBF	40	pink	5.6.09	50°20'4.73"N 4° 8'52.09"W	Bovisand, Plymouth Sound, Devon
U.K.	PlyMew	44	pink	3.7.09	50°18'38.00"N 4° 6'30.55"W	Mewstone Ledges, Plymouth Sound, Devon
U.K.	DevHD	36	pink	8.08.09	50°12'30.60"N 4°20'33.60"W	Hand Deeps, Plymouth Sound, Devon
U.K.	IoSHath	30	pink	4.8.10	49°52'57.12"N 6°20'59.91"W	Hathor, Isles of Scilly, Cornwall
U.K.	IoSLR	22	pink	11.06.09	49°58'60.00"N 6°18'48.00"W	Lion Rock, Isles of Scilly, Cornwall
U.K.	IoSnnw	23	20 pink, 3 white	11.06.09	49°58'7.20"N 6°15'19.20"W	NNW Flat Ledge, Isles of Scilly, Cornwall
U.K.	JTEten	7	pink	07/2009	50°39'11.27"N 2°53'10.65"W	East Tennents Reef, Lyme Bay, Dorset
U.K.	**Lundy**	22	pink	9.8.09 / 20.9.09	51°10'19.80"N 4°41'15.60"W	Lundy Island, Devon (Battery N=19, Jenny's Cove N=3)
U.K.	LymeHW	9	pink	22.4.09	50°40'31.80"N 2°56'7.50"W	Heroine (shipwreck), Lyme Bay, Dorset
U.K.	**ManMo**	30	pink	21.03.10	50° 2'45.66"N 5° 2'40.02"W	SS Mohegan (shipwreck) Manacles rocks, Cornwall
U.K.	**ManRR**	43	pink	23.3.09	50° 2'40.02"N 5° 2'32.22"W	Manacles, Raglan Rocks, Cornwall
U.K.	**ManV**	24	pink	23.3.09	50° 4'22.32"N 4°59'48.12"W	Volnay (shipwreck), Manacles, Cornwall
U.K.	Sawtooth	12	pink	07/2009	50°41'6.65"N 2°48'7.34"W	Sawtooth, Lyme Bay, Dorset
U.K.	Skomer	39	n/a	3.06.06, 2007	51°44'40.14"N 5°17'42.30"W	Skomer Island, Pembrokeshire
U.K.	WestTen	43	pink	18.08.09	50°38'52.80"N 2°57'46.80"W	West Tennents Reef, Lyme Bay, Devon
U.K.	nrPad	7	pink	6.10.05	50°35'40.98"N 4°56'54.12"W	Camel Estuary, nr Padstow, Cornwall
Ireland	Ire_BR	29	pink	16.5.12	54°34'37.20"N 8°25'44.58"W	Black Rock, St John's Head, co. Donegal
Ireland	**Ire_TR**	48	pink	16.5.12	54°28'17.88"N 8°26'41.40"W	Thumb Rock, Mullaghmore, co. Sligo
Portugal	EvARM	27	check	n/a	37° 5'25.34"N 8°20'45.06"W	Jardim de Veira, Armação de Pêra
Portugal	Faro1	41	pink	26.5.10	37° 2'15.84"N 8°21'21.90"W	Amazonia das Gorgónias, Portimão, Armação de Pêra
Portugal	Faro2	43	pink	26.5.10	37° 3'5.16"N 8°21'10.68"W	Poço, Portimão, Armação de Pêra,
Portugal	Faro3	42	5 brown, 37 white/brown	27.5.10	37° 6'5.94"N 8°34'35.70"W	Portimão, nameless site
Portugal	Faro4	35	pink	27.5.10	37° 6'15.48"N 8°33'33.96"W	Portimão, nameless site
Portugal	Faro5	44	pink	28.5.10	36°58'48.78"N 7°59'27.24"W	Pedra da Greta, Portimão
France	EvMai	13	n/a	n/a	n/a	Maire-Pharillons Fromages (N=10), Liban (N=3), Provence
France	Brest3	43	7 pink, 36 white	19.5.10	48°18'40.62"N 4°25'21.78"W	Pointe de Rozegat , Rade de Brest, Brittany
France	**LTGlen**	40	pink	12.5.11	47°43'38.39"N 4° 3'35.75"W	Laonégued Taër, Glenan Archipelago, Brittany
France	**MenGlen**	43	pink	12.5.11	47°41'19.86"N 3°59'31.70"W	Men Goé, Glenan Archipelago, Brittany
France	**Ros2**	36	29 pink, 6 white	21.5.10	48°42'33.71"N 3°54'11.78"W	La Vieille, Baie de Morlaix, Brittany
France	**Ros1**	40	33 pink, 7 white	20.5.10	48°44'49.50"N 3°57'42.24"W	Astan, Baie de Morlaix, Brittany

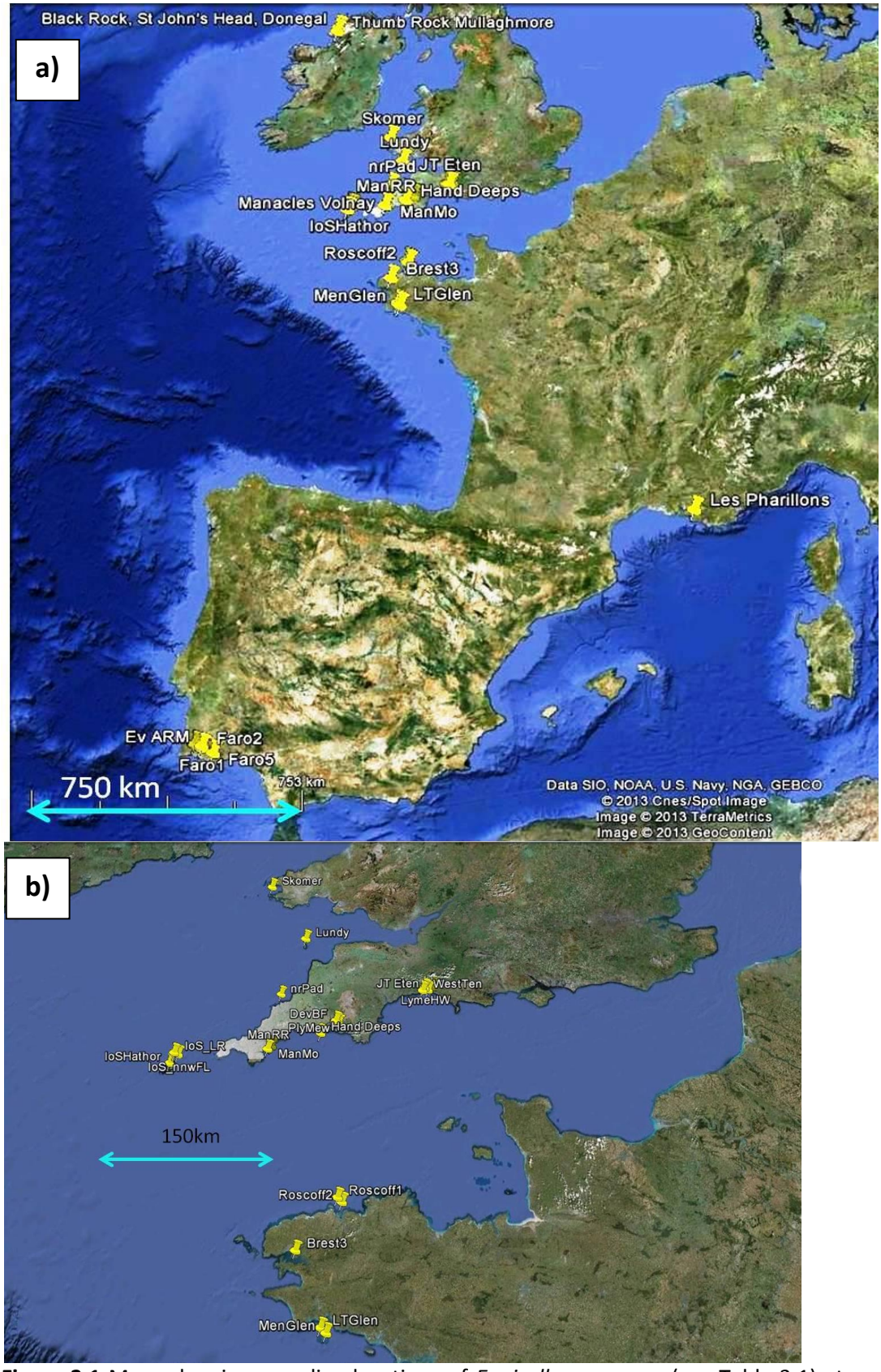


Figure 3.1 Maps showing sampling locations of *Eunicella verrucosa* (see Table 3.1) at various spatial scales; a) overview of locations of 30 sampled populations in the NE Atlantic and Mediterranean, b) more detailed map of South West UK and Wales and Brittany, c) more detailed map of Portuguese sampling scheme, and d), e) and f) are detailed maps of Lyme Bay, Plymouth Sound and Ireland sampling respectively. Scale bars as indicated.

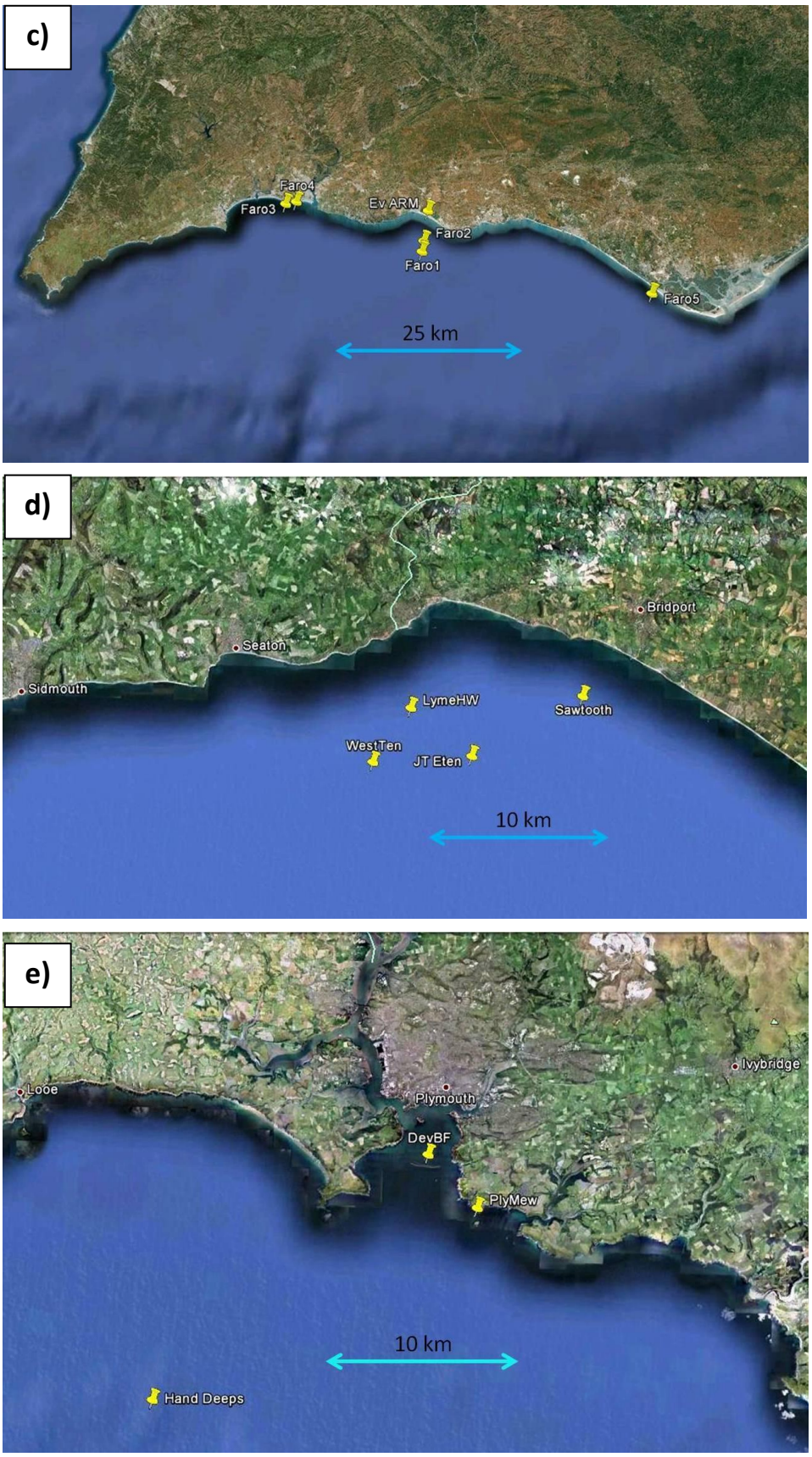


Figure 3.1 continued.

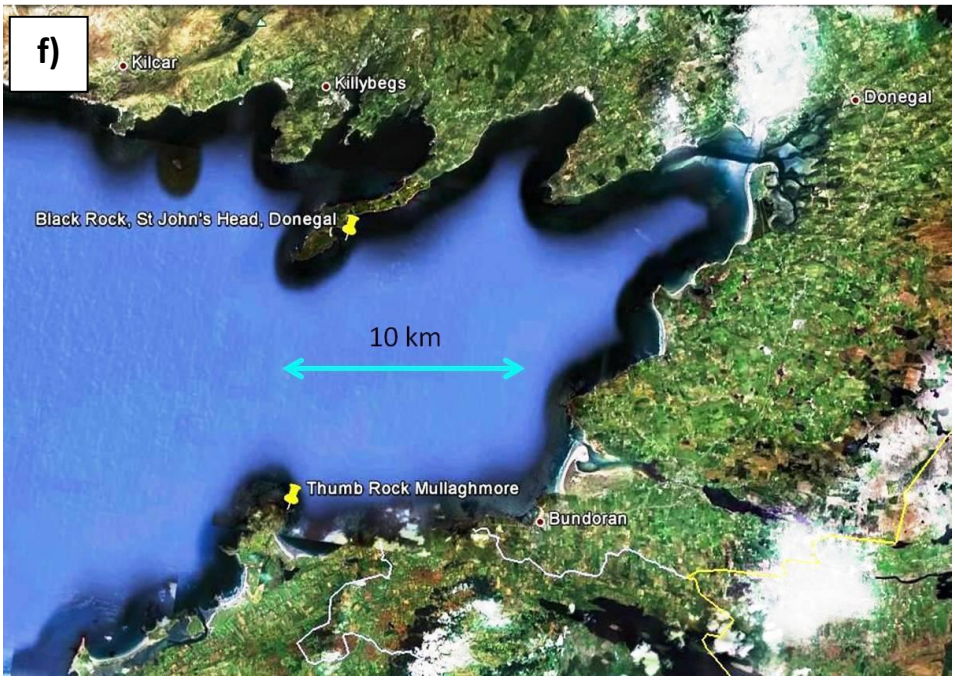


Figure 3.1 continued.

3.2.4 Linkage Disequilibrium

Linkage disequilibrium (LD) was tested between all loci pairs across all populations by permutation tests in Genepop v4.2 (Raymond and Rousset 1995, Rousset 2008). Both log-likelihood ratio statistics and probability tests were examined with 1000 dememorizations, and 100 batches with 1000 iterations per batch. Pairs of loci showing evidence for linkage disequilibrium at the 5% level were noted, and P-values were examined between these loci in each of the 30 study populations to see if they were linked in all populations or if LD was restricted to one or several of them. No pairs of loci showed evidence of linkage disequilibrium in all populations; the highest incidence observed in several loci pair combinations was significant LD in three populations. Therefore, as this was deemed to reflect an artefact of LD simulations in Genepop and not likely to reflect real and consistent genomic linkage disequilibrium, no loci were removed from the subsequent analyses on the basis of LD. As no pairs of loci were shown to have significant linkage disequilbrium across all populations at the 5% level in Genepop, and as no loci needed to be discarded, corrections for multiple tests were therefore not conducted.

3.2.5 Heterozygosity-Based Summary Statistics and Allelic Richness

Summary statistics and initial measures of population differentiation were calculated using Arlequin v3.5.1.2 (Excoffier 2005), Genepop v4.02 (Raymond and Rousset 1995) and Genodive v2.0b23 (Meirmans and van Tiendieren 2004). Raw data files were converted to Arlequin input files using Genepop (option 7). Firstly, to explore the distribution of differentiation across the

entire dataset and to gain an overview of the hierarchical population structure at different spatial scales, AMOVAs (Analysis of MOlecular VAriance) were conducted in Arlequin using the default settings (genotypic data = 1, gametic phase and recessive data = 0), and standard AMOVA computations (haplotypic format, selecting individual level, 1000 permutations, and number of different alleles as Fst-like with an allowable missing data level of 40%). The data were initially tested with all populations assigned to the same group, and subsequently divided into five separate groups roughly corresponding to preliminary results from Bayesian clustering analysis (STRUCTURE- see 3.2.10 below) as the Mediterranean, Portugal, Brittany, the UK and Ireland (Table 3.5).

Next, heterozygosity-based summary statistics were calculated on a population-by-population basis in Arlequin and Genodive (Table 3.6). This included measures such as, defined briefly (and including definitions from Meirmans and van Tienderen 2004) , the total number of alleles (k), the average number of all alleles in each locus, the observed proportion of heterozygosity (Ho, ranging from 0 when all individuals are homozygotes and 1 where all are heterozygotes), the expected heterozygosity proportion within populations (Hs; analogous to He, which measures expected heterozygosities assuming HWE, including a correction for sampling bias where not all individuals in a population are represented, according to Nei 1987), Gis and Fis (analogous measures of the degree of deviation from HWE, aka inbreeding coefficients, Wright 1922). The inbreeding coefficient, Fis, was calculated in Arlequin with an associated P-value, to which a correction for multiple tests was applied using the false discovery rate method (FDR, Benjamini and Hochberg 1995). The remaining statistics were calculated in Genodive; this program calculates standard errors based upon a jack-knifing approach and 95% confidence intervals are obtained by bootstrapping the data (in this case 999 permutations); therefore a FDR correction was not applied to them. For a more detailed examination of locus-by-locus deviations from HWE within each population, observed vs. expected heterozygosities were calculated for each locus using Arlequin and are shown in Appendix 3**.** The P-values were corrected using the FDR method as above. The number of alleles per locus was also calculated within each population. Allelic richness (Ar) was calculated in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003) as a further measure of genetic diversity between populations.

3.2.6 Testing For Evidence of Genetic Bottlenecks

Evidence for genetic bottlenecks was tested using the program BOTTLENECK v1.2.02 (Cornuet and Luikart 1996). This software manipulates the assertion that populations that have recently experienced a reduction in effective population size exhibit correlated reductions in allele

numbers (k) and gene diversity (He), although k declines faster than He. Therefore, observed gene diversity (Hobs) is higher than the expected equilibrium gene diversity (Heq) in recently bottlenecked populations, and BOTTLENECK computes this from observed k under Hardy-Weinberg Equilibrium assumption of a constant-size equilibrium population (Luikart *et al.* 1998). The software was run under the infinite alleles, stepwise mutation and two-phase models assuming mutation-drift equilibrium. In the two-phase model simulation, the proportion of SMM permitted in the TPH was set at 10% following the author's recommendations for microsatellites. For all runs 1000 iterations were calculated, and sign tests and Wilcoxon sign rank tests were used to determine a P value for the likelihood of a bottleneck for each population (Table 3.7).

3.2.7 Fixation Indices and Pair-wise Population Indices

To assess population genetic structure, I used measures of differentiation based upon both Wright's fixation indices (pairwise Fst) and Dest- a measure of differentiation that is independent of intra-population diversity ('Jost's D'; Jost 2008). Pairwise Fst was calculated in Arlequin and P-values were corrected using FDR (Table 3.8). As a comparison to Fst, Dest values were calculated in GenAlEx (with 999 permutations) and the extent of differences in significant P-values on the resulting pairwise matrices was examined. Due to computational processing limitations in this software posed by some populations having insufficient data at some loci, three problematic loci (with the most missing data) were removed from the analysis; 01C02 (Ever001), Exe47 (Ever 012) and Exe50 (Ever013). Therefore, Dest calculations were based upon eleven as opposed to fourteen loci; consequently, Fst values were also recalculated in GenAlEx based upon the same reduced dataset for comparative purposes. This is the only analysis for *Eunicella verrucosa* with a reduced microsatellite panel. Subsequently, pairwise Fst matrices were calculated using all loci in Arlequin, with 1023 permutations at the 5% significance level (Table 3.9a and 3.9b).

3.2.8 Principal Component Analysis

Principal component analyses were performed on a covariance matrix of Nei's unbiased genetic distances, which is a suitable measure of genetic diversity if sample and/or loci numbers used is small (Nei 1978), and pairwise Fst matrices (e.g., Wright 1951) in GenAlEx v6.5b3, omitting the standardize data option (Peakall and Smouse 2012). Although initial PCA runs successfully distinguished three regional clusters (see 3.3.4 below), a Lyme Bay population (JT East Tennents) was also isolated. As this population consisted of only seven sampled individuals, it was removed from the analysis along with two other populations of less than ten

individuals (nr Padstow and Heroine Wreck, Lyme Bay) to avoid spurious results due to insufficient sampling (Figure 3.4).

3.2.9 Isolation by distance

Mantel tests were used to examine potential correlation between geographic distances and genetic differences. To assess geographic distance between sites, shortest distance in a straight line or manually drawn lines following coastlines were measured using Google Earth, following Amaral *et al.* 2012. These distances are likely to represent conservative measures of hydrodynamic movement between sites. Tests were conducted on linearized Fst values and log distances between sampling sites in Genepop v4.2 (Rousset 2008, using Option 6 'Fst and other correlations') and sub-option 9; geographic distances were log transformed due the two dimensional sampling scheme according to Rousset (1997) ('analysis of isolation by distance', Figure 3.5). Significance was tested with 1000 permutations, the minimum distance between samples to be taken in account for regression set at 0.0001 and the regression coefficient calculated using the Spearman Rank correlation coefficient. All samples from Marseilles (EvMai) were removed from the analysis to avoid spurious results based upon the large geographic and genetic distances between this site and all others noted in preliminary analyses, and lack of sampling elsewhere in the Mediterranean at similar spatial scales to elsewhere in the study. Mantel tests were conducted on all remaining data (Fig 3.5a), and subsequently on only the UK sites (Figure 3.5b) as there appeared to be little differentiation between them as observed from Fst values and STRUCTURE (and therefore an analysis using only this data might show fine scale patterns of genetic and geographic distance at a more local scale).

3.2.10 Bayesian Clustering Analyses

Population clusters were estimated using STRUCTURE v2.3.4 (Pritchard *et al.* 2000) - a model-based Bayesian clustering approach, whereby individuals are probabilistically assigned to a known or unknown population (K) based upon shared allele frequencies (Figure 3.6). Simulations were run on all data initially and subsequently with only the UK and Brittany samples, in order to avoid structure noted from other samples overwhelming the detection of finer-scale structure within this region. An admixture ancestry model with correlated allele frequencies was used, under the assumption that there would be at least some migration between sites, which was indicated by results from Fst analysis. As preliminary data demonstrated relatively weak population structure, the LOCPRIOR option was selected which can help detect structure and assist clustering at lower levels of divergence (Hubisz *et al.* 2009). The LOCPRIOR was set to each sampling location (N=30). For comparison, a run without

this option selected was done (Appendix 8). Simulations were replicated twice, with an initial burnin of 10,000 iterations and 10^6 MCMC (Markov Chain Monte Carlo) repetitions after burnin. In all cases convergence was verified by graphing MCMC progress *a posteriori*. Initial runs highlighted a lack of clustering at the level of each sampled site; therefore K values were limited to 2-6. The selection of an optimal K value from all simulations was assisted with the correction of Evanno *et al.* (2005), which plots the log probability [L(K)] of the data and compares it to delta K , as implemented in STRUCTURE HARVESTER (Earl and von Holdt 2012, Appendix 7).

3.2.11 Coalescent Approaches

To estimate migration rates between regions, the software MIGRATE 3.4.4 (Beerli & Felsenstein 2001) was used (Table 3.10). MIGRATE uses a coalescence approach to calculate the population size parameter *theta* θ ($4Ne\,\mu$) (where Ne is the effective population size and μ is a constant per-locus mutation rate per site) and migration rates ($M \diagup \mu$) (where M is the immigration rate per generation). A Bayesian likelihood inference was used in conjunction with a Brownian motion model of stepwise mutation. I used one long-chain search with 1×10^6 generations sampled every 100^{th} step (10,100 recorded steps), with 10% (100,000 steps) discarded as burn-in. These settings were used with default parameters and several runs were completed to determine optimal prior distributions for theta and M, which were subsequently determined to uniform distributions with 0-50, delta=5 and 0-150, delta=10 respectively. The relative mutation rate for each locus was estimated from the data using the Watterson estimator in MIGRATE and the program was run on a dataset structured regionally according to results from STRUCTURE analyses (Cluster 1 – Brittany, 5 populations, Cluster 2 – UK, 16 populations, Cluster 3 – Portugal and the Mediterranean, 7 populations and Cluster 4 – Ireland, 2 populations). The analysis was repeated twice to ensure consistency. Theta values were converted into effective population size values ($Ne = \theta/4\,\mu$) assuming a microsatellite mutation rate of 10^{-4}; actual mutation rates for each locus used in this study are unknown and generally, mutation rates vary between 10^{-2} to 10^{-6} (Li *et al.* 2002). I therefore chose a mid-point estimate that has been used elsewhere to infer mutation rates in a panel of scleractinian coral microsatellites (Casado-Amueza *et al.* 2012).

3.3 Results

3.3.1 Allelic Patterns

The distribution of loci showing evidence for null alleles, showing potential scoring error due to stutter peaks, with evidence for large allelic dropout, and where more than 50% of alleles belong to one size class for all loci in all populations according to Microchecker v2.2.3 (van Oosterhout *et al.* 2004) are shown in Table 3.2. One locus, 06E10 (Ever009) shows evidence for null alleles in 25 out of 30 populations; this locus also has significant heterozygote deficiencies and therefore shows deviation from HWE expectations in 22 of these 25 populations (after FDR correction, Appendix 3). No loci show evidence for null alleles in all populations, but Exe15/Ever002, Exe49/Ever006, Exe47/Ever012 and Exe50/Ever013 did in eight, eight, seven and eight populations, respectively (out of 30 total) in which null alleles may be present. There does not appear to be a geographic correlation with evidence for null alleles, nor a link with sample size; the smallest population (nrPad, N=7) did not show any evidence for null alleles (or significant HWE deviation), whereas the largest sample (IreTR N=48) did at three loci. There was some evidence for stutter peaks, primarily in 06E10/Ever009 in five populations, Exe49/Ever006 in one population (Ire_TR), Exe47/Ever012 in four populations and Exe50/Ever013 in three populations. Incidentally, of these four loci, 06E10/Ever009 is a tetranucleotide, whereas the other three are trinucleotides; stutter peaks are most commonly seen in dinucleotide repeats when a deficiency of heterozygotes with one motif difference between the alleles is detected or when there is a large excess of large homozygote classes (Microchecker User Guide, van Oosterhout 2003-2005). Scoring of raw genotypic data was checked as necessary, and I did not deem this evidence of stutter peaks to be problematic. Evidence for large allelic dropout is thought to occur due to increase amplification efficiency of smaller alleles and no evidence of this was implied in any loci.

The results from Microchecker also show a striking pattern of more than 50% of alleles belonging to just one size class in the majority of populations for eight of the fourteen loci (01C02/Ever001, Exe15/Ever002, Exe21/003, Exe41/005, Exe48/008, Exe17/010, Exe34/011 and Exe47/012), and for half of the populations at locus 06E10/Ever009. This trend is also evident in the bubble plots, which graphically show the distribution of alleles across populations for each locus (Figure 3.2). It is apparent that there are very few obvious visual differences in the distribution of allele frequencies between populations, as the size and frequency of loci appears nearly uniform in all populations at most loci. A notable exception is at locus Exe50/Ever013, which has an obvious difference in the small number of alleles and their size and frequency in the two Irish sites, Thumb Rock and Black Rock. This locus is monomorphic for the allele 149 at Thumb Rock (N=29), and has just two alleles at Black Rock (N=19, alleles 149 and 144, of which neither are private alleles, Table 3.3). The Marseilles population (EvMai) also has only two alleles at this locus, again which are not private alleles

(N=12). These plots also indicate the prevalence of monomorphy at some loci in most populations, for example Exe41/Ever005 was monomorphic for the allele 235 in 21/30 populations. As expected following my selection of microsatellites (Chapter 2) no loci were monomorphic across all populations, although Exe48/Ever008 only has two alleles of 151 and 154bp, representing a single motif difference. Many loci appear to have one dominant, most frequent allele plus several other alleles of rare occurrence, typified by Exe47/Ever012, Exe17/Ever010 and Exe41/Ever005.

Further investigation into the anomalous allele frequencies indicated in the bubble plots for the Irish samples at Ever013/Exe50 revealed that this locus could be under positive selection, under both the infinite allele and stepwise mutation models of microsatellite evolution, as calculated in LOSITAN (Appendix 4). It also has the highest overall Fst of all loci (and a relatively high allelic richness, Table 3.6). I decided to keep this locus in subsequent analyses for several reasons. Firstly, aberrant allele sizes make it challenging to determine if my loci fit either the IAM or SMM model (Figure 3.3). Secondly, the neutrality of microsatellites is often questioned and non-neutral markers can be informative in molecular ecology and population genetics (Li *et al.* 2002). Indeed, in my data allelic richness calculations suggest that diversity is reduced at this locus in both Irish populations and could therefore be informative (Appendix 6). Finally, the use of loci under selection has proven more informative than neutral loci in elucidating patterns concerning genetic diversity and connectivity in other benthic marine invertebrates (Wei *et al.* 2013). Therefore I considered it to be useful.

Nineteen private (i.e. unique) alleles were detected, distributed across sixteen populations (Table 3.3). By population, Faro 4 (Portugal) and Thumb Rock (Ireland) had three and two private alleles, respectively, whereas each of the remaining fourteen private alleles occurred within fourteen populations. By locus, 06E10/Ever009 had the most private alleles (five), the loci 01C02/Ever001, Exe33/Ever004 and Exe49/Exe006 had three private alleles each, Exe24/Ever014 had two private alleles and Exe17/Ever010, Exe34/Ever011 and Exe10/Ever007 had one private allele each. At most, two individuals from the same population had the same private allele, but most occurrences were confined to one individual. Locus 06E10/Ever009 has private alleles in five geographically disparate populations; I observed eighteen alleles at this locus across the entire dataset and therefore 28% of these are unique to a particular population and a single individual. Of the total number of alleles detected in each locus, the percentage of private alleles is 20% (3 out of 15 loci, 01C02/Ever001), 23% (3 out of 13 loci, Exe33/Ever004), 17% (3 out of 18 loci, Exe49/Exe006), 22% (2 out of 9 loci, Exe24/Ever014), 11% (1 out of 9 loci, Exe17/Ever010), 20% (1 out of 5 loci, Exe34/Ever011) and 9% (1 out of 11 loci, Exe10/Ever007). In summary, private alleles appear to be rare among populations of

Eunicella verrucosa and occur rarely in more than one individual, but represent a significant proportion of the allelic diversity seen in most microsatellite loci. By locus, Fst values ranged between 0.001 (Ever008/Exe48) and 0.151 (Ever013/Exe50), with the second highest value at 0.037 (Ever014/Exe24M, Table 3.4). Both of these loci show some evidence that they are under positive selection (Exe50 especially so, Appendix 4). By locus, allelic richness varied between 5.9 (Ever006/Exe49) and 1.1 (Ever005/Exe41) with a mean value of 3.3 (Table 3.4).

3.3.2 Heterozygosity, Summary Statistics and Allelic Richness

Results from AMOVA analyses indicate that almost all of the variation is explained by variation within individuals (91.99 and 91.12% with data undivided and split into five clusters respectively), whereas variation among individuals within populations accounts for a small proportion of genetic structure observed (6.59 and 6.52% of the variation, undivided and five data clusters respectively, Table 3.5). Variation among populations accounted for 1.43% of variation when data was assigned to one group, whereas adding five regional groupings did not result in strong differentiation among regions and this explained 3.29% of the variation. Therefore, it appears that most differences in *Eunicella verrucosa* in my data are at the individual level across all sampled regions and populations although there are some weak demarcations between sampled regions in this study.

Six out of 14 loci showed significant deviation from HWE in at least one population (following correction with the false discovery rate method, Benjamini and Hochberg 1995). These are Exe15/Ever002, Exe49/Ever006, 06E10/Ever009, Exe47/Ever012, Exe50/Ever013 and Exe24/Ever014- all of which displayed significant heterozygote deficiencies (Appendix 3). Loci 06E10/Ever009 and Exe50/Ever013 showed HWE deviation in the most populations (9 and 8 out of 30, respectively), although no loci showed significant HWE departures across a majority of populations and therefore no loci were removed from further analyses on this basis. Several loci were monomorphic in at least one population; 01C02/Ever001, Exe21/Ever003, Exe41/Ever005, Exe17/Ever010, Exe47/Ever012 and Exe50/Ever013, with the highest extent observed in 21 populations in locus Exe41/Ever005 and in locus Exe47/Ever012 in 16 populations. As monomorphy was not consistently observed in all populations at the same locus, I concluded that all loci were polymorphic and informative and none were removed from further analyses.

Evidence for inbreeding indicated by a heterozygote deficit, was further supported by significant inbreeding coefficients at the 5% level in sixteen out of thirty populations (Table 3.6). The overall range of significant Fis values was low and ranged between 0.05 to 2.0, and some values were higher than the lower end of the significant range yet were insignificant

(e.g., Marseilles EvMai had an Fis value of 0.09 and an insignificant P value at the 5% level). There was usually no discernible spatial distribution of populations with significant inbreeding; Roscoff2 (Fis = 0.09), but not the adjacent Roscoff1, had significant inbreeding as did Isles of Scilly Hathor (Fis = 0.09), but not Isles of Scilly Lion Rock. Of the samples from the range limits of this study, Ireland Thumb Rock had significant inbreeding (Fis = 0.12), but not Ireland Black Rock which is across Galway Bay from Thumb Rock and is thought to be the most northerly *Eunicella verrucosa* population in its entire range. The population from Sawtooth was my most easterly sample and had the highest significant Fis (0.02); of the other easterly populations in Lyme Bay, West Tennents (Fis = 0.05) but not JT_East Tennents or the Heroine Wreck had a significant value. Of the Manacles sites in Cornwall, the Mohegan was the only population to exhibit inbreeding (Fis = 0.15) and in Plymouth Sound, the Mewstone had a significant Fis value (0.06) whereas the other sites there did not (Breakwater Fort and Hands Deep). In southern Brittany, Brest3 had significant high inbreeding rates, whereas Men Glen and LT Glen from the Glenan archipelago did not. Lundy, one of my most geograghically isolated sites, had a high but insignificant Fis (0.06). Interestingly, in Portugal, all sites sampled there had high and significant Fis coefficients (EvArm, and Faro1-5, with Fis values ranging from0.06 in Faro2 to 0.13 in Faro5). This demonstrates high rates of inbreeding across all sites sampled within that region. On a locus-by-locus basis, significant heterozygote deficits were never detected in all loci within a single population; the highest number of errant loci observed was in the Faro4 population, where four loci showed significant HWE deviations, and Faro5, where three loci showed significant HWE deviations (and two more were monomorphic, Appendix 3).

The total number of alleles across all loci ranged between 39 and 83 (Table 3.6). The average total number of alleles in a population was 67, it appears that approximately 30 individuals need to be sampled per population to attain the average total number of alleles. However, there is some indication that representative genetic diversity was not sampled in its entirety as correlated allelic numbers and samples size failed to plateau and lower allele numbers tended to coincide with lower sample numbers (Appendix 5). Allelic richness varied between 2.5 and 3.48 (mean 3.1), with the highest and lowest values found in populations from Marseilles and Lyme Bay respectively (Table 3.6). When Ar values were ordered in ascending size in the context of sampling location (data not shown), both Irish samples had the lowest Ar (2.78 and 2.92 for Black Rock and Thumb Rock respectively). These sites are at the northerly extreme of my sampling scheme and represent the most northerly populations of *Eunicella verrucosa* in its range. On the contrary, two of the highest values were found in the Lyme Bay area in Devon, UK, the area thought to contain the most easterly sea fans in their range (West Tennents reef,

3.32 and the Lyme Bay Heroine Wreck, 3.48). Otherwise there were no clear patterns of allelic richness variation, and the overall variation was small.

3.3.3. Evidence for Genetic Bottlenecks

Under the stepwise mutation model, almost all *Eunicella verrucosa* populations show evidence of being in bottleneck conditions, although there are very few such instances under both the infinite alleles and two phase models (Table 3.7). Under the latter two models, the probability that the 'near Padstow' population was bottlenecked had support from the Wilcoxon sign-rank test, which is thought to have more statistical power than sign tests (Luikart *et al*. 1997). Given that uncertainty remains about which is the most likely mutation model for these loci, and that allele frequency distribution graphs suggest that stepwise mutation may be inappropriate (Figure 3.3), it is difficult to ascertain the likelihood of bottlenecks in the dataset. Furthermore, the program BOTTLENECK v1.2.02 (Cornuet and Luikart 1996) assumes a constant-size equilibrium population (Luikart *et al.* 1998), which is unlikely to be the case for my populations. Results here also suggest that the Irish Thumb Rock population does not show evidence of a bottleneck, despite having a lower allelic richness and a lower than expected heterozygosity (Table 3.6), both of which may indicate a bottleneck. Therefore results from BOTTLENECK cannot be interpreted unequivocally.

3.3.4 Pairwise Population Indices

Using 14 loci, pairwise Fst values ranged between zero and 0.1155 (Ireland Thumb Rock and Ev Marseilles, Table 3.8). There is a striking regional genetic differentiation demonstrated by the prevalence of significant Fst values between samples from Portugal and Ireland with almost all other sites, but not within these regions (i.e, the two Irish sites do not show a significant Fst to each other, the Portuguese sites do not show a significant Fst among them and the sampled sites in Brittany are not significantly different to one another with the exception of a northern Brittany site, Roscoff 1, and a southern Brittany site, Men Glenan, 0.0103, P value = 0.018). There is some lesser differentiation between samples from Brittany and elsewhere, however, the UK sites appear to show no significant structure across that region (with the exception of the Heroine Wreck in Lyme Bay and Lion Rock in the Isles of Scilly).

A comparison between Fst and Dest pairwise comparisons shows a much greater overall extent of significant values for Dest , especially within the Portuguese samples (albeit based upon 11/14 loci, Tables 3.9a and 3.9b). This which may be expected given that Fst comparisons are thought to underestimate the level of population differentiation when using hypervariable

markers, as it depends on within-population heterozygosity, as opposed to actual differentiation (Jost 2008). In both tables, it is clear that there are very few significant pairwise comparisons with the UK samples, the most notable exception being Isles of Scilly Lion Rock, which is significantly different to three other populations in both analysis (with no striking geographic pattern). When comparing the two Fst results, there are less significant comparisons here using eleven loci than when calculated with fourteen loci in Arlequin, and the range of Fst values is generally much lower (between 0.063 and 0.005). Nonetheless, in all pairwise comparisons it is evident that most of the significant variation is also at the regional scale here and is much more apparent between the Portuguese and Irish samples and all other samples, than it is within samples from the UK and Brittany.

3.3.5 Principal Component Analysis

The first two coordinates of the principal components analysis of covariance explained 67.23% of the variation based upon Nei's genetic distance, and 58.66% of the variation based upon pairwise Fst as calculated in GenAlEx v6.5b.3 (Peakall and Smouse 2012) and shown in Figure 3.4. In this program, the relationship between elements of the matrix table and their first two principal components are plotted, although six axes are considered and the percentage of variation given by the first three axes is given. Cumulative variation explained by the first three axes was 84.04% for Nei's distance (35.89+31.34+16.8 for axes 1, 2 and 3 respectively) and 78.44% for pairwise Fst (32.6+ 26.06+19.78 for axes 1, 2 and 3 respectively, data not shown). In both plots, there is a clear separation between the samples from Ireland, the samples from Portugal and all other sites, forming three distinct clusters. The samples from the UK and Brittany form a single cluster with the sites in Brittany relatively indistinguishable from the UK (and the one Mediterranean site), although sites from northern Brittany (Roscoff 1 and Roscoff 2) are slightly more separated from the UK samples than samples from the other southern sites in Brittany (Brest3, LT Glenan and Men Glenan). The Mediterranean sample (Ev Marseilles) is more distant to other sites in the Brittany / UK cluster in the PCA based upon pairwise Fst than in the Nei's-distance PCA. However, in the Nei's PCA, the Isles of Scilly Hathor population appears to cluster nearer to the Irish samples in the Nei's distance PCA than in the Fst PCA , an intriguing result given that Hathor is the nearest site sampled to the western Irish populations. Overall, the PCA plots imply that the data is well defined into clusters at the regional scale and that Ireland, Portugal and the UK-plus-Brittany form three distinct groups. Within these clusters, there is no clear geographical correlation or resolution at smaller spatial scales. For example, in Portugal, samples from Faro3 and Faro5 are the most distant sites sampled from within this region (approximately 55km distant), yet they are adjacent in the Nei's distance PCA and close to each other in the Fst PCA, and within the UK cluster, Skomer

samples from Pembrokeshire in Wales are near to samples from the Mewstone in Plymouth Devon. As might be expected, the farthest east sites sampled, West Tennents and Sawtooth in Lyme Bay, are relatively distant to the Skomer samples, the most westerly population, in each of the PCA plots.

3.3.6 Isolation-by-Distance

Results from Mantel tests for isolation-by-distance (IBD) can be found in Figure 3.5, tested initially across the entire dataset (Fig. 3.5a) and subsequently within the UK and Brittany samples to detect finer-scale patterns among sites that were not well differentiated in Fst, PCA or STRUCTURE analyses (Fig 3.5b) (it is worth reiterating that geographic distances here were calculated in a simple linear fashion are not based upon currents, as such they may under-represent actual distances travelled by larvae). An increase in mean and variance of Fst with geographic distance would be expected if populations showed isolation by distance, corresponding to a stepping stone model (Figure 1.1). Although there is a weak, yet highly-significant correlation between Fst values and distance in the entire dataset, there is no clear pattern of IBD and only 4% of the variation in the genetic data is explained by log transformed geographic distances for the whole sampled range of *E. verrucosa* (R^2= 0.049, P=0.001). This correlation is lost within the UK and Brittany subset of data and a non-significant regression is observed (R^2=0.0007, P=0.157), indicating that genetic patterns observed in this region are not due to an isolation-by-distance pattern (maximum distance between sites being 507km, Skomer in Wales to LT Glenan in southern Brittany) and that none of the genetic variation is explained by distance. There are nonetheless some interesting patterns. Across the entire dataset, in comparison to similar scatter plots illustrated in Figure 1.1, it is possible that at scales of 10-100km, and island model predominates in which populations are more influenced by gene flow than drift (as in Figure 1a; this implies connectivity between populations at local scales). At scales from 100 to 100km, the range of Fst values is more scattered, reminiscent of patterns observed in Figure 1.1c, where populations are more separated and where genetic drift is more influential than gene flow. This indicates more isolation between populations. At large distances, samples continue to mimick Figure 1.1c; i.e., they are out of equilibrium and drift is more relevant then gene flow in shaping populations. There is an apparent break in data between 550-800km, likely representing a lack of sampling anywhere between Portugal and Brittany.

3.3.7 Bayesian Clustering Analyses

Results from Bayesian clustering analysis indicate extensive admixture within each of three regional clusters (the most likely number of population clusters K=3, STRUCTURE and Structure

Harvester, Figure 3.6), with differentiation between them. The clusters clearly support the clusters separated in the PCA analysis of Portugal, Ireland and the UK plus Brittany (Figure 3.6a). Within the UK plus Brittany cluster, there is similarity between the Brittany samples and Devon Plymouth Mewstone samples, all of which generally show a higher probability of belonging to the same cluster as the Portuguese samples than the UK samples, as illustrated by the higher (but still low) proportion of green colour (which matches Portugal, Fig. 3.6a).

The same analysis using a no admixture model (Fig. 3.6b) also shows a strong Portuguese, Irish and UK structuring (red, yellow and blue respectively) with a distinct signature for the southern Brittany samples and a more admixed pattern for the northern Brittany samples (Roscoff 1 and 2). Interestingly, in simulations using both admixture and no admixture models, samples from the Isles of Scilly Hathor site show a higher affinity to the Irish cluster than other populations, corroborating a similar pattern in the Nei's genetic distance-based PCA analysis (Fig 3.4a). Samples from the Marseilles area did not form a strongly differentiated cluster as expected given their geographic distance to all other samples; under admixture they are equally as likely to belong to the Portuguese or UK / Brittany cluster and without they are assignable to any one of the three Portugal, UK or Brittany clusters. In both cases they show no affinity to the Irish samples. The samples from Ireland appear to be the most unique cluster in the full dataset. When a subset of data comprising only UK and Brittany samples was tested, the most likely K value was two (Fig. 3.5c, although a K=1 is also a possibility but incomputable in Evanno corrections, Appendix 7). A division between the Breton and UK samples can be seen, so although highly admixed, it appears that there is some divergence between French and British samples. An analysis including samples from the UK only shows no resolution at this scale, and therefore STRUCTURE analyses strengthen the regional scale of variation noted in PCA and pairwise statistical comparisons.

3.3.8 Coalescent Analyses

Bayesian estimates of the value of θ ranged from 0.75 (Ireland cluster) to 1.05 (Brittany cluster), which were transformed into effective population sizes of 1875, 2375, 2625 to 2958 for Ireland, UK, Brittany and Portugal & Med clusters respectively (Table 3.10). Estimated migration rates ranged between lowest values of 0.95 (Ireland to Brittany and Brittany to Portugal), to highest values of 21.55 (UK to Portugal and the Med) and 24.95 (Brittany to the UK). Some striking asymmetrical rates of migration were suggested, notably that very little migration occurs in a northward direction from Portugal to the UK (0.85) or from Portugal to Ireland (1.65), yet in the opposite direction migration is much higher (21.55 and 8.45 for the UK and Ireland to Portugal respectively). On the contrary, northward migration appears to be

three times higher from Brittany to the UK (24.95) than in the opposite direction (8.05), and is also higher from the UK and Brittany into Ireland than vice versa (5.45 vs. 2.85 and 1.55 vs. 0.95 respectively), and is also higher in a northward direction from Portugal to Brittany (2.25 vs. 0.95). No clusters were clearly 'sinks' or 'sources' for all of the other clusters (i.e., migration was not always higher into or higher out of the other three clusters), although it is of note that each cluster represents a relatively crude group containing sites that may be more geographically disparate within the cluster than they are to other sites in other clusters. The smallest effective population size is Ireland, and the largest the Portugal and Mediterranean cluster; however, Ne was calculated using a mutation rate of $\mu=10^{-4}$,an estimate for these loci, therefore these values must be considered with relation to each other as opposed to their absolute values.

Table 3.2. Results from Microchecker for all loci and populations for *Eunicella verrucosa*. Columns correspond to: 1= evidence of null alleles, 2=evidence for stutter peaks, 3=evidence for large allelic dropout and 4=more than 50% of alleles belong to one size class. Shaded cells indicate positives.

Locus		01C02.				Exe15.				Exe21.				Exe33.				Exe41.				Exe49.				Exe10.			
(clone name)		01_C02				tet_1				tet_16				tri_22				tet_67				tri_38				di_17			
(new name)		Ever001				Ever002				Ever003				Ever004				Ever005				Ever006				Ever007			
Population	**N**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Brest3	43	N	N	N	Y	N	N	N	y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
DevBF	40	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N
EvARM	27	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
EvMAI	13	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y
Faro1	41	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
Faro2	43	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
Faro3	42	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
Faro4	35	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
Faro5	44	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
HandsDeep	36	N	N	N	N	N	N	N	Y	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
IoSHath	30		N/A				N/A				N/A				N/A				N/A				N/A				N/A		
IoSLR	22	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	Y
IoSnnw	23	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
IreBR	29	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
IreTR	48	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	Y	N	N	N	N	N	N
JTEten	7		N/A				N/A				N/A				N/A				N/A				N/A				N/A		
LTGlen	40	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N
LundyBatt	22	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N
LymeHW	9	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
ManMo	30	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N
ManRR	43	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	Y	N	N	N	N	N	N	N
ManV	24	N	N	N	N	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
MenGlen	43	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
nrPad	7	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
PlyMew	44	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	Y	N	N	N	N	N	N	Y
Ros1	40	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
Ros2	36	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N
Sawtooth	12	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
Skomer	39	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
WestTen	43	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N

Table 3.2 continued.

Locus (clone name) (new name)		Exe48. tri_33 Ever008				06E10. 06_E10. Ever009				Exe17. tet_3 Ever010				Exe34. tet_28 Ever011				Exe47. tri_32 Ever012				Exe50. tri_45 Ever013				Exe24Mplex11. tri_1 Ever014			
Population	**N**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Brest3	43	N	N	N	y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
DevBF	40	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y
EvARM	27	N	N	N	Y	Y	Y	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
EvMAI	13	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N
Faro1	41	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N
Faro2	43	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	Y	N	N	N	N	N	N	Y
Faro3	42	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	Y	Y	N	Y	N	N	N	Y	N	N	N	N
Faro4	35	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	Y	N	N	Y	Y	N	N	N	N	N	N	Y
Faro5	44	N	N	N	Y	Y	Y	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	Y	Y	N	N	N	N	N	N
HandsDeep	36	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	Y	N	N	N	N	N	N	N
IoSHath	30		N/A				N/A				N/A				N/A				N/A				N/A				N/A		
IoSLR	22	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
IoSnnw	23	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N
IreBR	29	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N
IreTR	48	N	N	N	Y	Y	Y	N	Y	N	N	N	Y	N	N	N	Y	Y	Y	N	Y	N	N	N	Y	N	N	N	N
JTEten	7		N/A				N/A				N/A				N/A				N/A				N/A				N/A		
LTGlen	40	N	N	N	Y	Y	Y	N	Y	N	N	N	Y	N	N	N	Y	Y	N	N	Y	Y	N	N	N	N	N	N	N
LundyBatt	22	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N
LymeHW	9	N	N	N	Y	Y	N	N	N	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	Y
ManMo	30	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	Y	Y	N	Y	N	N	N	N
ManRR	43	N	N	N	Y	Y	Y	N	N	N	N	N	Y	N	N	N	Y	Y	Y	N	Y	N	N	N	Y	N	N	N	N
ManV	24	N	N	N	Y	N	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y
MenGlen	43	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	Y	N	N	N	N	N	N	Y
nrPad	7	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
PlyMew	44	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	Y	Y	N	Y	N	N	N	N	N	N	N	N
Ros1	40	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N
Ros2	36	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	Y	N	N	Y	N	N	N	N	N	N	N	N
Sawtooth	12	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	Y	Y	N	Y	N	N	N	N
Skomer	39	N	N	N	Y	Y	N	N	N	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N
WestTen	43	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N	N

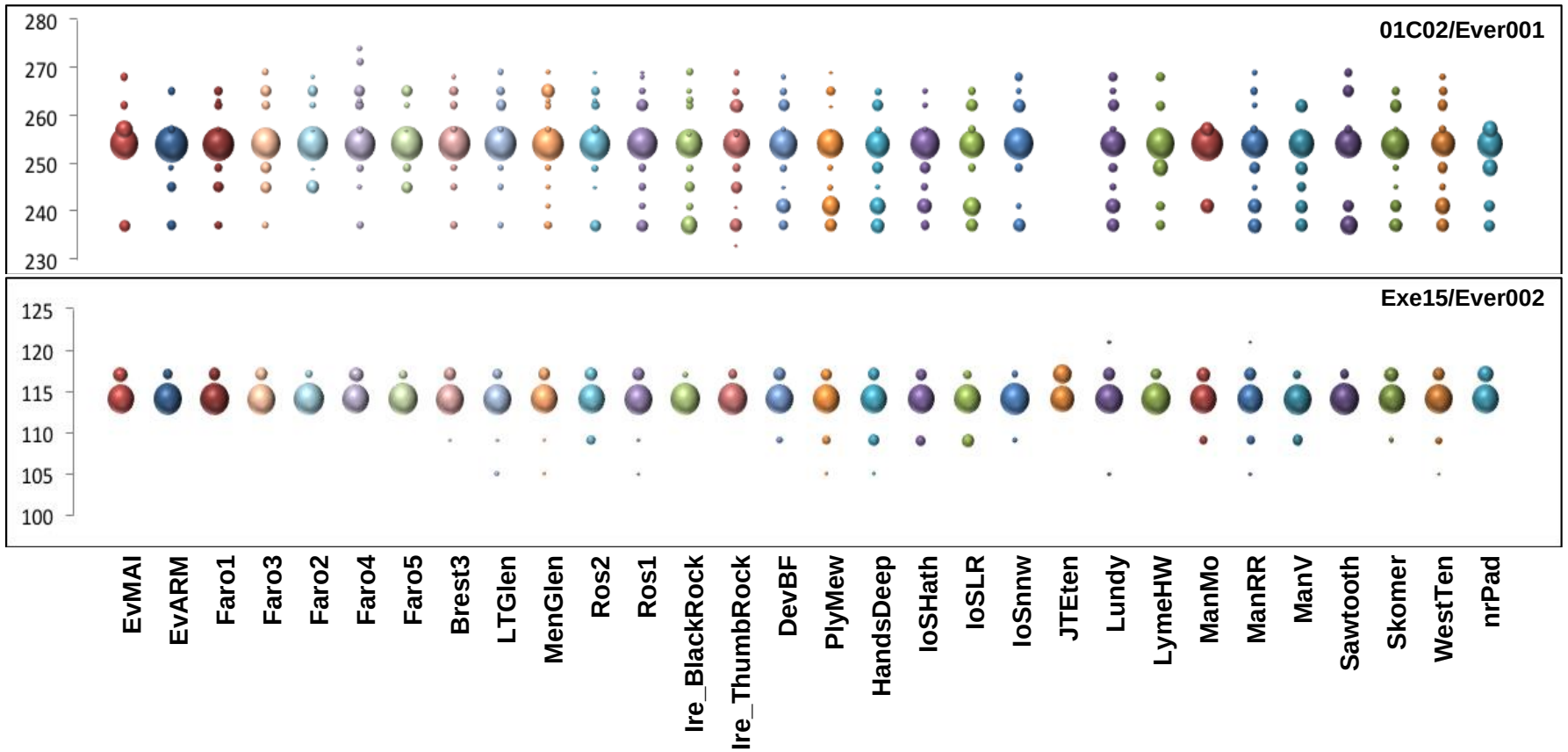


Figure 3.2. *Eunicella verrucosa* bubble plots showing distribution of alleles across all populations for all loci. Y-axis represents size of alleles (bp) and X-axis shows allele frequency by population in the order indicated underneath (a different colour per population). The larger the circle, the more frequent the allele by proportion within that particular population (i.e., two circles of the same size within a population would represent the presence of two alleles with a 50% frequency each). A missing bubble at a population indicates that no individuals amplified from that site at that particular locus (e.g., 01C02/Ever001 in the JT_Eten population).

Figure 3.2 continued.

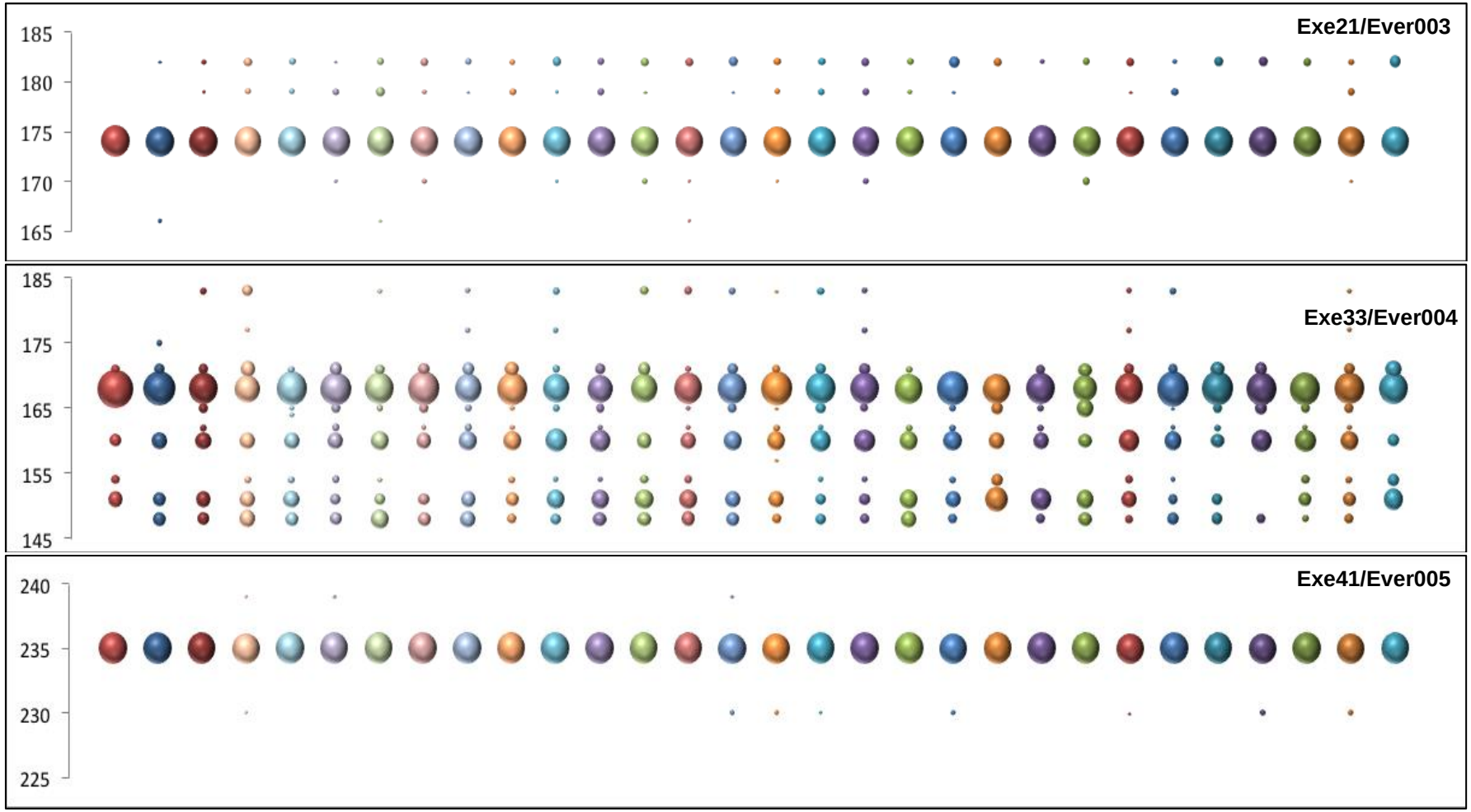


Figure 3.2 continued.

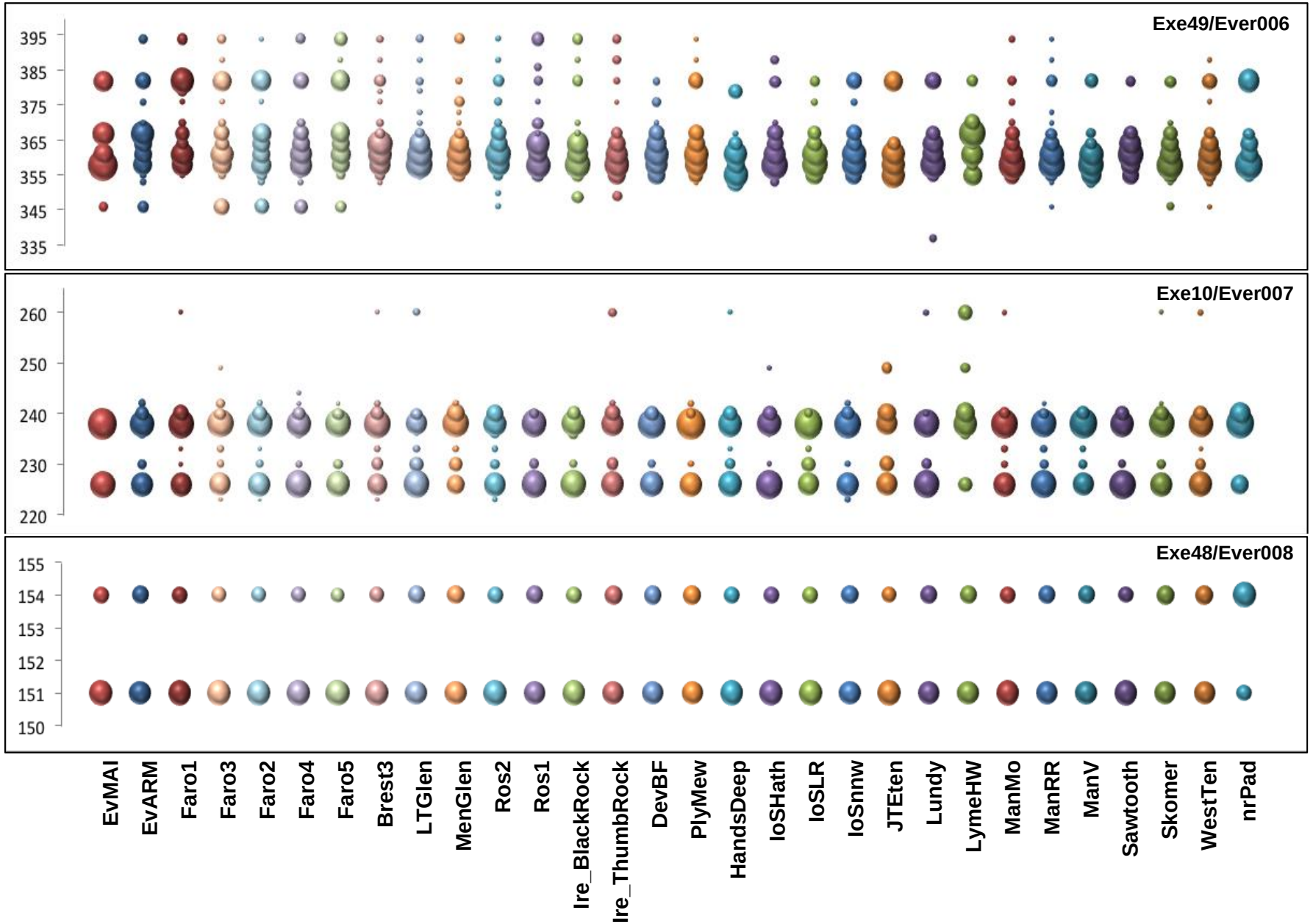


Figure 3.2 continued.

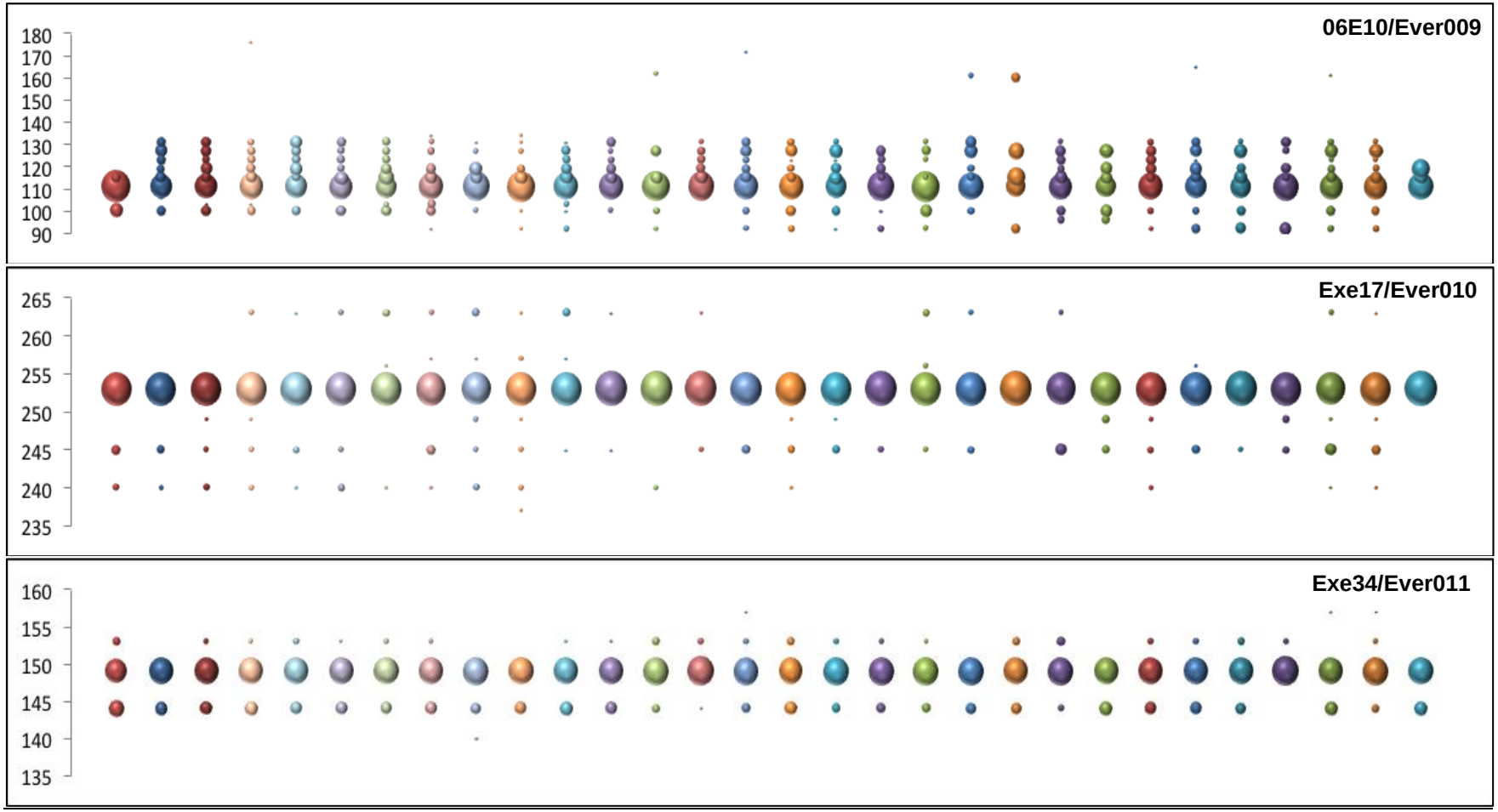
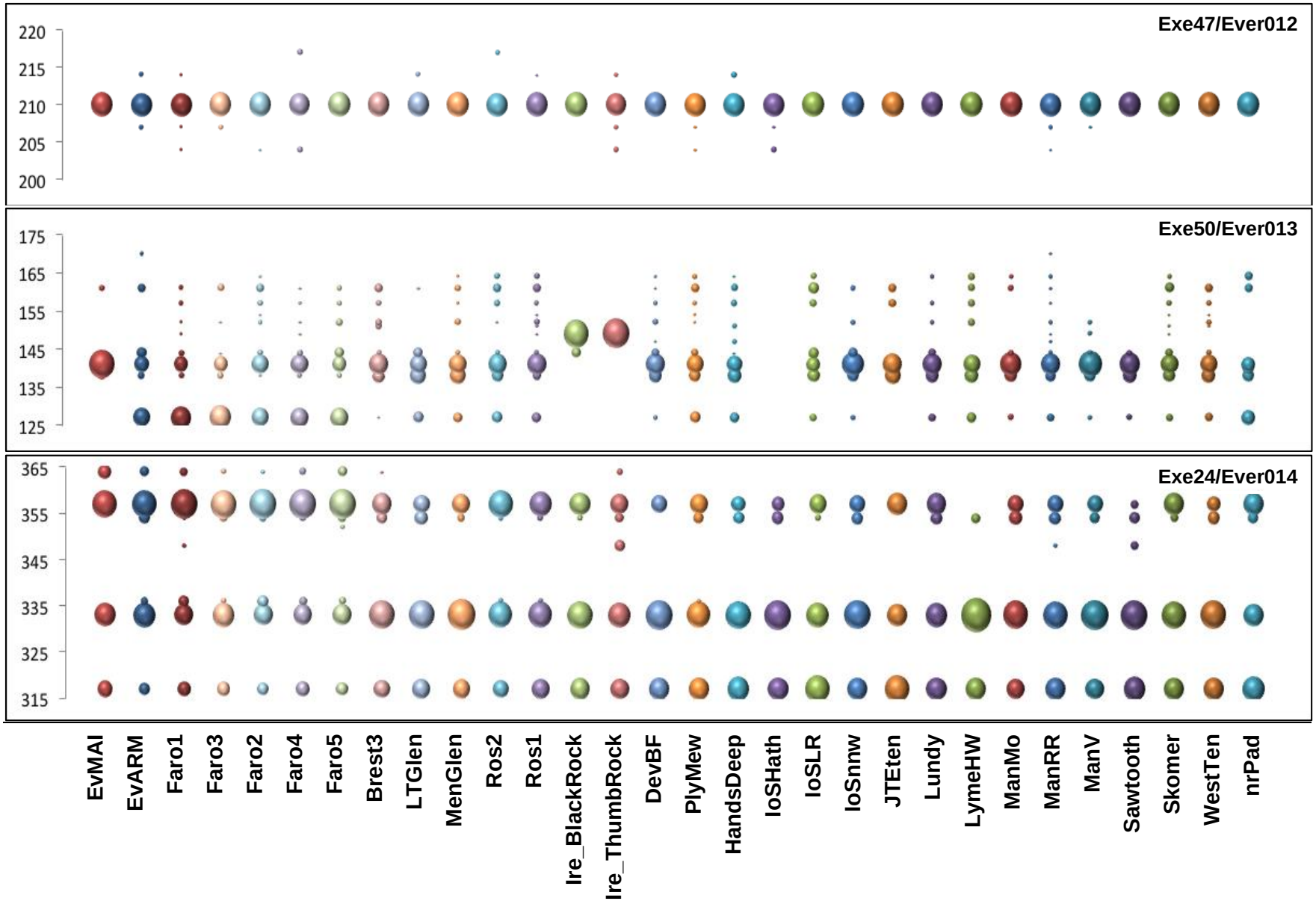
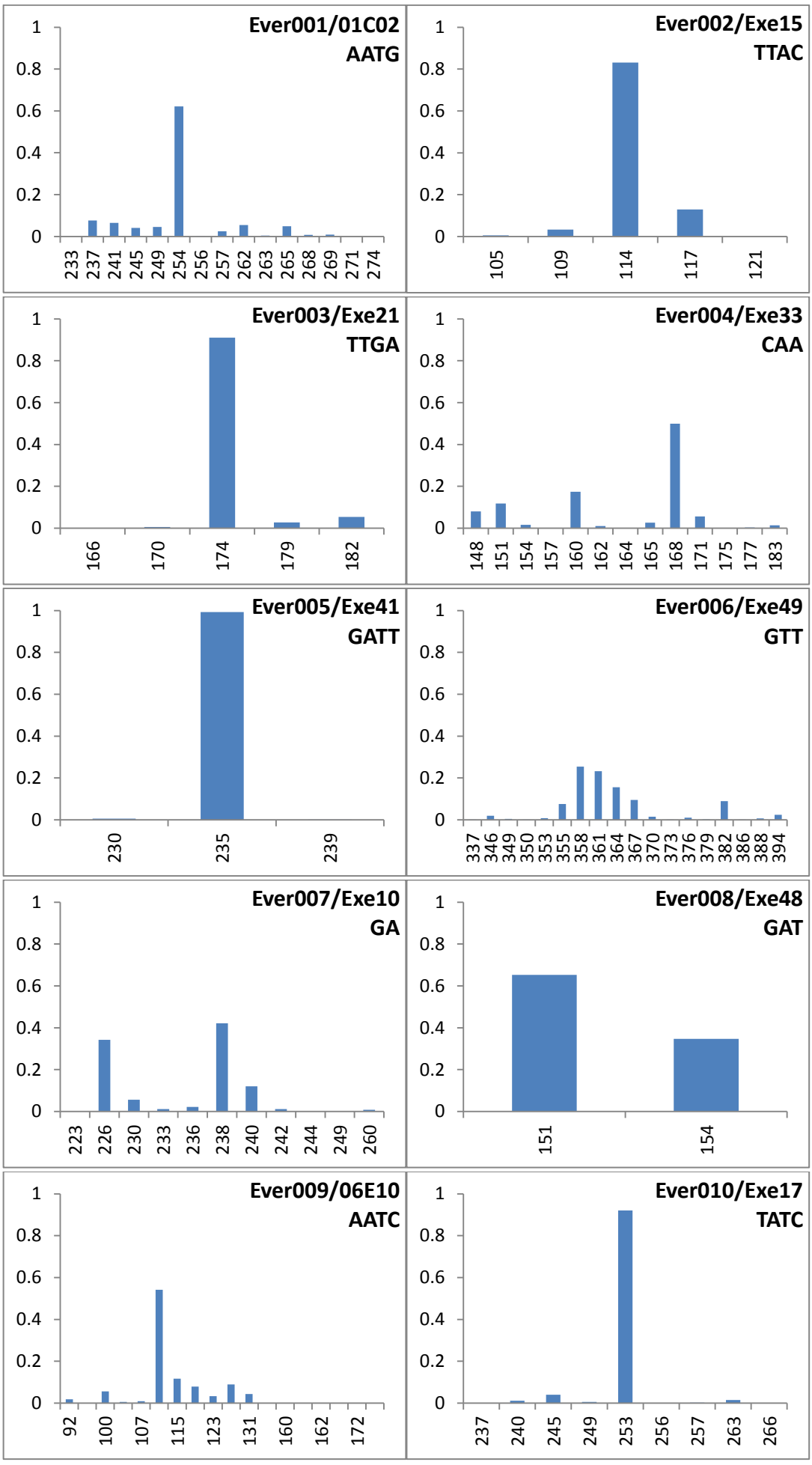


Figure 3.2 continued.




Ever001/01C02
AATG
1
0.8
0.6
0.4
0.2
0
233 237 241 245 249 254 256 257 262 263 265 268 269 271 274
Ever002/Exe15
TTAC
105 109 114 117 121
Ever003/Exe21
TTGA
166 170 174 179 182
Ever004/Exe33
CAA
148 151 154 157 160 162 164 165 168 171 175 177 183
Ever005/Exe41
GATT
230 235 239
Ever006/Exe49
GTT
337 346 349 350 353 355 358 361 364 367 370 373 376 379 382 386 388 394
Ever007/Exe10
GA
223 226 230 233 236 238 240 242 244 249 260
Ever008/Exe48
GAT
151 154
Ever009/06E10
AATC
92 100 107 115 123 131 160 162 172
Ever010/Exe17
TATC
237 240 245 249 253 256 257 263 266

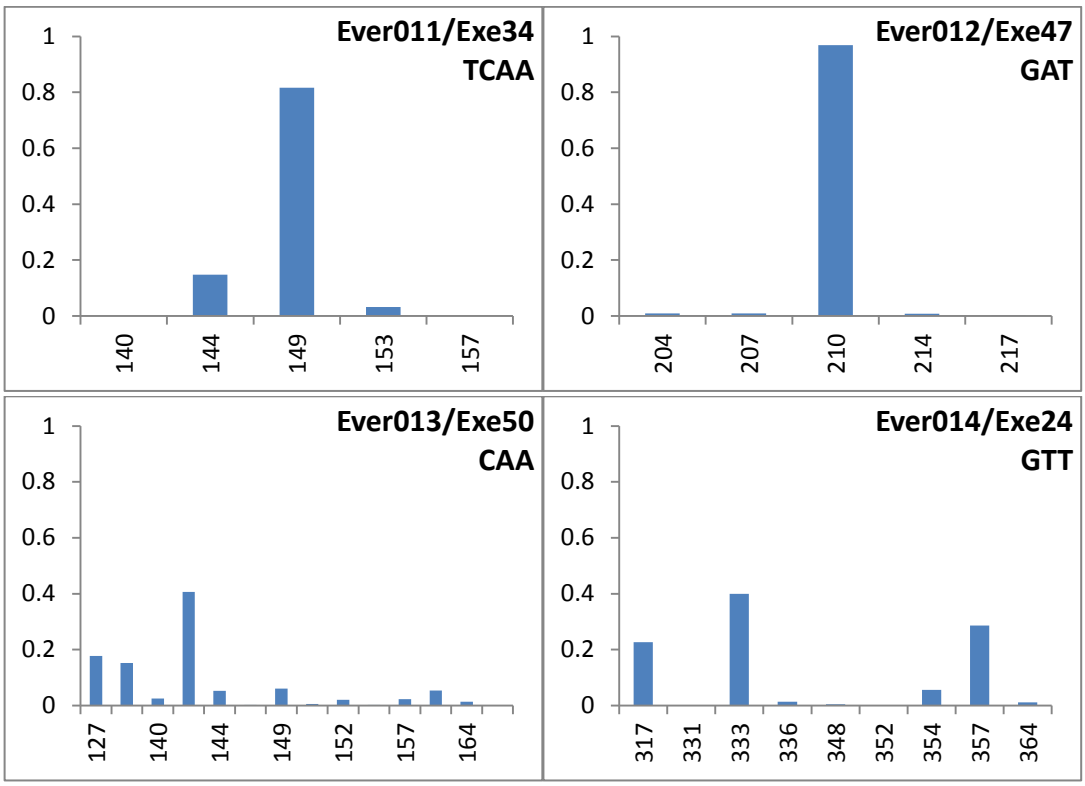


Figure 3.3. Total allele frequencies shown by locus across all *Eunicella verrucosa* data (N=955), calculated in Microsatellite Analyser (MSA) v.4.05 (Dieringer and Schlötterer 2003). All alleles detected at each locus are shown (X axis) and cumulative frequency = 1 (Y axis). Scale is the same on all Y axes; as some alleles were found in extremely low frequencies, bars for them are indiscernible (e.g., for Ever002 allele 105, fq = 0.005394, data not visible). Repeat motif for each locus is given, to illustrate prevalence of imperfect repeats and aberrant allele sizes compared to expectations based upon models of microsatellite evolution (e.g., infinite alleles and stepwise mutation models).

Table 3.3 Summary of private alleles by population in *Eunicella verrucosa* as calculated in GenAlEx v. 6.5b3 (Peakall and Smouse 2012). No distinction is made between heterozygotes and homozygotes in allele frequencies and therefore number of individuals containing the allele was counted manually as indicated.

Population	Locus	Clone	Allele	Freq	# Indivs with allele
DevBF	Ever009	06E10.	172	0.013	1
EvARM	Ever004	Exe33.	175	0.019	1
Faro2	Ever004	Exe33.	164	0.012	1
Faro3	Ever009	06E10.	176	0.012	1
Faro4	Ever001	01C02.	271	0.036	2
Faro4	Ever001	01C02.	274	0.018	1
Faro4	Ever007	Exe10.	244	0.014	1
Faro5	Ever014	Exe24.	352	0.011	1
Ire_BlackRock	Ever009	06E10.	162	0.018	1
Ire_ThumbRock	Ever001	01C02.	233	0.011	1
Ire_ThumbRock	Ever014	Exe24.	331	0.013	1
JTEten	Ever009	06E10.	160	0.071	1
LTGlen	Ever011	Exe34.	140	0.025	2
Lundy	Ever006	Exe49.	337	0.024	1
ManRR	Ever009	06E10.	165	0.013	1
MenGlen	Ever010	Exe17.	237	0.012	1
PlyMew	Ever004	Exe33.	157	0.011	1
Ros1	Ever006	Exe49.	386	0.025	2
Ros2	Ever006	Exe49.	350	0.015	1

Table 3.4: *Eunicella verrucosa* heterozygosity summary statistics by locus. *H*o observed heterozygosity, *H*s heterozygosit, Fst values, Ar allelic richness. Calculated in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003).

Locus	Ho	Hs	Fst (theta)	Ar
Ever001/01C02	0.616	0.591	0.028	3.542
Ever002/Exe15	0.213	0.296	0.011	2.220
Ever003/Exe21	0.160	0.166	-0.002	1.978
Ever004/Exe33	0.673	0.693	0.005	4.906
Ever005/Exe41	0.014	0.015	0.002	1.078
Ever006/Exe49	0.715	0.826	0.009	5.857
Ever007/Exe10	0.699	0.686	0.003	4.153
Ever008/Exe48	0.451	0.454	0.001	1.998
Ever009/06E10	0.394	0.666	0.010	5.167
Ever010/Exe17	0.142	0.146	0.003	1.959
Ever011/Exe34	0.289	0.311	0.008	2.303
Ever012/Exe47	0.019	0.054	-0.004	1.245
Ever013/Exe50	0.522	0.642	0.151	5.309
Ever014/Exe24M	0.668	0.678	0.037	3.928
Overall	0.398	0.445	0.029	3.260

Table 3.5 Analysis of Molecular Variance (AMOVA) tests to determine hierarchical population structure of *Eunicella verrucosa* (tested in Arlequin v. 3.5.1.2, Excoffier 2010). Top: all data was assigned to one group, bottom: data was split into five groups based upon preliminary STRUCTURE results (Marseilles, Portugal, Brittany, Ireland and the UK).

One Group

Source of variation	Percentage of Variation	d.f.	Fstat	Fvalue	P-value	Sum of squares	Variance components
Among populations	1.43	29	FST	0.01425	0	162.584	0.04089 Va
Among individuals within populations	6.59	925	Fis	0.06681	0	2790.327	0.18891 Vb
Within individuals	91.99	955	Fit	0.08011	0	2520	2.63874 Vc
Total		**1909**				**5472.911**	**2.86854**

Five Groups

Source of variation	Percentage of Variation	d.f.	Fstat	Fvalue	P-value	Sum of squares	Variance components
Among groups	3.29	4	F_{CT}	0.03292	0	129.874	0.09533 Va
Among populations within groups	-0.94	25	FST	0.00128	1	32.71	-0.02710 Vb
Among individuals within populations	6.52	925	Fis	0.10481	0	2790.327	0.18891 Vc
Within individuals	91.12	955	Fit	0.10595	0	2520	2.63874 Vd
Total		**1909**				**5472.911**	**2.89588**

Table 3.6: Summary statistics by population for *Eunicella verrucosa*. *N* number of individuals included from each population, *k* (total) all alleles in all loci totalled, *k* (mean) average number of alleles per locus, *Ar* average allelic richness, *Ho* observed heterozygosity, *Hs* heterozygosity within populations, *G*is inbreeding coefficient, *F*is inbdreeding coeffiecient, P value of F*is* value(Rand FIS>=Obs FIS). * values calculated in Arlequin v. 3.5.1.2 (Excoffier 2010, 1023 permutations), Ar calculated in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003) and all other values derived from Genodive v2.0b23 (Meirmans and van Tiendieren 2004). Populations in **bold type** had 13 usable loci as calculated in Arlequin, in all others 14 loci were useable. Standard errors of F-statistics in Genodive are obtained via jack-knifing over loci and 95% confidence intervals through bootstrapping (data not shown). For a detailed presentation of summary statistics by locus for all populations, see Appendix 3. Samples are colour coded regionally: red = Mediterranean, yellow = Portugal, green = Brittany, pink = Ireland, blue = UK.

Populations	N	*K** (total)	*K** (mean)	*Ar*	*Ho*	*Hs*	*G*is	*FIS**	*P*-value*
Ev Marseilles	13	41	2.9	2.50	0.34	0.38	0.11	0.09	0.12
Ev Armacao	27	65	4.6	3.16	0.36	0.44	0.17	**0.16**	**0**
Faro1	41	75	5.4	3.13	0.39	0.44	0.12	**0.07**	**0.03**
Faro3	42	77	5.5	3.26	0.38	0.46	0.16	**0.09**	**0**
Faro2	43	72	5.1	3.06	0.39	0.42	0.08	**0.06**	**0.03**
Faro4	35	77	5.5	3.21	0.39	0.46	0.15	**0.09**	**0.01**
Faro5	44	70	5.0	3.17	0.37	0.43	0.14	**0.13**	**0**
Brest3	43	78	5.6	3.15	0.4	0.44	0.09	**0.06**	**0.04**
LTGlen	40	71	5.1	3.01	0.38	0.43	0.13	0.02	0.29
MenGlen	43	77	5.5	2.99	0.39	0.43	0.08	0.03	0.21
Roscoff2	40	74	5.3	3.09	0.41	0.44	0.08	0.05	0.11
Roscoff1	36	79	5.6	3.25	0.42	0.47	0.12	**0.09**	**0.01**
Ire_BlackRock	29	59	4.2	2.78	0.37	0.38	0.04	0.01	0.39
Ire_ThumbRock	48	71	5.1	2.92	0.33	0.40	0.17	**0.12**	**0**
DevonBF	40	70	5.0	3.09	0.4	0.45	0.11	**0.07**	**0.02**
PlymouthMew	44	78	5.6	3.20	0.43	0.47	0.09	**0.06**	**0.02**
HandsDeep	36	73	5.2	3.35	0.44	0.49	0.11	**0.06**	**0.04**
IoScilly Hath	30	61	4.4	3.02	0.36	0.43	0.16	**0.09**	**0.02**
IoScilly LR	22	61	4.4	3.10	0.43	0.45	0.04	0.03	0.28
IoScilly nnw	23	61	4.4	2.96	0.41	0.42	0.04	0.01	0.46
JT Eest Ten	7	39	2.8	3.00	0.41	0.47	0.13	0.10	0.16
Lundy	22	65	4.6	3.17	0.41	0.46	0.1	0.06	0.11
Lyme Bay HW	9	55	3.9	3.48	0.42	0.49	0.15	0.10	0.07
Manacles Mo	30	63	4.5	3.08	0.35	0.44	0.19	**0.15**	**0**
Manacles RR	43	83	5.9	3.27	0.43	0.47	0.08	0.03	0.15
Manacles V	24	59	4.2	3.07	0.42	0.44	0.05	-0.03	0.76
Sawtooth	12	51	3.6	2.94	0.33	0.42	0.21	**0.20**	**0**
Skomer	39	72	5.1	3.16	0.45	0.47	0.04	0.01	0.37
West Ten	43	80	5.7	3.32	0.44	0.48	0.07	**0.05**	**0.05**
nr Padstow	7	43	3.1	2.99	0.50	0.48	-0.06	-0.10	0.87

Table 3.7: Tests for evidence of genetic bottlenecks according to the software BOTTLENECK v1.2.02 (Cornuet and Luikart 1996) under the infinite alleles (IAM), two phase model (TPM) and stepwise mutation model (SMM) assuming mutation-drift equilibrium. He E expected number of loci with heterozygosity excess, E/D numbers of loci with heterozygosity excess / heterozygosity deficiency. Shaded cells represent significant P values at the 5% level (i.e., showing evidence of a bottleneck) under Sign or Wilcoxon sign ranked tests.

	IAM				TPM				SMM			
Population	**He E**	**E/D**	**Sign Test**	**Wilcoxon Test**	**He E**	**E/D**	**Sign Test**	**Wilcoxon Test**	**He E**	**E/D**	**Sign Test**	**Wilcoxon Test**
EvMai	6.61	7/5	0.530	0.677	6.65	7/5	0.539	1.000	6.93	7/5	0.604	0.519
EvARM	7.14	8/5	0.424	0.685	7.27	8/5	0.454	0.893	7.57	2/11	0.002	0.004
Faro1	7.28	8/5	0.455	0.839	7.39	7/6	0.520	0.685	7.58	1/12	0.000	0.001
Faro3	7.91	8/6	0.591	0.855	7.96	7/7	0.398	0.502	8.32	1/13	0.000	0.000
Faro2	7.23	7/6	0.555	1.000	7.28	5/8	0.159	0.497	7.55	3/10	0.011	0.003
Faro4	7.82	7/7	0.428	0.542	7.99	5/9	0.089	0.268	8.12	2/12	0.001	0.001
Faro5	7.15	9/4	0.225	0.588	7.23	7/6	0.556	1.000	7.47	3/10	0.013	0.004
Brest3	7.25	6/7	0.334	0.636	7.41	3/10	0.014	0.191	7.53	3/10	0.012	0.003
LTGlen	7.80	7/7	0.432	0.715	7.91	6/8	0.221	0.787	8.17	4/10	0.023	0.004
MenGlen	7.59	7/7	0.477	0.502	7.68	7/7	0.459	0.326	7.83	3/11	0.009	0.003
Ros2	7.79	9/5	0.354	1.000	7.90	7/7	0.410	0.715	8.17	2/12	0.001	0.000
Ros1	7.76	6/8	0.247	0.761	7.79	6/8	0.241	0.173	8.19	3/11	0.005	0.001
Ire_BlackRock	6.95	7/6	0.601	1.000	7.03	7/6	0.600	0.636	7.40	3/10	0.014	0.048
Ire_ThumbRock	7.14	7/6	0.576	0.946	7.10	6/7	0.365	0.636	7.41	3/10	0.014	0.998
DevBF	7.60	6/8	0.274	0.903	7.70	6/8	0.257	0.715	8.04	4/10	0.027	0.020
PlyMew	7.87	6/8	0.228	0.542	8.00	6/8	0.208	0.194	8.18	2/12	0.001	0.001
HandsDeep	7.75	8/6	0.557	0.626	7.94	7/7	0.401	0.761	8.24	4/10	0.021	0.003
IoSHath	6.64	7/7	0.252	0.677	6.69	4/8	0.100	0.301	6.91	2/10	0.005	0.005
IoSLR	7.35	8/5	0.472	0.839	7.39	8/5	0.480	0.946	7.60	5/8	0.119	0.080
IoSnnw	7.59	6/8	0.276	0.626	7.55	5/9	0.134	0.391	7.91	4/10	0.032	0.013
JTEten	6.14	9/3	0.084	0.077	6.42	9/3	0.112	0.129	7.05	7/5	0.598	0.569
Lundy	7.07	6/7	0.373	0.787	7.23	4/9	0.062	0.893	7.42	4/9	0.051	0.005
LymeHW	7.32	9/4	0.256	0.685	7.42	8/5	0.487	0.893	7.48	5/8	0.132	0.414
ManMo	7.60	7/7	0.475	1.000	7.63	6/8	0.270	0.463	8.03	3/11	0.007	0.005
ManRR	7.46	7/6	0.504	0.787	7.55	6/7	0.275	0.244	7.69	2/11	0.002	0.001
ManV	7.30	6/7	0.325	0.839	7.41	5/8	0.142	0.455	7.68	4/9	0.037	0.021
Sawtooth	7.40	5/9	0.153	0.326	7.60	4/10	0.046	0.194	7.75	4/10	0.038	0.003
Skomer	7.06	7/6	0.594	0.588	7.12	5/8	0.181	0.839	7.30	3/10	0.016	0.027
WestTen	7.80	8/6	0.569	0.952	7.93	6/8	0.217	0.542	8.02	3/11	0.007	0.002
nrPad	6.25	2/10	0.026	0.002	6.71	9/3	0.148	0.006	7.03	8/4	0.399	0.176

Table 3.8 Matrix of pairwise population Fst values calculated in Arlequin (negative values generated by this software are effectively zero). Values significant after correction with the false discovery rate method are highlighted. Populations are listed in a regional organization as follows: Mediterranean (EvMai, red), Portugal (yellow), Brittany (green), Ireland (pink), UK (blue, for specific details see Table 3.1).

	EvMAI	EvARM	Faro1	Faro3	Faro2	Faro4	Faro5	Brest3	LTGlen	MenGlen	Ros2	Ros1	Ire_BlackRock	Ire_ThumbRock
EvMAI														
EvARM	**0.046**													
Faro1	**0.061**	-0.002												
Faro3	**0.059**	-0.004	-0.007											
Faro2	**0.043**	-0.004	-0.002	-0.002										
Faro4	**0.032**	0.001	0.001	-0.004	0.002									
Faro5	**0.065**	-0.002	-0.002	-0.007	-0.001	0.001								
Brest3	**0.027**	**0.020**	**0.036**	**0.025**	**0.025**	**0.025**	**0.030**							
LTGlen	**0.052**	**0.015**	**0.031**	**0.013**	**0.025**	**0.023**	**0.024**	-0.001						
MenGlen	**0.041**	**0.029**	**0.042**	**0.028**	**0.039**	**0.035**	**0.042**	0.001	-0.007					
Ros2	**0.029**	0.012	**0.018**	0.007	**0.015**	0.010	**0.019**	0.004	-0.006	0.007				
Ros1	**0.034**	**0.023**	**0.030**	**0.018**	**0.023**	**0.016**	**0.028**	0.006	-0.007	**0.010**	-0.001			
Ire_BlackRock	**0.113**	**0.068**	**0.073**	**0.060**	**0.072**	**0.066**	**0.069**	**0.054**	**0.028**	**0.049**	**0.049**	**0.045**		
Ire_ThumbRock	**0.115**	**0.065**	**0.073**	**0.070**	**0.071**	**0.066**	**0.070**	**0.057**	**0.030**	**0.054**	**0.053**	**0.050**	-0.002	
DevBF	**0.037**	**0.031**	**0.045**	**0.031**	**0.037**	**0.034**	**0.045**	0.004	-0.004	0.003	0.006	0.002	**0.046**	**0.046**
PlyMew	**0.029**	**0.023**	**0.028**	**0.018**	**0.030**	**0.023**	**0.038**	0.006	-0.010	0.005	**0.010**	**0.008**	**0.050**	**0.052**
HandsDeep	**0.060**	**0.036**	**0.043**	**0.031**	**0.045**	**0.032**	**0.045**	**0.014**	-0.005	**0.016**	**0.015**	**0.013**	**0.048**	**0.049**
IoSHath	0.003	-0.050	-0.026	-0.019	-0.034	-0.042	-0.039	-0.049	-0.053	-0.039	-0.061	-0.056	-0.006	0.006
IoSLR	**0.052**	**0.047**	**0.047**	**0.031**	**0.052**	**0.040**	**0.055**	**0.017**	-0.019	0.005	**0.016**	**0.016**	**0.042**	**0.043**
IoSnnw	**0.031**	**0.015**	**0.032**	**0.027**	**0.019**	**0.026**	**0.028**	-0.005	-0.008	0.003	0.000	0.000	**0.052**	**0.054**
JTEten	-0.003	-0.002	0.004	-0.014	-0.003	-0.008	-0.001	-0.019	0.010	-0.015	-0.042	-0.034	0.000	0.002
Lundy	**0.029**	**0.033**	**0.038**	**0.029**	**0.034**	**0.024**	**0.043**	0.011	-0.006	**0.018**	0.009	0.004	**0.052**	**0.050**
LymeHW	**0.084**	**0.032**	**0.048**	**0.032**	**0.050**	**0.053**	**0.049**	0.014	0.004	0.017	0.014	**0.029**	**0.076**	**0.072**
ManMo	-0.017	-0.006	0.011	-0.007	-0.002	0.001	0.005	-0.020	-0.001	-0.019	-0.030	-0.034	0.011	**0.016**
ManRR	**0.027**	**0.029**	**0.038**	**0.028**	**0.033**	**0.026**	**0.041**	0.008	0.006	**0.016**	**0.011**	**0.008**	**0.054**	**0.051**
ManV	**0.022**	**0.033**	**0.047**	**0.034**	**0.034**	**0.035**	**0.043**	0.005	**0.027**	**0.016**	0.009	0.006	**0.057**	**0.056**
Sawtooth	**0.053**	**0.051**	**0.065**	**0.050**	**0.055**	**0.046**	**0.062**	0.007	-0.016	0.008	0.019	0.012	**0.057**	**0.061**
Skomer	**0.024**	**0.021**	**0.030**	**0.023**	**0.029**	**0.021**	**0.039**	0.003	-0.008	0.003	0.001	0.004	**0.050**	**0.048**
WestTen	**0.046**	**0.039**	**0.045**	**0.032**	**0.046**	**0.036**	**0.054**	**0.009**	-0.018	0.005	**0.013**	**0.012**	**0.048**	**0.046**
nrPad	**0.063**	**0.037**	**0.035**	0.029	**0.045**	**0.037**	**0.054**	**0.034**	0.018	0.021	0.018	0.023	**0.072**	**0.060**

Table 3.8 continued.

	DevBF	PlyMew	HandsDeep	IoSHath	IoSLR	IoSnnw	JTEten	Lundy	LymeHW	ManMo	ManRR	ManV	Sawtooth	Skomer	WestTen	nrPad
EvMAl																
EvARM																
Faro1																
Faro3																
Faro2																
Faro4																
Faro5																
Brest3																
LTGlen																
MenGlen																
Ros2																
Ros1																
Ire_BlackRock																
Ire_ThumbRock																
DevBF																
PlyMew	-0.007															
HandsDeep	0.001	0.005														
IoSHath	-0.070	-0.077	-0.070													
IoSLR	0.000	0.000	0.005	-0.090												
IoSnnw	-0.017	-0.009	0.004	-0.029	0.001											
JTEten	-0.031	-0.056	-0.055	-0.097	-0.062	-0.048										
Lundy	-0.004	-0.004	0.000	-0.061	0.003	-0.003	-0.055									
LymeHW	0.000	0.015	0.010	-0.042	**0.030**	0.004	-0.050	0.022								
ManMo	-0.031	-0.056	-0.037	-0.084	-0.059	-0.050	-0.002	-0.042	-0.032							
ManRR	-0.001	-0.006	-0.001	-0.076	0.009	-0.012	-0.024	-0.004	0.016	-0.027						
ManV	-0.008	-0.017	-0.005	-0.048	-0.001	-0.024	-0.006	-0.009	-0.005	-0.008	-0.009					
Sawtooth	-0.009	0.008	0.003	-0.074	0.012	-0.014	-0.042	0.002	0.019	-0.050	-0.005	-0.018				
Skomer	-0.004	-0.004	0.003	-0.070	0.008	-0.010	-0.054	-0.002	0.011	-0.046	-0.004	-0.011	0.010			
WestTen	-0.007	0.000	-0.005	-0.075	0.005	-0.008	-0.066	-0.007	0.006	-0.053	-0.006	-0.021	-0.003	-0.001		
nrPad	0.016	0.000	0.017	-0.039	0.021	0.024	-0.038	0.017	0.024	-0.014	0.015	0.024	**0.048**	0.007	0.008	

Table 3.9 Comparison of Jost's D estimates and pairwise Fst values in all populations using 11 out of 14 loci (following removal of 01C02 / Ever001, Exe47 / Ever 012 and Exe50 / Ever013 due to missing data) a) Pairwise Dest matrix and b) Pairwise Fst. Significant comparisons are highlighted, in pairwise Fst this is following FDR correction.

a) Pairwise Dest Matrix	EvMai	EvARM	Faro1	Faro3	Faro2	Faro4	Faro5	Brest3	LTGlen	MenGlen	Ros2	Ros1	Ire_ BlackRock	Ire_ ThumbRock	DevBF
EvMai															
EvARM	0.009														
Faro1	0.012	-0.004													
Faro3	0.014	-0.003	-0.003												
Faro2	0.017	-0.006	-0.004	0.000											
Faro4	0.006	-0.003	-0.002	-0.002	0.001										
Faro5	0.025	-0.003	-0.001	-0.002	-0.002	-0.002									
Brest3	0.019	0.004	0.011	0.006	0.013	0.007	0.012								
LTGlen	0.030	0.018	0.023	0.015	0.027	0.017	0.026	0.005							
MenGlen	0.021	0.016	0.023	0.014	0.027	0.020	0.031	0.000	0.003						
Ros2	0.021	0.004	0.004	-0.002	0.010	0.002	0.010	0.003	0.006	0.007					
Ros1	0.024	0.009	0.012	0.005	0.014	0.006	0.013	0.004	0.001	0.006	0.000				
Ire_BlackRock	0.025	0.015	0.018	0.009	0.018	0.014	0.019	0.008	0.001	0.006	0.008	-0.001			
Ire_ThumbRock	0.033	0.014	0.020	0.016	0.020	0.018	0.023	0.011	0.002	0.007	0.010	0.004	-0.002		
DevBF	0.027	0.010	0.018	0.012	0.021	0.017	0.024	0.001	0.004	0.001	0.004	0.001	0.004	0.002	
PlyMew	0.010	0.008	0.013	0.012	0.019	0.016	0.023	0.004	0.012	0.007	0.009	0.007	0.012	0.012	-0.003
HandsDeep	0.036	0.014	0.023	0.021	0.026	0.017	0.024	0.004	0.007	0.012	0.008	0.005	0.011	0.009	-0.002
IoSHath	0.022	0.017	0.024	0.018	0.028	0.019	0.029	0.007	-0.001	0.003	0.005	0.004	0.002	0.004	-0.003
IoSLR	0.019	0.022	0.023	0.016	0.027	0.022	0.030	0.009	0.006	0.005	0.010	0.007	0.004	0.005	0.000
IoSnnw	0.021	0.005	0.013	0.011	0.013	0.018	0.019	0.001	0.005	0.003	0.007	0.005	0.002	0.001	-0.007
JTEten	0.026	0.010	0.004	0.004	0.017	0.000	0.015	0.004	0.013	0.012	-0.013	0.001	0.010	0.001	-0.002
Lundy	0.014	0.003	0.007	0.007	0.011	0.004	0.014	0.003	0.001	0.010	0.003	-0.002	0.002	-0.001	-0.004
LymeHW	0.058	0.016	0.031	0.021	0.038	0.043	0.037	0.012	0.021	0.016	0.015	0.026	0.027	0.021	0.000
ManMo	0.011	0.008	0.013	0.007	0.018	0.011	0.021	0.000	0.001	0.000	-0.002	-0.001	0.005	0.005	-0.006
ManRR	0.020	0.009	0.015	0.016	0.020	0.012	0.021	0.003	0.006	0.012	0.008	0.007	0.015	0.011	0.000
ManV	0.025	0.010	0.016	0.015	0.021	0.020	0.023	0.002	0.012	0.008	0.011	0.012	0.012	0.009	-0.004
Sawtooth	0.037	0.017	0.026	0.022	0.028	0.022	0.027	0.001	-0.005	0.003	0.012	0.005	0.000	-0.002	-0.008
Skomer	0.013	0.005	0.011	0.012	0.017	0.009	0.022	0.003	0.008	0.005	0.001	0.004	0.014	0.010	-0.002
WestTen	0.032	0.014	0.021	0.019	0.027	0.020	0.029	0.005	0.003	0.005	0.010	0.007	0.008	0.003	-0.005
nrPad	0.028	0.022	0.020	0.023	0.030	0.028	0.042	0.022	0.023	0.014	0.016	0.014	0.028	0.014	0.008

Table 3.9 continued.

a)

	PlyMew	HandsDeep	IoSHath	IoSLR	IoSnnw	JTEten	Lundy	LymeHW	ManMo	ManRR	ManV	Sawtooth	Skomer	WestTen	nrPad
PlyMew															
HandsDeep	0.005														
IoSHath	0.003	-0.003													
IoSLR	0.000	0.007	0.000												
IoSnnw	-0.005	0.001	-0.004	-0.001											
JTEten	0.002	-0.013	0.004	0.004	0.005										
Lundy	-0.002	-0.002	0.002	-0.001	-0.002	-0.014									
LymeHW	0.014	0.008	0.012	0.024	0.004	0.000	0.018								
ManMo	-0.004	-0.003	-0.008	-0.004	-0.006	-0.009	-0.005	0.007							
ManRR	-0.002	-0.003	0.000	0.009	-0.001	-0.004	-0.002	0.017	-0.004						
ManV	-0.003	-0.002	0.002	0.007	-0.006	-0.005	0.002	0.001	-0.004	-0.006					
Sawtooth	0.004	-0.005	-0.012	-0.001	-0.010	0.008	-0.001	0.007	-0.004	-0.001	0.000				
Skomer	-0.002	0.000	0.003	0.007	-0.001	-0.009	-0.003	0.013	-0.005	-0.002	0.001	0.007			
WestTen	0.000	-0.005	-0.004	0.004	-0.005	-0.007	-0.005	0.004	-0.004	-0.003	-0.004	-0.007	-0.002		
nrPad	0.004	0.021	0.020	0.026	0.014	-0.001	0.008	0.024	0.009	0.014	0.015	0.033	0.004	0.007	

Table 3.9 continued.

b) Pairwise Fst Matrix (FDR Corrected)	EvMai	EvARM	Faro1	Faro3	Faro2	Faro4	Faro5	Brest3	LTGlen	MenGlen	Ros2	Ros1	Ire_ BlackRock	Ire_ ThumbRock	DevBF
EvMai															
EvARM	0.023														
Faro1	0.023	0.007													
Faro3	0.024	0.008	0.005												
Faro2	0.026	0.005	0.004	0.007											
Faro4	0.020	0.008	0.006	0.006	0.008										
Faro5	0.031	0.007	0.006	0.006	0.005	0.006									
Brest3	0.027	0.011	0.013	0.010	0.014	0.012	0.014								
LTGlen	0.035	0.020	0.022	0.016	0.024	0.018	0.023	0.010							
MenGlen	0.029	0.019	0.021	0.015	0.024	0.020	0.026	0.006	0.008						
Ros2	0.029	0.012	0.010	0.007	0.013	0.009	0.013	0.009	0.011	0.011					
Ros1	0.031	0.015	0.014	0.010	0.015	0.011	0.015	0.009	0.008	0.011	0.007				
Ire_BlackRock	0.034	0.020	0.021	0.014	0.020	0.018	0.020	0.013	0.009	0.012	0.014	0.008			
Ire_ThumbRock	0.038	0.018	0.019	0.016	0.019	0.018	0.021	0.013	0.008	0.011	0.013	0.010	0.007		
DevBF	0.033	0.015	0.018	0.014	0.020	0.018	0.021	0.008	0.009	0.007	0.010	0.007	0.011	0.008	
PlyMew	0.021	0.014	0.015	0.014	0.018	0.016	0.020	0.008	0.014	0.011	0.012	0.011	0.015	0.014	0.005
HandsDeep	0.038	0.018	0.021	0.019	0.023	0.017	0.021	0.009	0.012	0.014	0.012	0.010	0.015	0.012	0.006
IoSHath	0.032	0.022	0.024	0.019	0.026	0.021	0.026	0.013	0.008	0.010	0.012	0.011	0.011	0.011	0.007
IoSLR	0.031	0.026	0.025	0.020	0.027	0.024	0.028	0.015	0.014	0.013	0.016	0.014	0.014	0.013	0.010
IoSnnw	0.032	0.015	0.018	0.016	0.018	0.021	0.021	0.010	0.012	0.011	0.014	0.012	0.012	0.009	0.005
JTEten	0.049	0.034	0.027	0.028	0.034	0.026	0.033	0.027	0.032	0.031	0.019	0.026	0.032	0.026	0.024
Lundy	0.027	0.014	0.014	0.014	0.017	0.013	0.018	0.011	0.010	0.016	0.012	0.009	0.012	0.009	0.008
LymeHW	0.063	0.032	0.038	0.032	0.042	0.044	0.041	0.027	0.032	0.029	0.029	0.035	0.038	0.033	0.020
ManMo	0.024	0.015	0.016	0.013	0.019	0.016	0.021	0.008	0.009	0.008	0.008	0.008	0.013	0.011	0.005
ManRR	0.028	0.014	0.016	0.016	0.018	0.014	0.019	0.008	0.011	0.014	0.012	0.011	0.018	0.013	0.007
ManV	0.033	0.018	0.019	0.018	0.022	0.022	0.023	0.010	0.017	0.014	0.016	0.016	0.018	0.015	0.007
Sawtooth	0.051	0.030	0.033	0.030	0.034	0.031	0.034	0.016	0.013	0.017	0.024	0.019	0.017	0.014	0.011
Skomer	0.023	0.012	0.014	0.013	0.017	0.013	0.020	0.008	0.012	0.010	0.008	0.009	0.017	0.013	0.006
WestTen	0.035	0.017	0.019	0.017	0.023	0.019	0.024	0.009	0.008	0.009	0.013	0.011	0.013	0.008	0.004
nrPad	0.048	0.038	0.034	0.036	0.040	0.039	0.047	0.035	0.036	0.030	0.033	0.031	0.041	0.031	0.028

Table 3.9 continued.

b)

	DevBF	PlyMew	HandsDeep	IoSHath	IoSLR	IoSnnw	JTEten	Lundy	LymeHW	ManMo	ManRR	ManV	Sawtooth	Skomer	WestTen	nrPad
DevBF																
PlyMew	0.005															
HandsDeep	0.006	0.010														
IoSHath	0.007	0.010	0.007													
IoSLR	0.010	0.010	0.015	0.011												
IoSnnw	0.005	0.006	0.010	0.008	0.011											
JTEten	0.024	0.025	0.018	0.030	0.030	0.030										
Lundy	0.008	0.008	0.009	0.013	0.012	0.011	0.021									
LymeHW	0.020	0.027	0.024	0.029	0.037	0.025	0.039	0.033								
ManMo	0.005	0.006	0.007	0.005	0.009	0.007	0.022	0.009	0.026							
ManRR	0.007	0.005	0.005	0.008	0.015	0.009	0.023	0.009	0.029	0.006						
ManV	0.007	0.007	0.008	0.012	0.016	0.008	0.024	0.014	0.023	0.009	0.005					
Sawtooth	0.011	0.018	0.013	0.009	0.018	0.011	0.040	0.019	0.034	0.015	0.015	0.018				
Skomer	0.006	0.005	0.007	0.010	0.014	0.008	0.020	0.008	0.027	0.005	0.006	0.009	0.020			
WestTen	0.004	0.006	0.004	0.006	0.012	0.006	0.021	0.007	0.022	0.006	0.005	0.007	0.011	0.005		
nrPad	0.028	0.024	0.035	0.037	0.041	0.033	0.040	0.030	0.049	0.030	0.030	0.033	0.053	0.024	0.026	

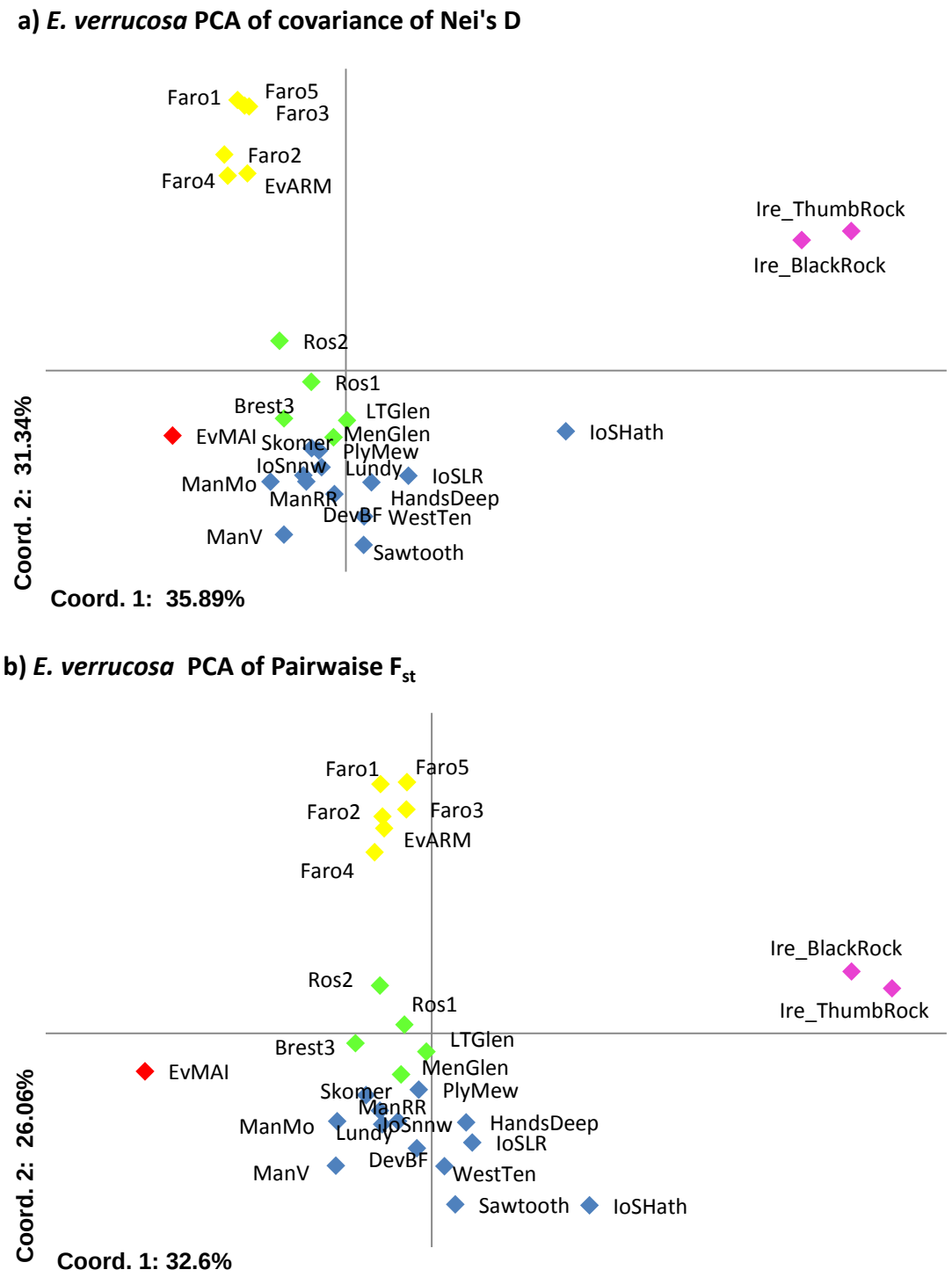
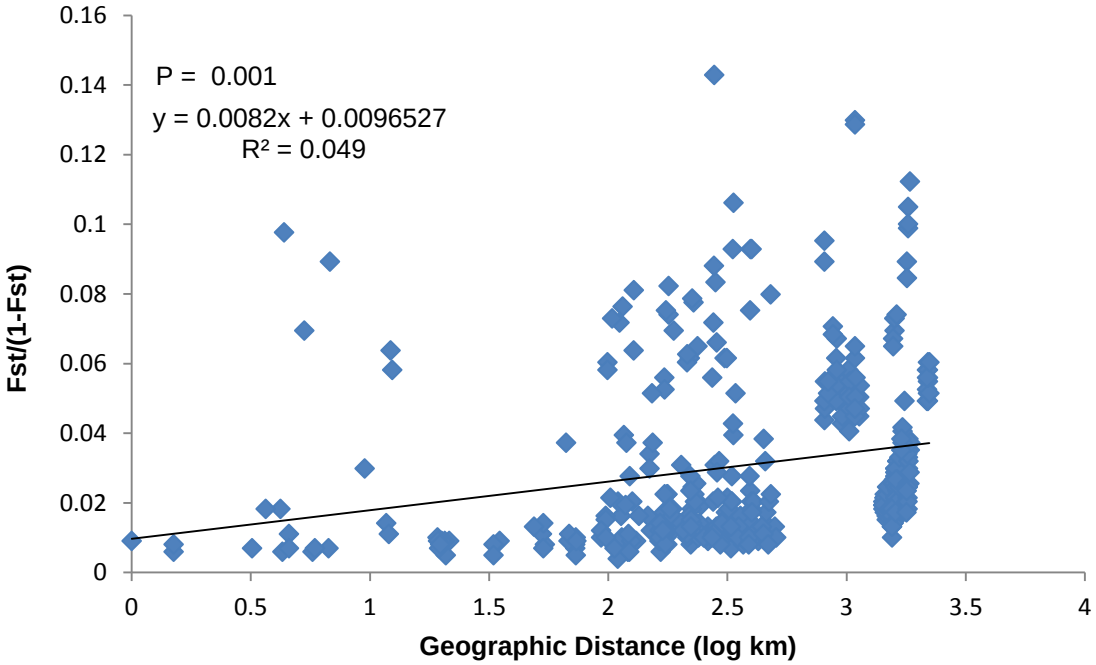


Figure 3.4 Principal coordinates analyses (PCA) of: a) covariance matrices based upon Nei's unbiased genetic distance and b) pairwise Fst for *Eunicella verrucosa*. Populations are colour coded as follows: blue = UK sites, green = Brittany, yellow = Portugal, pink = Ireland and red = Mediterranean (Marseilles). Small samples were removed from the analysis (JTEten, LymeHW and nr Pad). The first two coordinates are plotted here, although percentage variation explained by the first three axes is 84.04% for Nei's distance (35.89+31.34+16.8 for axes 1, 2 and 3 respectively) and 78.44% for pairwise Fst (32.6+ 26.06+19.78 for axes 1, 2 and 3 respectively). PCA analyses were conducted in GenAlEx v6.5b.3 (Peakall and Smouse 2012).



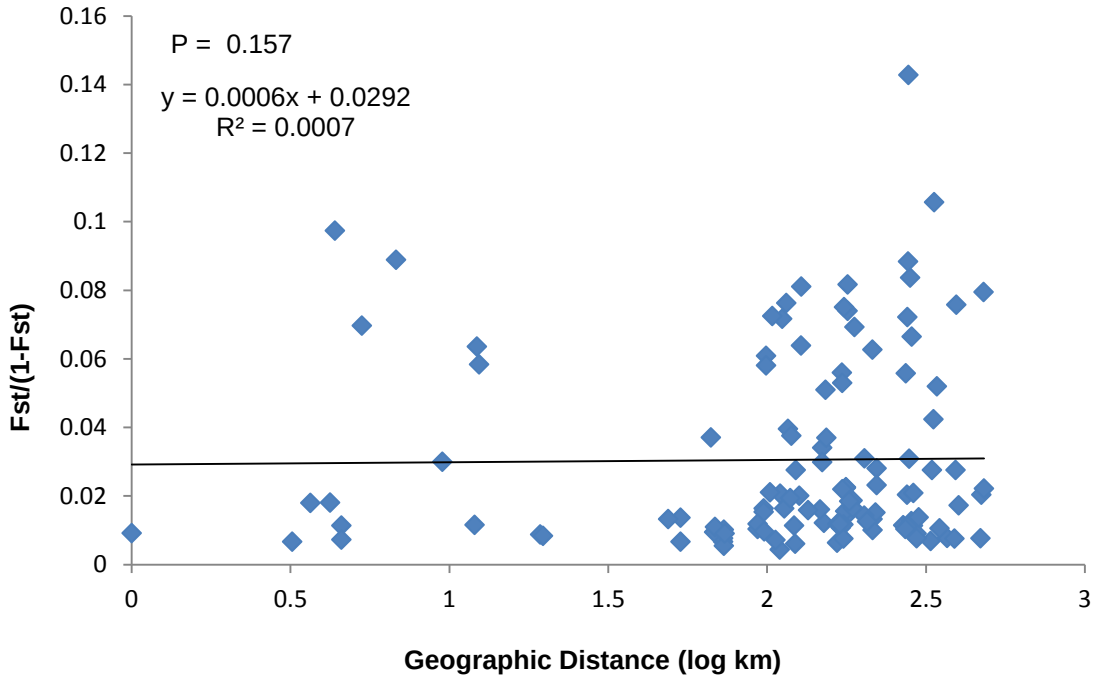


Figure 3.5. Mantel tests to determine Isolation by Distance (IBD) in *Eunicella verrucosa* across a) the entire range sampled in this study (Portugal, Brittany, Ireland and UK), and b) between UK samples only. Geographical distance explained 4% of the variation in genetic difference (linearised Fst) by log transformed geographic distance across the whole dataset, but almost none of the variation within the UK. P values correspond to one tailed P-value Pr (correlation > observed correlation) as calculated in Genepop v4.2 under the null hypothesis of independence of geographic and genetic distance (Raymond and Rousset 1995, Rousset 2008).

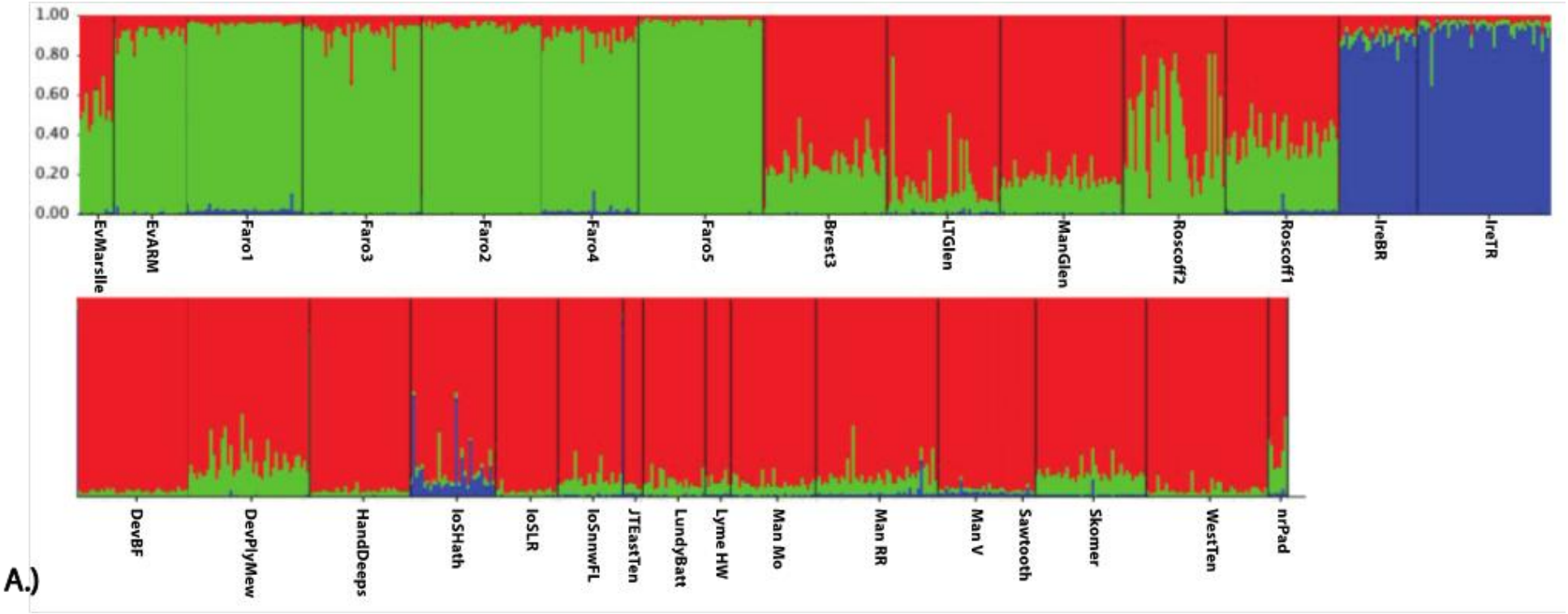


Figure 3.6. Bar plots of population clusters from STRUCTURE analysis for *Eunicella verrucosa*, showing decreasing structure with decreasing spatial scale. Y-axis represents the average probability of membership of individuals based upon 14 loci, X-axis represents individuals (grouped into populations). Populations are grouped regionally, in green are the Mediterranean and Portugal, subsequently Brittany in green and red, Ireland in blue, and the remaining populations are all from the UK (red,bottom half). **a)** clustering pattern across all data (N=955, 30 populations), showing K=3, the most likely K suggested by STRUCTURE HARVESTER (Appendix **8**), with the following settings: 10k burnin, 10^6 MCMC, using sampling location as a prior (LOCPRIOR option), correlated allele frequencies, and admixed populations, with each run replicated 2 times, **b)** data and settings as above, this time without the application of an admixture model and one replication (K=4 shown, STRUCTURE HARVESTER not conducted as only one replicate was done). Results in a) and b) are cut into two halves for clarity**, c)** UK and Brittany data only (N=633, 21 populations), settings as above, two replicates, K=2 following recommended value from STRUCTURE HARVESTER (Appendix 7) and **d)** data from the UK only (N=431, 16 populations).

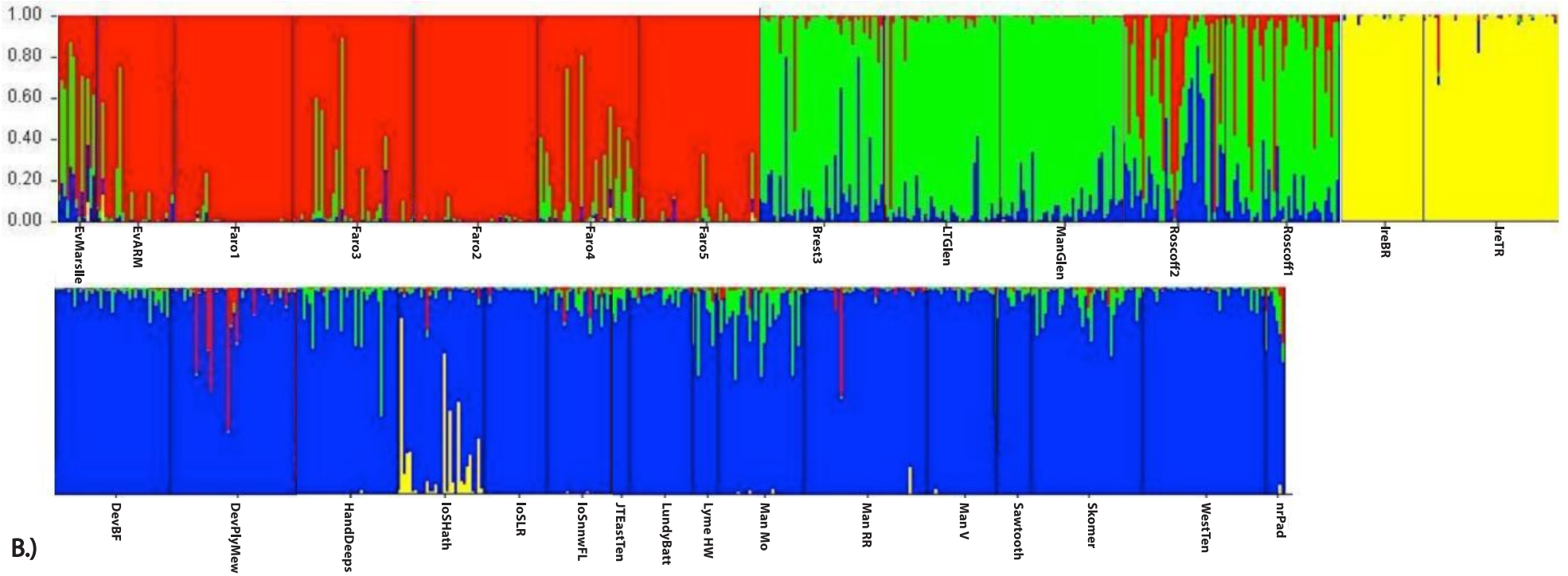


Figure 3.6 continued.

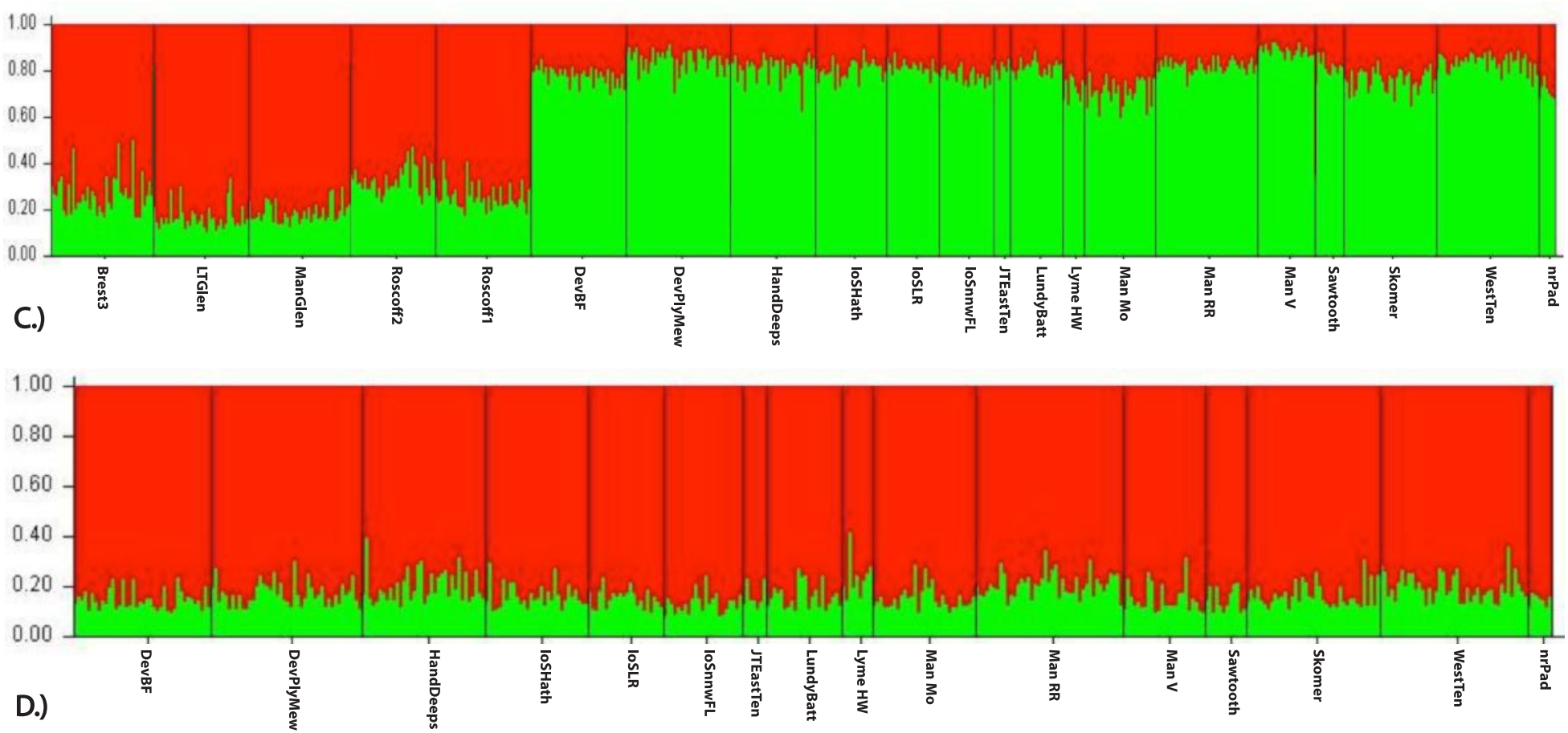


Figure 3.6 continued.

Table 3.10: Gene flow estimates among the clusters of *Eunicella verrucosa* populations suggested by STRUCTURE analyses, based on a Bayesian likelihood inference calculated using the software MIGRATE (Beerli & Felsenstein 2001). Theta (θ) values are shaded in bold on the diagonal and were used to calculate Ne assuming a mutation rate of $\mu=10^{-4}$ per locus per generation ($Ne = \theta/ 4\mu$). Rows and columns represent source and sink populations, respectively, and all populations (N=30) were grouped according to the four regional clusters as indicated.

		Source:				
		"Brittany" (1)	**"UK" (2)**	**"Portugal & Med" (3)**	**"Ireland" (4)**	**Ne**
Sink:	**"Brittany" (1)**	**1.05**	8.05	2.25	0.95	2625
	"UK" (2)	24.95	**0.95**	0.85	2.85	2375
	"Portugal & Med" (3)	0.95	21.55	**1.18**	8.45	2958
	"Ireland" (4)	1.55	5.45	1.65	**0.75**	1875

3.4 Discussion

3.4.1 Overview

Using fourteen novel microsatellites, I found high levels of genetic connectivity between populations of *Eunicella verrucosa* sampled within each of the regions of the UK, Ireland, France and Portugal, with evidence for disparate genetic clusters in western Ireland, southern Portugal, and Brittany plus the southwest UK and Wales, with some divergence between the UK and France. Therefore, the overall genetic structure seen here supports regional divergence of *E. verrucosa* in the NE Atlantic. These clusters are supported by multivariate clustering (PCA), Bayesian admixture (STRUCTURE) and isolation by distance (Mantel) analyses, although overall low Fst and Dest values suggest that there is little differentiation within and between each of these regions, and it appears that a large proportion of the variation is at the individual level and is driven by a few loci that may be subject to positive selection. There was no indication of poly-modal larval dispersal from the genetic data obtained in this study (as suggested by Shanks *et al.* 2003). Coalescent analyses suggest that gene flow is often asymmetrical between regions, yet contrary to expected patterns, may occur in a southward direction. Irish samples of *E. verrucosa* form a distinct cluster, with a corresponding lowered allelic richness as may be expected for range-limit populations, yet only one out of two Irish samples demonstrated lower than expected heterozygosity and a high likelihood of inbreeding, and bottleneck tests proved inconclusive given uncertainty over microsatellite evolution patterns in this species. Very few duplicate genotypes affirm the hypothesis that *E. verrucosa* reproduces sexually. More than half of all sampled populations show heterozygote deficits indicative of inbreeding; as isolation by distance analyses indicate that populations at local to regional scales are not at equilibrium, the apparently high extent of connectivity indicated here may not be representative of demographic connectivity.

3.4.2 Patterns of Genetic Diversity in *Eunicella verrucosa*

Across my sampled range, *E. verrucosa* shows evidence for high gene flow at local population scales, yet significant differentiation between regions and some localized areas. This suggests that the population genetic structure of *E. verrucosa* might be more appropriately described as a metapopulation- defined as “an assemblage of discrete local populations with some measure of shared migration among them” (Cowen and Sponaugle 2009). Originally, metapopulation models were developed in ecology to explain interactions of conspecific population networks that showed no structure, but that shared migrants at some level and therefore whose dynamics were shaped by local colonisation and extinction (Mumby 1999). Metapopulation models have since been applied with increasing frequency to describe dynamics between

marine populations, especially for patchy habitats and animals depending upon pelagic larval dispersal, as such Caribbean coral reefs (Mumby 1999). The predictive value of metapopulation models are also valuable in the context of marine protected areas network design in terms of size and spacing of them to maximise connectivity (e.g., Botsford *et al.* 2009, Kininmonth *et al.* 2011). In the North East Atlantic, connectivity patterns have been ascribed to a metapopulation dynamic in many sessile taxa, such as brittle stars (*Ophiothrix fragilis*, Lefebvre *et al.* 2003), and freshwater bryozoans (*Cristatella mucedo*, Freeland *et al.* 2000).

Eunicella verrucosa appears to have high rates of inbreeding, and was noted at sixteen out of thirty sites. The prevalence of inbreeding is surprising given the low population structure observed (and hence implication of high connectivity). This could be indicative of a high level of self-seeding. Interestingly, all Portuguese samples are likely to be inbred, but elsewhere in the data the distribution of inbreeding did not follow a particular geographic pattern. Reasons for this are unclear, and suggest that despite evidence for migration from other more northerly populations in Europe (i.e., Ireland and the UK, Table 3.10), populations in that region may be isolated. One alternative hypothesis for elevated rates of inbreeding could be that it results from anthropogenic activity. Elsewhere, heterozygote deficiencies have been observed in populations subject to overharvesting or to fishing damage. For example, Henry & Kenchington (2004) found increased rates of clonality following trawling damage in the hydroid *Sertularia cupressina*, which over time could reduce genetic diversity, and Costantini *et al.* (2007) attributed reduced genetic diversity in *Corallium rubrum* to overharvesting. Trawling damage is likely to have considerable negative effects on benthic fauna, including that found in the Lyme Bay area of my study (Hinz *et al.* 2011). Two of my four samples from Lyme Bay also show evidence for inbreeding (Sawtooth and Lyme Bay), although trawling activity cannot be unequivocally blamed as rocky outcrops typical of *E. verrucosa* habitat in that area are less likely to be trawled. Whether trawling activity, as opposed to local hydrodynamic regimes or biogeographic barriers, is the cause of elevated inbreeding in the southern Portuguese samples, and indeed all inbred samples here, is unclear, but is an interesting hypothesis which may provide collateral in conservation contexts.

Samples collected from two sites in Donegal Bay- Thumb Rock on the south side and Black Rock on the North side (Fig. 3.1f), are the most divergent samples in my study based upon Bayesian admixture and PCA clustering analyses, where they are distinct to any other sampled population. This is interesting given that Donegal Bay represents the most northerly limit of the distribution of *E. verrucosa* (marlin.ac.uk) and as such these populations may be considered marginal to my other samples and to populations further south in its range, extending to Angola (Grasshoff 1992). Without the benefit of additional genotypic sampling

from its southern extremities, and given its large range, it is difficult to infer the origins of the Irish *E. verrucosa* populations or to determine the extent of their isolation. However, it does appear from coalescent analyses (Table 3.10) that there is some connection between Irish and UK and Irish and Portuguese sea fans.

The Thumb Rock site shows evidence for high levels of inbreeding, and both sites contain private alleles (one in Black Rock and two in Thumb Rock), indicating some degree of isolation. There is also reduced allelic richness in both of these samples. Marginal populations are typically characterised by reduced and/or divergent genetic diversity and this has been demonstrated repeatedly for numerous marine invertebrates. Divergence in peripheral populations, usually evidenced by reduced haplotypic diversity compared to adjacent populations or to the metapopulation as a whole, is attributed to one of two scenarios; recent range expansions made possible by, for example, favourable shifts in environmental conditions (which may correlate to a latitudinal gradient) or alternatively, to population fragmentation due to habitat disturbance or vicariant events. Hellberg *et al.* (2001) detected a reduced diversity of mtCO1 haplotypes in northerly Californian populations of the gastropod *Acanthinucella spirata*, which they attributed to a post-last glacial maximum (LGM) range expansion. Sotelo *et al.* (2009) also suggest the velvet swimming crab *Necora puber* has undergone a recent demographic expansion after the LGM in the NE Atlantic (based upon C01 and 16S data). In a coral species with a trans-Atlantic range, Nunes *et al.* (2009) found reduced haplotypic diversity in peripheral populations in Brazil and West Africa which they attributed to founder effects. On a larger scale, Johannesson and Andre (2006) reviewed genetic diversity of 29 species in the Baltic Sea and noted such extensive losses of diversity that this entire area could be considered a marginal ecosystem. Discontinuous populations may warrant extra attention in conservation management strategies if they are genetically compromised, as decreases in allelic richness in marginal populations can be indicative of a genetic bottleneck, which can lead to elevated rates of inbreeding and a reduction in genetic fitness and adaptive potential (Broquet *et al.* 2010, Wright *et al.* 2008; here I found inconclusive evidence for bottlenecks in this study, but did detect a low effective population size relative to other regions).

Divergent populations in western Ireland have been documented previously in other marine invertebrates. Sotelo *et al.* (2008) found that spiny spider crabs (*Maja brachydactyla)* were most divergent in the NE Atlantic in their most southerly and northerly sampled sites, the latter being Galway Bay. Using microsatellite data and mitochondrial 16S and COI DNA, the authors found strong differentiation of Irish crabs in both datasets, but interestingly also found some affinity between them and samples from Galicia (in the mitochondrial dataset) as well as

to the island of Ushant off the west coast of Brittany (in the microsatellite dataset). Haplotype clustering between Fowey in Cornwall, Biscay and southern Iberia (but not Brittany) was also seen in *Carcinus maenas* crabs (see 3.4.2 above, Roman and Palumbi 2004). In this study, there was no clear pattern between the Irish and Breton samples in pairwise comparisons (although in the case of Brest3, Ireland and the Isles of Scilly appear to be the most divergent to it; Table 3.8). However, it is interesting that a site in the Isles of Scilly (Hathor) was the only site with some level of similarity to the Irish cluster in STRUCTURE, which might be expected given its geographical proximity to Ireland compared to my other samples. The study of Sotelo *et al.* (2008) and Roman and Palumbi (2004) indicate that samples obtained from Galicia and/or the Bay of Biscay would be a valuable addition to my dataset and may elucidate the origins of Irish *E. verrucosa.* There is also the possibility that Irish *E. verrucosa* originated much farther south, as other marine invertebrates are not differentiated across large ranges in the Atlantic, including the shrimp *Crangon crangon* that shares the same COI haplotype from Morocco to Iceland (Luttikhuizen *et al.* 2008). Gene flow between Cornwall and southern Ireland is also sustained in the sea star *Asterina gibbosa* (Baus *et al.* 2005) and western Ireland is a hypothetical cryptic refuge with unusual diversity as suggested by Provon and Bennett (2008).

3.4.3 Population Genetic Spatial Structure of *Eunicella verrucosa*

There is a weak but highly significant (R^2 = 0.049, P=0.001 Fig. 3.4) pattern of isolation-by-distance (IBD) across the entire sampled range, spanning over 2200km from southern Portugal to western Ireland. This is not surprising given the regional-level structure described above. At smaller scales, such as among the UK samples, this correlation is lost, which supports PCA and STRUCTURE analyses. For comparison, Baus *et al.* (2005) described a strong correlation of IBD in the sea star *Asterina gibbosa* from the Mediterranean to Ireland, although no IBD was detected in Atlantic populations of the green crab *Carcinus maenas* by Roman and Palumbi (2004). This analysis infers high levels of connectivity in *Eunicella verrucosa* at scales of several hundred kilometres. However, it is of note that the linear marine distances calculated for Mantel tests may be conservative estimates of the distances travelled by larvae, whereas distances based on more realistic hydrodynamic or Lagrangian modelling of tidal residual or wind-driven currents would more accurately explain dispersal patterns in the English Channel (Lefebvre *et al.* 2003).

An alternative explanation to limited spatial genetic structure within regional clusters could be that they show non-equilibrium conditions. Patterns of isolation by distance, demonstrated by a positive correlation between genetic and geographic distances, are considered to be indicative of populations at equilibrium of dispersal and genetic drift, whereas no discernible

correlation can be indicative of the contrary (Beebee and Rowe 2004, Slatkin 1993). The time required to reach genetic equilibrium is inversely proportional to migration rates between populations, but in some cases, such as poleward recolonizations at high latitudes following glacial retreat, may not occur for 1000s of generations (Hellberg 2009). Estimated times to reach equilibrium also depend on migration models in question; Crow and Aoki (1984) determined that time taken to reach Gst (an analog of Fst that considers several loci) was slightly slower in a stepping stone than an island model and that reaching equilibrium is also slower where migration rates are low and population sizes are large. As a corollary to this assertion, Whitlock and McCauley (1999) suggest that numbers of generations required for metapopulations to reach equilibrium and time for Fst values to reach halfway to a new equilibrium are also large if population sizes are big and migration rates are low. Data obtained during this research suggest that the sampled area of *Eunicella verrucosa* may not yet have reached equilibrium, which may be expected given that my samples are from the northerly limits of its range. This assertion stems from scatter plot distributions of genetic vs. geographic distances outlined in Figure 1.1, the pattern of which follows my data at local spatial scales; in other words, Fst variation is narrow over a large spatial scale which can suggest a recolonization event (Beebee and Rowe 2004). Using microsatellite data, it is difficult to calibrate range expansions in the NE Atlantic with the retreat of the ice sheet at the end of the last glacial maximum, as has been attempted with DNA sequence data (e.g., Hoarau *et al.* 2007); nonetheless a northward range extension of *E. verrucosa* in the NE Atlantic from southerly refugia is feasible and has been shown for other marine taxa (Provan and Bennett 2008).

3.4.4 Connectivity and Biology of *Eunicella verrucosa*

Although this study is the first to examine octocoral connectivity in the UK and NE Atlantic, there have been a multitude of similar studies in overlapping areas for a range of temperate marine taxa including plants, invertebrates and vertebrates. The overall picture of gene flow in marine invertebrates across the Atlantic coast of Europe is typically portrayed as high in extent, with regional subdivisions of the Mediterranean, Western Europe and Northern Europe (Roman and Palumbi 2004). Within the Mediterranean basin, connectivity among octocorals appears to be more limited than in the Atlantic, and have shown regional-level isolation-by-distance patterns with gene flow concordant with surface currents (*Paramuricea clavata*, Mokhtar-Jamai *et al.* 2011), and strong genetic structuring even at small spatial scales (*Corallium rubrum*, Costantini *et al.* 2007). Patterns of hexacoral connectivity in the Mediterranean also suggest that regional currents coupled with reproductive ecology determine the extent of gene flow, as evidenced by stepping-stone patterns (e.g., *Astroides*

calycularis, Casado-Amueza *et al.* 2011), genetic structure within patches at small scales (e.g, *Leptosammia pruvoti*, Goffredo *et al.* 2009) and genetic fragmentation due to limited dispersal (e.g, *Balanophyllia europaea*, Goffredo *et al.* 2004b).

My samples collected from the Marseilles area, EvMai (N=13) are different to other samples according to admixture analysis and are equally likely to be from the UK/Brittany cluster as they are to the Portuguese cluster; they are also disparate to Portugal and the UK/Brittany in Fst-based PCA analyses and are significantly different to almost all other populations in pairwise comparisons (Figs. 3.6, 3.4 and Table 3.8 respectively). The Strait of Gibraltar has historically been considered a geographic barrier to gene flow for Atlanto-Mediterranean invertebrate species, as evidenced by the occurrence of divergent haplotypes in Mediterranean vs. Atlantic populations in sponges (*Crambe crambe*, Duran *et al.* 2004a), shrimp (*Crangon crangon* , Luttikhuizen *et al.* 2008), sea stars (*Asterina gibbosa*, Baus *et al.* 2005) and crabs (*Carcinas maenas*, Roman and Palumbi 2004). This barrier is likely augmented by the Almeria-Oran front at the eastern end of the Alboran Sea, where salinity, temperature and turbulence fluctuations limit planktonic dispersal (Casado-Ameuza *et al.* 2012). However, discontinuity and genetic structure is not always observed and in some cases there is little variation between populations spanning the both basins, such as in the urchin *Paracentrotus lividus* (Duran *et al.* 2004b). In this study, the lack of a totally separate admixture cluster for the Mediterranean population (e.g., such as that for the Irish samples) indicates that there may be continued gene flow between the Mediterranean and Atlantic areas, although this sample includes only thirteen individuals and therefore this hypothesis warrants more rigorous sampling. Of the *Eunicella* species present in the North East Atlantic, *E. verrucosa* has the most extensive range overall and the most northerly range limits, but two other species are present in western Africa in addition to the western Mediterranean, *E. gazella* and *E. filiformis,* whereas *E. cavolini* and *E. singularis* are Mediterranean only (Grasshoff 1992). This implies that the Gibraltar Straits represent a biogeographic barrier for at least some congeners.

There is some evidence that the western entrance to The English Channel is a biogeographic barrier to colonisation in some species, which corresponds to borders between Boreal (temperate northern Atlantic) and Lusitanian provinces (Spalding *et al.* 2007). This has been observed genetically in, for example, the polychaete worm *Pectinaria koreni*-clade 1 (Jolly *et al.* 2006), although migration and mating between French and British cuttlefish (*Sepia officinalis*) populations in the Hurd Deep area seems likely, indicating that this area is not a barrier to all species (Wolfram *et al.* 2006). For some taxa, a dispersal barrier may occur further south in Galicia, as suggested for the brittle star *Ophiothrix fragilis* (Muths *et al.* 2009) and supported by the presence of a southern clade of *Carcinus maenas* crabs (Roman and Palumbi 2004).

Interestingly, in the latter study a haplotype more typically found in the North Sea was also found in Fowey, Cornwall, but never in France, and an additional haplotype was shared between samples from Fowey with Biscay and southern Iberia but never with Brittany, implying some movement between the south west UK and locations to the North and (not immediate) south.

3.4.5 Conservation Implications for *Eunicella verrucosa*

Developing conservation efforts for marine species with metapopulation characteristics and no clear geographically defined connectivity patterns is challenging due to the confounding effects of ecological connectivity (i.e., contemporary gene flow from ongoing larval import and export) and evolutionary processes (i.e., rare migration events that homogenize populations coupled with mutation and drift, Marko and Hart 2011). This can be particularly troublesome in long-lived species with overlapping generations and high levels of clonality, such as sponges and corals, as genotypes may persist for decades to centuries and traditional F-statistics may not be sensitive enough to be indicative of present-day patterns of connectivity (Botsford *et al.* 2009). Coalescent approaches, which infer allelic evolution through time, can be used to estimate population divergence and infer effective population (Ne) size (provided the rates of mutation of the markers being used is known; e.g., Ayre *et al.* 2009). Isolation-with-migration (IM) coalescent models are being applied and are useful for non-equilibrium populations (i.e., where allelic gain through migration and gene flow are unequal to the loss of alleles through genetic drift) and which may also account for asymmetric migration and overlapping generations (Marko and Hart 2011). By using such analyses here, I have demonstrated that there is substantial association between geographically distant populations across the sampled range of *Eunicella verrucosa* (e.g., inferred migration between the UK and Ireland to Portugal). Value can therefore be applied to using such approaches in management strategies that require international effort. Parentage analysis is also informative as it can assign recruits to an individual or population, with the caveat being that the sampling effort required must cover a significant fraction of source adults and recruits must be sampled across most of the assumed range; understandably this approach has had more limited impact in the marine environment (Botsford *et al.* 2009).

Eunicella verrucosa may be considered a 'poster-child' for UK-based marine conservation efforts; it garners huge conservation interest, is monitored annually by amateur divers and has its own website (http://pinkseafan.wildlifetrusts.org/index1.html). *E. verrucosa* is deemed 'vulnerable' by the IUCN Red-List, and in the United Kingdom is protected by Schedule 5 of the 1981 Wildlife and Countryside Act (whereby killing, injuring or selling colonies is prohibited),

and has status as a Biodiversity Action Plan Priority species (Hall-Spencer *et al.* 2007,www.naturalengland.org.uk). However, *E. verrucosa* is still threatened by increased intensity of inshore fishing effort (Lumbis 2008); resultant degradation of reef habitats in the UK has led to closures of some areas to benthic trawling activity to protect *E. verrucosa* and it therefore warrants protection (Atrill *et al.* 2011).

There is considerable evidence from this study and similar research in the North East Atlantic that connectivity in this area is governed by regional level patterns at large spatial scales and that consequently, inter-governmental collaboration is needed to effectively conserve regional biodiversity. The need for international collaboration in conservation is not a novel concept and has been recognised in connectivity studies elsewhere; Ridgway *et al.* (2008) examined genetic diversity of the coral *Pocillopora verrucosa* in MPAs in South Africa and found that conservation efforts might be futile there without adequate protection of the source populations in neighbouring Mozambique. In my study area, international efforts directed by the OSPAR Commission (www.ospar.org) have resulted in development of an MPA Network that spans international borders and offers protection in three regions (defined by them) that overlap the range of *Eunicella verrucosa*; region II (Greater North sea), region III (Celtic Seas) and region IV (Bay of Biscay and Iberian Coast). However, *E. verrucosa* is not one a species targeted by this framework and although it *is* targeted specifically by the UK MPA network, how exactly it will be protected by it remains unclear. International efforts have been made previously to specifically protect other sessile species; the deep water coral *Lophelia pertusa* was targeted by closure of the Rockhall Bank in the NE Atlantic to fishing in 2007 (Hall-Spencer *et al.* 2009).

To conclude, microsatellite markers developed for this species have proven useful in delineating genetic connectivity patterns in *Eunicella verrucosa* in the NE Atlantic, the most northerly part of its range. Despite overall low genetic structure, at scales spanning more than 2000km, regional variation is observed and distinct clusters can be detected, in particular Portugal, Ireland, the UK and to a lesser extent, Brittany. The Irish samples show a genetic signature typical of marginal sites (e.g., lowered allelic richness), corresponding to their status as the most northerly *E. verrucosa* populations. However, MIGRATE analyses suggest that this area may be a source for areas further south, as well as a sink. Many populations are likely to be inbred. Additional research would benefit from current and hydrodynamic modelling around some of these populations to determine if, for example, self-seeding may be predicted despite expectations of relatively high connectivity along an (assumed) continuous habitat in this species in this area.

Chapter 4. Genetic Assessment of Connectivity and Population Structure in the Ubiquitous Octocoral *Alcyonium Digitatum* in the UK and NE Atlantic

Abstract

Outside of the Mediterranean and Caribbean regions, there are few studies of genetic population structure or connectivity in a prevalent yet poorly-understood group of invertebrates, the octocorals, which constitute an important component of the benthic fauna in the provision of structural complexity and habitat for numerous taxa. Octocorals are prevalent globally, from polar regions to the tropics, and they display a considerable intertidal-to- abyssal depth range. In the British Isles, the study area for this research, there is a limited subset of octocoral species diversity compared to lower latitudes. Nonetheless, the octocoral *Alcyonium digitatum* has a ubiquitous presence there where it can be found all around the coast. Globally, *A. digitatum* spans the Atlantic but in Europe, it has a wide Lusitanian and Boreal distribution between Portugal, Norway, and Iceland. Therefore the British Isles are centrally positioned in its range.

Alcyonium digitatum is a gonochoric broadcast spawner, unusual in that it releases gametes in winter, which suggests that larvae may be able to disperse long distances on hibernal wind-driven currents. As little is known about the genetic connectivity of this species, I developed a panel of eleven microsatellites and used them to assess population structure among and between populations in Brittany, western Ireland, South West England and Wales and the North Sea, comprising 655 individuals from 20 sites. Based on the reproductive mode and the timing of it in this species, I hypothesized that connectivity would be pronounced. Some of these sites coincide with proposed conservation areas under current plans for a UK marine reserve network. The resulting pattern of connectivity suggests admixture, little structure over large spatial scales, and therefore conforms to the expectation of high levels of gene flow in this area. However, and despite little evidence for asexual reproduction, almost all populations have significantly high inbreeding coefficients. This suggests that isolation between populations may be higher than expected under an admixture model and indicates that self-seeding populations coupled with rarer dispersal events may explain the apparent genetic homogeneity observed in this part of its range.

4.1 Introduction

4.1.1 The Importance of Baseline Genetic Data in Marine Communities

Understanding genetic diversity is essential for understanding ecosystem functionality, as the extent of it correlates with population resilience, interspecific competition, community structure, nutrient fluxes and primary productivity (reviewed in Hughes *et al.* 2008). In the UK, which is currently in the process of designating its first network of marine reserves, there is a paucity of baseline genetic data that can give an indication of marine ecosystem health, especially for sessile communities. The lack of baseline data with which to compare contemporary patterns and changes in ecosystem or species health and abundance is well recognised in coral reefs (Knowlton and Jackson 2008). Some UK marine communities are threatened, such as at Lundy, where coral recruitment is declining in the protected coral *Leptopsammia pruvoti* (Irving 2004), in Lyme Bay where trawling activity has decimated epifaunal communities (Hinz *et al.* 2011), and potentially in the English Channel where pollution events and changing temperatures have altered animal communities (Southward *et al.* 2005). The need for an ecosystem-based management approach in these areas is well recognised, and conservation efforts in the UK rely upon ecosystem 'indicators' that give a measure of damage or potential damage to particular areas or groups of animals to inform policy (Rogers and Greenaway 2005). There are few (if any) genetic assessments in the current guidelines for the new marine reserve network, and baseline data could prove invaluable for monitoring population expansions or declines, as has been done for several invasive species (e.g., the slipper limpet *Crepidula fornicata*, Dupont *et al.* 2007 and the ascidian *Styela clava*, Dupont *et al.* 2009). In this study, a genetic assessment of the ubiquitous octocoral *Alcyonium digitatum* is presented. This species is found all around the UK where it is often the dominant component of the benthic biomass that provides structural complexity and habitat for other invertebrates (e.g., Hartnoll 1975).

4.1.2 *Alcyonium* Linnaeus, 1758

Members of the order Alcyoniina are commonly termed 'true soft corals' and lack an internal proteinaceous axis such as that found in holaxonians (i.e. sea fans and sea rods). As such, they are usually lobate or digitate fleshy or encrusting forms and rely upon their sclerite mass and hydrostatic pressure for support (Fabricius and Alderslade 2001). *Alcyonium* (F. Alcyoniidae) is a highly speciose genus with a circum-global distribution spanning polar and tropical regions, including the Mediterranean, Pacific, Atlantic and Mediterranean Oceans (van Ofwegen *et al.*2001). There are between 75 and 135 species, all of which are thought to be heterotrophic suspension feeders typically found on rocky overhangs and ledges up to 40m deep, although

some species may occur in soft sediment (McFadden *et al.* 2001). Taxonomic relationships within this genus are often poorly resolved (e.g., McFadden 1999), and as is the case for most coral and octocoral taxa, species descriptions have typically been based upon morphological characterisation of their sclerites. It is clear that the full extent of molecular and morphological diversity within this genus is still unknown and a large depth distribution of some species of over 1000m has meant that some species have only recently been discovered and described, such as *Alcyonium megasclerum* at 1000-1350m and *A. profundum* at 2200 - 2600m in Cape Verde (Stokvis and van Ofwegen 2006).

4.1.3 Distribution of *Alcyonium digitatum*

In the shallow waters in the Northeast Atlantic, the most prevalent benthic taxa are found subtidally on rocky substrata and include *Alcyonium (=Bellonella) bocagei* (Kent, 1870), *A. acaule* Marion, 1878, *A. coralloides* (Pallas, 1766), *A. digitatum* Linnaeus, 1758, *A. glomeratum* (Hassal, 1843), *A. hibernicum* (Renouf, 1931) and *A. palmatum* Pallas, 1766. The distribution of these species is predominantly Lusitanean-Meditteranean and/or Boreal (Watling and Auster 2005). *Alcyonium acaule* is common between 10-90m in the NW Meditearranean (Fiorillo *et al.*2012) and *A. coralloides* extends from the Mediterranean into the Atlantic as far north as Brittany (McFadden and Hutchinson 2004). *Alcyonium, coralloides* was previously thought to also be common in the western British Isles and Ireland (McFadden 1999), but subsequent research has shown that this is likely to be misidentified *A. hibernicum*, which is also found in Brittany (McFadden and Hutchinson 2004). *Alcyonium glomeratum* is thought to have a limited distribution in the south and western British Isles and Ireland (www.marlin.ac.uk), but likely is also found in Atlantic Portugal (including the Azores), Spain and France (e.g., World Register of Marine Species website, marinespecies.org). *Alcyonium digitatum* apparently has a much more extensive range than these other congeners, extending between Portugal, Norway and Iceland and throughout the North Sea in the NE Atlantic, but is also found on the continental shelf and slope of eastern Canada south to Cape Hatteras in the USA (Hartnoll 1975, Watling and Auster 2005). This species has a ubiquitous presence in the UK and can be found continually along all coastlines in rocky upper - and circalittoral zones, typically to '35-40 fathoms' (i.e. up to 200 metres, Hickson 1901) and in strong to moderate tidal regimes with varying wave exposure preferences (from very sheltered to very exposed, marlin.ac.uk). It is known to readily colonise artificial structures including ship wrecks (Hiscock *et al.* 2010) and is so widespread in the UK that it may even be considered one of Britain's most abundant animals (Hickson 1901).

Alcyonium digitatum is commonly named 'dead man's fingers' due to its digitate branching morphology (incidentally, the binomen may be linked to the Greek myth of Alcyone, who

allegedly threw herself into the sea on learning of the shipwreck that caused the death of her husband Ceyx, Hickson 1895). Like *Eunicella verrucosa* (Chapter 3), colour dimorphism is characteristic of *A. digitatum* and colonies are predominantly white or orange. This is due to orange pigmentation (or lack of) in spicules of the two morphs and there is no evidence for ecological or genetic differentiation of the two morphs (this study, but see d'Hondt and d'Hondt 2003). The two colour morphs vary in distribution (Hartnoll 1975, this study). Yellow and yellow-orange specimens can allegedly be found in western Scotland, the Shetlands, Port Erin and the Bristol Channel (Hickson 1895, 1901), but generally, white colonies appear to be rarer in more southerly areas outside of the UK (such as Brittany; personal observation), whereas white colonies predominate in southern England and brown or orange colonies are more common in the north and in the east of the UK (Hiscock *et al.* 2004; personal observation).

4.1.4 Reproductive Ecology of *Alcyonium digitatum*

The reproductive strategies displayed by *Alcyonium* are exceptionally diverse, with reports of both gonochoric and hermaphroditic sexes and brooding and broadcasting spawning modes (McFadden *et al.* 2001). *Alcyonium coralloides* is a gonochoric internal brooder, *A. acaule* broods larvae externally and *A. digitatum, A. glomeratum* and *A. palmatum* broadcast-spawn with planktonic development, although all are believed to produce lecithotrophic larvae (McFadden *et al.* 2001). In some cases, population growth and persistence of *Alcyonium* spp. may be a combination of both asexual propagation of ramets by binary fission and rarer long-distance dispersal of genets (e.g., *A. rudyi*, McFadden 1997). Atypically, parthenogenesis has been suggested for *A. hibernicum*, which broods larvae (Fautin 2002). This unusual reproductive strategy supports the hypothesis of a hybrid origin in this species (McFadden and Hutchinson 2004). It appears that gonochoric internal brooding is the reproductive mode most common in *Alcyonium* species studied to date, which has also been noted in the family Xeniidae in the Red Sea (also members of the order Alcyoniina); this strategy contrasts markedly with scleractinian corals, in which hermaphroditic spawning is most common (see McFadden *et al.* 2001). From a recent review, there appears to be a relatively even split between the use of broadcasting and brooding strategies in octocorals, although there is a high likelihood of gonochorism (up to 89%, Kahng *et al.* 2011).

The reproductive cycle of *Alcyonium digitatum*, a broadcast spawner, has been relatively well characterised. The timing of gamete development and spawning was first demonstrated experimentally in aquaria by Hickson (1895), who dissected hundreds of colonies at the Marine Biological Association (MBA) laboratories in Plymouth and observed a nine month gametogenic period followed by the release of gametes in December and January, noting that

egg release occurred throughout the day and night (in aquaria). He never found spermatozoa or ova simultaneously in the same colony, and therefore concluded that *A. digitatum* was gonochoric. Similar research was repeated by Hartnoll (1975), who examined nearly 1000 colonies of each colour morph in Port Erin, and confirmed the gonochoric sexuality of *A. digitatum.* However, he also observed hermaphroditism in 0.5% of colonies, similar to McFadden et al 2001, who noted one hermaphrodite in 333 sexually mature colonies (0.3%). Hartnoll observed a 12 month cycle of gametogenesis and a corresponding feeding cycle whereby polyps retract and stop feeding for the latter half of the year, culminating with spawning in December to January. He also described the production of neutrally buoyant ova that are fertilised in the water column and suggested that colonies are likely to be at least 2-3 years old before reaching sexual maturity. Unfortunately, Hickson (1895) and Hartnoll (1975) failed to settle any juveniles in their observations, although Matthews (1917), who also worked at the MBA in Plymouth, managed to observe artificially fertilised larvae swimming in aquaria for 14 weeks, with some cases of survivorship until 35 weeks, supporting a very long pelagic duration *in vitro* and potentially a high dispersive capacity (although some larvae were ready to settle as early as seven days post-fertilisation). Winter spawning is unknown for other members of this genus and its rationale is unclear, although it could be a means to avoid predatory planktotrophs associated with the spring bloom and/or to profit from an abundance of its diet of phytoplankton and zooplankton following seasonally early settlement (Hartnoll 1975).

4.1.5 Ecological Importance of *Alcyonium digitatum*

Chemical ecology and bioprospecting appears to be less explored in *Alcyonium digitatum* than for other octocoral taxa, although the secondary metabolites it produces appear to act as a feeding deterrent to fish (e.g., to the Dover sole *Solea solea*, Mackie 1987). Other compounds of unknown ecological and pharmaceutical function have also been isolated from the genus *Alcyonium* and may potentially be of bioprospecting interest (e.g., Diaz-Marrero *et al.* 2009).

Due to the high abundance *of Alcyonium digitatum* in the UK, threatening anthropogenic activity is unlikely to endanger the species across its range, although trawling activity has been shown to decimate populations locally, such as in Lyme Bay, Devon, where fished areas contained 67% lower colony abundance than un-trawled areas, and where protected areas were shown to enhance their biomass (Hinz *et al.* 2011). Anecdotal evidence suggests longevity in this species (and genus) likely exceeds 20 years (marlin.ac.uk, *A. acaule* Fiorillo *et al.* 2012); if they also take 2-3 years to attain sexual maturity (Hartnoll 1975), this implies that population recovery may be very slow in areas of trawling damage (assuming that sufficient adults remain locally to seed the damaged population).

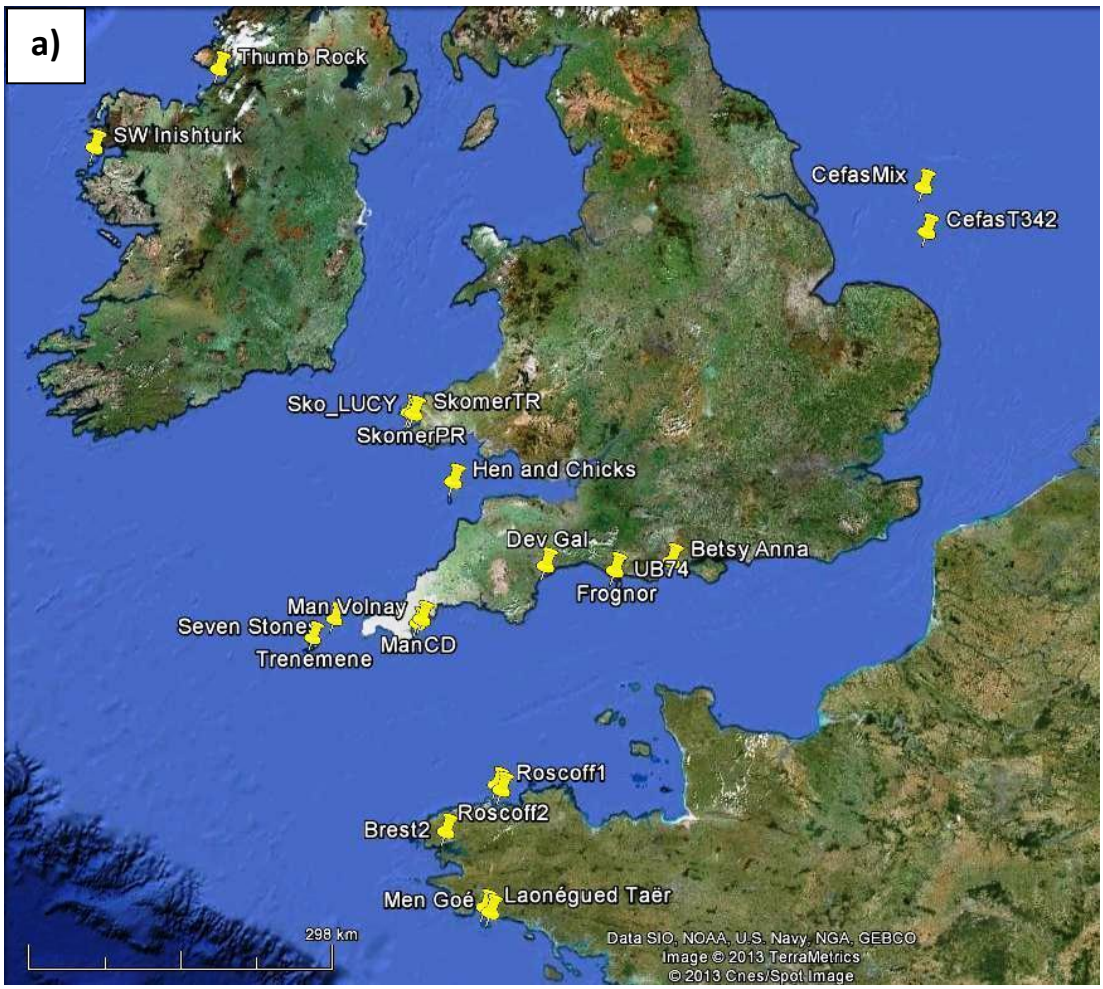
4.2 Methodology

4.2.1 Sample Collection

Alcyonium digitatum samples were collected between March 2009 and May 2012 from 21 sites in South West England, the North Sea, western Ireland and Brittany (Table 4.1 and Figure 4.1). All samples were collected by SCUBA with the exception of CEFAS T342 and CEFAS Mix from the North Sea- these samples were bycatch of sediment samples that were collected by benthic trawlers and donated to me by the Centre for Environment, Fisheries & Aquaculture Science (CEFAS). Many samples were collected on our behalf by amateur divers, hence a high proportion of sampling sites on or near shipwrecks. Samples were collected at three scales to test connectivity at fine spatial scales (e.g., within a locality such as Skomer Marine Reserve, sites separated by 0-10km), local spatial scales (e.g., within Lyme Bay, sites 10-25km apart) and regionally (e.g., Brittany to SW England, sites more than 25km apart). Protocols for *in situ* sample collection are outlined in Chapter 2. As is the case for *E. verrucosa* (Chapter 3), lack of larval dispersal data and connectivity patterns are poorly understood for this species; despite the use of the term 'population' being used to describe a geographically-defined sampling site in this chapter, I appreciate that each site may not necessarily represent a genetically distinct population.

Table 4.1. List of sampling sites for *Alcyonium digitatum*. Bold type indicates sites or areas in which *Eunicella verrucosa* was also sampled. [1] colonies with the orange colour morph were sampled at some sites as indicated, otherwise all colonies were white. [2] CEFAS samples were from benthic trawls; 'CefMix' was a combination of 4 adjacent trawls of 300m each, where N=25/11/8/4 respectively, 'CefT342' was one trawl of 600m (only the coordinates of the start of the trawl for T342 and where N=25 (T86) are given here). N represents samples included in the final dataset and not the total numbers collected or extracted; these individuals amplified in at least 9/11 loci.

Country	Code	N	Orange?[1]	Collected	GPS	Site Name
U.K.	**Dgal**	7		7.5.09	50°33'18.72"N 3°26'21.43"W	The Galicia (shipwreck), Devon
U.K.	**DorBA**	24		10.5.09	50°36'58.74"N 1°49'57.84"W	The Betsy Anna (shipwreck), Dorset
U.K.	**Frog**	18		25.9.10	50°32'2.00"N 2°33'6.00"W	Frognor 1 (Norwegian steamship shipwreck), Lyme Bay, Dorset
U.K.	**HC**	36	N=1	27.7.10	51°12'12.51"N 4°40'50.29"W	Hen and Chickens, Lundy, Devon
U.K.	**Tren**	42		1.8.10	49°51'54.00"N 6°23'9.00"W	Trenemene reef, Isles of Scilly, Cornwall
U.K.	**Stone**	40		1.8.10	50° 1'58.80"N 6° 7'7.20"W	Seven Stones reef, Isles of Scilly
U.K.	**ManCD**	33		22.3.09	50° 2'43.39"N 5° 2'44.99"W	Carn-du rocks, The Manacles, Cornwall
U.K.	**ManV2**	28		23.3.09	50° 4'22.32"N 4°59'48.12"W	Volnay (shipreck) Manacles, Cornwall
U.K.	**Lucy**	22		13.6.09	51°44'28.08"N 5°16'36.54"W	The Lucy (shipwreck), Skomer, Pembrokeshire
U.K.	**PR**	51		13.6.09	51°44'40.25"N 5°18'27.18"W	Payne's Rock, Skomer, Pembrokeshire
U.K.	**TR**	21		15.6.09	51°44'17.52"N 5°15'21.54"W	Tusker Rock, Skomer, Pembrokeshire
U.K.	**UB74**	19		25.9.10	50°31'50.21"N 2°33'19.26"W	UB74 (shipwreck, 1st WW German U-boat), Lyme Bay, Dorset
U.K.	**CefMiX**	27	N=6	5/2009	53°38'40.55"N 1°32'49.96"E	[2]CEFAS trawls (4 adjacent sites), Humberside, the North Sea
U.K.	**CefT342**	33	N=19	05/2009	53°16'33.86"N 1°34'9.29"E	[2]CEFAS trawl (T3-42), nr Norfolk, the North Sea
France	**Bre2**	43	N=43 (all)	19.5.10	48°20'20.94"N 4°34'32.52"W	"Mengam", Rade de Brest, Brittany
France	**LTGlen**	29	N=29 (all)	12.5.11	47°43'38.39"N 4° 3'35.75"W	Laonégued Taër, Glenan Archipelago, Brittany
France	**Mglen**	34	N=33	12.5.11	47°41'19.86"N 3°59'31.70"W	Men Goé, Glenan Archipelago, Brittany
France	**Ros1**	41		20.5.10	48°44'49.50"N 3°57'42.24"W	"Astan", Baie de Morlaix, Brittany
France	**Ros2**	41		21.5.10	48°42'33.71"N 3°54'11.78"W	"La Vieille", Baie de Morlaix, Brittany
Ireland	**IreIT**	48	N=48	12.5.12	53°43'8.50"N 10°7'19.14"W	SW Inisturk island, co. Sligo, Ireland
Ireland	**IreTR**	18	N=48	15.5.12	54°28'17.88"N 8°26'41.40"W	Thumb Rock, Mullaghmore, co. Donegal,


a)
Thumb Rock
SW Inishturk
CefasMix
CefasT342
Sko_LUCY
SkomerTR
SkomerPR
Hen and Chicks
Dev Gal
Betsy Anna
UB74
Frognor
Man Volnay
Seven Stone
ManCD
Trenemene
Roscoff1
Roscoff2
Brest2
Men Goé
Laonégued Taër
298 km
Data SIO, NOAA, U.S. Navy, NGA, GEBCO
Image © 2013 TerraMetrics
© 2013 Cnes/Spot Image


b)
Sko_Lucy
SkomerTR
SkomerPR
Hen and Chicks
Dev Gal
Betsy Anna
UB74
Frognor
Seven Stones
Man Volnay
ManCD
Trenemene
100 km
Data SIO, NOAA, U.S. Navy, NGA, GEBCO
Image © 2013 TerraMetrics
Google earth

d)

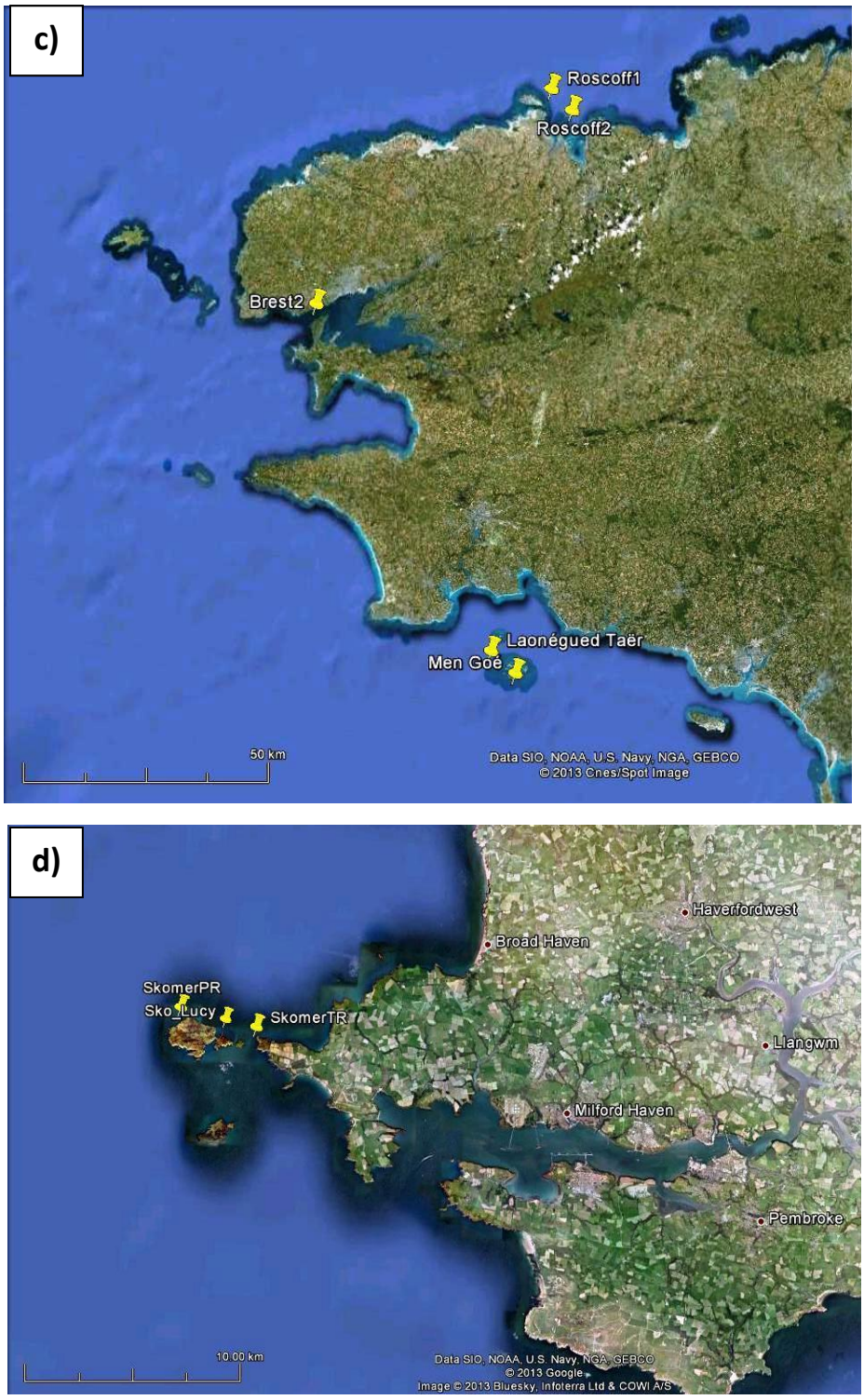


Figure 4.1. Maps showing sampling locations of *Alcyonium digitatum* (see Table 4.1) in this study at various spatial scales; a) all 21 sampling sites in this study in Great Britain, Brittany and Ireland, b) detailed map of SW England sites, c) detailed map of samples collected in North and South Brittany and d) detailed map of samples taken at Skomer Marine Nature Reserve. All maps were created in Google Earth (©Google Inc. 2011), scale bars as indicated. 'CefasT342' samples were collected from a 300m benthic trawl, shown here is the start of the trawl. 'CefasMix' samples were collected from four proximal benthic trawls; this data point shown here is the start of one of them. Exact coordinates were not available at all sites and some points may be approximate.

4.2.2 Allelic patterns in *Alcyonium digitatum* data

Chapter 2 describes the protocols for microsatellite development and for lab-based genotyping methods. Therefore, in this chapter methodology will focus on describing statistical and population genetic data analyses (i.e., all methods except laboratory-based procedures). All genotyping data was compiled into a matrix and all individuals with a failure rate of three or more loci were manually removed from the matrix. Subsequently, data was checked for duplicate genotypes using the 'Identity Analysis' in Cervus v3.0 (Kalinowski 2007), with a minimum number of matching loci set at one and disallowing fuzzy matching. Duplicate genotypes found within the same population were removed from the data matrix. This resulted in a final dataset of 655 genotyped individuals from 21 sites (numbers from each site are given in Table 4.1).

Allelic patterns for all loci within each population were visually examined using 'bubble' plots (Figure 4.2), and subsequently allele frequencies were summed across the entire dataset and graphed by locus. This exercise was a way to visualise the spread of alleles, to determine the extent of imperfect repeat distributions, and to get an idea of the likelihood that loci conformed to a stepwise mutation-like or infinite allele-like pattern of evolution (Figure 4.3). Evidence for null alleles, stutter peaks and large allelic dropout and to determine if the majority of alleles belonged to one size class were checked in each population using Microchecker v2.2.3 (van Oosterhout *et al.*2004, Table 4.2), with the maximum allele size set at 400bp, a 95% confidence interval and 1000 iterations. A list of private alleles present in each population was constructed in GenAlEx v6.5b.3 (Peakall and Smouse 2012, Table 4.3).

4.2.3 Exploring Locus Diversity Indices and Testing For Outliers

In order to determine if the relative effects of each locus on genetic diversity were due to one or several, or all, of them (i.e., a genomewide effect), average Fst (theta) and heterozygosity metrics were calculated for each locus from the entire dataset using FSTAT v2.9.3.2 (Goudet 2002) following Weir and Cockerham's 1984 estimation, using a 5% nominal level for multiple tests. Allelic richness (Ar) was calculated per locus by rarefaction algorithms implemented in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003) to examine how many alleles were present at each locus independently of the sample size. Allelic richness values are presented by locus in Table 4.5 and by population in Table 4.6.

The software LOSITAN was used to detect loci that may be subject to selection (Antao *et al.* 2008), following default and/or the author's recommended settings; 50,000 simulations, Neutral mean Fst" and "Force mean Fst" options selected, a 95% confidence interval and false discovery rate correction set at 0.1 for both infinite alleles (IAM) and stepwise mutation (SMM)

models. Graphical outputs from LOSITAN simulations and loci lists highlighting candidate loci under balancing and positive selection are shown in Appendix 4.

4.2.4 Linkage Disequilibrium

Evidence for linkage disequilibrium (LD) was tested between all loci pairs across all populations in Genepop v4.2 (Raymond and Rousset 1995, Rousset 2008) with both log likelihood ratio statistics and probability tests set at 1000 dememorizations, 100 batches and 1000 iterations per batch. Results were examined to determine how many populations showed significant ($P \leq 0.05$) LD for the same pairs of loci. No pairs of loci showed evidence of linkage disequilibrium in all populations; at most, two (by probability test) or three (by log likelihood ratio) populations had significant LD between the same loci pair. This was deemed not likely to reflect true and consistent linkage disequilibrium and therefore no loci were removed from the subsequent analyses on the basis of LD. As no pairs of loci were shown to have significant linkage disequilbrium across all populations, and as no loci were under consideration to be discarded, corrections for multiple tests were not conducted.

4.2.5 Heterozygosity-Based Summary Statistics and Allelic Richness

Summary statistics and initial measures of population differentiation were calculated using Arlequin v3.5.1.2 (Excoffier 2010) and Genodive v2.0b23 (Meirmans and van Tienderen 2004). Raw data files were converted to Arlequin input files using Genepop v4.02 (Raymond and Rousset 1995). In Arlequin, AMOVAs (analysis of molecular variance) were calculated using the default options (genotypic data = 1, gametic phase and recessive data = 0), and standard AMOVA computations (haplotypic format, selecting individual level, 1000 permutations and number of different alleles as Fst-like with an allowable missing data level of 40%). This gave an overview of the hierarchical distribution of genetic variation across the entire dataset at different spatial scales. AMOVAs were conducted on individuals, populations and with all populations assigned to a single group and not subdivided further into regions following little clustering observed in preliminary Bayesian admixture analyses (see 4.2.10 below).

Heterozygosity-based summary statistics (e.g., *k*, *H*o, *H*s, *G*is and *F*is) were calculated on a population-by-population basis in Arlequin and Genodive, as described for *Eunicella verrucosa* (Chapter 3). The number of alleles, expected and observed heterozygosities, associated *P*-values and their significance after correction using the false discovery rate (FDR) method (Benjamini and Hochberg 1995) were also calculated on a locus-by-locus basis for each population (Appendix 3). To overcome the effects of variable sample sizes, allelic richness (Ar) was calculated in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003) in order to facilitate comparisons of genetic diversity between populations (Table 4.6).

4.2.6 Testing For Evidence of Genetic Bottlenecks

The program BOTTLENECK v1.2.02 (Cornuet and Luikart 1996) was used to detect evidence of genetic bottlenecks. All populations were tested using the infinite alleles, stepwise mutation and two-phase models assuming mutation-drift equilibrium, however, in the two-phase model simulation, the proportion of SMM permitted in the TPM was set at 30%. This is higher than the 5-10% the authors recommended for microsatellite data and higher than that permitted for similar analyses of *Eunicella verrucosa* in Chapter 3; however, I decided to increase this given the higher likelihood of the *Alcyonium digitatum* allele frequency data fitting a stepwise-like mutation model (Figure 4.3). For all runs 1000 iterations were calculated, and sign tests and Wilcoxon sign rank tests were used to determine a P value for the likelihood of a bottleneck for each population.

4.2.7 Fixation Indices and Permutation Tests

Population differentiation was calculated using pairwise Fst comparisons and Jost's D in GenAlEx. P-values from Fst analyses were adjusted using the false discovery rate method (Benjamini and Hochberg 1995) while those from Dest were not, due to bootstrapping replication inherent in this analysis (99 permutations). All loci and all sampled populations were included in these analyses (Tables 4.8 and 4.9). Following little resolution within the dataset following these analyses, populations were grouped by sampling region to test diversity between them and permutation tests were carried out in FSTAT v2.9.3.2 (Goudet 2002) with 1000 permutations (Table 4.10).

4.2.8 Principal Components Analysis

To detect spatial clustering within the *Alcyonium digitatum* data, principal coordinates analyses based upon covariance of Nei's genetic distance and pairwise *F*st values were conducted in GenAlEx (Figure 4.4). Samples collected from the Devon Galicia site were removed prior to analysis due to the small sample size from this population (N=7) in order to avoid false sample clustering. All other sites were included.

4.2.9 Isolation by Distance

To determine if any correlation was present between geographic distances and genetic differences, Mantel tests were conducted on linearized Fst and log distances between sampling sites in Genepop v4.2 (Rousset 2008), using Option 6- 'Fst and other correlations' and sub-option 9- 'analysis of Isolation by distance' (Figure 4.5). Distances between sites were measured as the smallest linear distance between sites following Amaral *et al.* (2012) and as in Chapter 3. Significance was tested with 1000 permutations, the minimum distance between

samples to be taken in account for regression set at 0.0001 and the regression coefficient was calculated using the Spearman Rank correlation coefficient. Samples from the Devon Galicia (DevGal) site were removed from IBD tests due to its small sample size compared to all other sampling sites (N=7 cf. 18-51). As no hierarchical population structure had been observed previously, all samples were analysed concurrently with no further data partitioning.

4.2.10 Bayesian Clustering Analyses

Population clusters were estimated using STRUCTURE v2.3.4 (Pritchard *et al.* 2000, Figure 4.6). Simulations were run on all data and initial results indicated a lack of clustering across the sampled range of *A. digitatum*, therefore the data were not subdivided further. Parameters were set to use an admixture model, correlated allele frequencies and sampling location as a prior. Simulations were replicated three times, with an initial burnin of 100,000 iterations and 10^6 MCMC (Markov Chain Monte Carlo) repetitions after burnin. K values ranged from 1-10 and poor resolution of clusters at these levels meant that the K value was not increased further. Furthermore, as population clusters were unresolved in this species, Evanno corrections (Evanno *et al.* 2009) were not used to find the most likely K value.

4.2.11 Coalescent Approaches

The software MIGRATE 3.4.4 (Beerli & Felsenstein 2001) was used estimate migration rates between regions and to calculate effective population sizes, using a Bayesian likelihood inference in conjunction with a Brownian motion model of stepwise mutation. I used one long-chain search with 1 x 10^6 generations sampled every 100^{th} step (10,100 recorded steps), with 10% (100,000 steps) discarded as burn-in. These settings were used with default parameters to determine optimal prior distributions for theta and M, which were subsequently determined to uniform distributions with 0-150, delta=15 and 0-100, delta=10 respectively. The analysis was repeated twice to ensure consistency. Theta values were converted into effective population size values (Ne = $\theta/4\ \mu$) assuming a microsatellite mutation rate of 10^{-4}; actual mutation rates for each locus used in this study are unknown and generally, mutation rates vary between 10^{-2} to 10^{-6} (Li *et al.* 2002). I therefore chose a mid-point estimate as in Chapter 3.

4.3 Results

4.3.1 Allelic Patterns

Evidence for null alleles, likely scoring errors due to stutter peaks, evidence for allelic dropout of large alleles and loci that have more than half of their alleles in one size class as calculated in Microchecker is shown in Table 4.2. Three loci show considerable evidence for null alleles,

including Adig003, Adig004 and Adig010. There was evidence that Adig004 has null alleles at all loci, and correspondingly, this locus is out of HWE in twenty out of twenty one populations (Appendix 3). Adig003 and Adig010 showed evidence for null alleles in all but three and four populations respectively. There did not appear to be a correlation between the likely presence of null alleles and geographic sampling location or sample size. Adig003, a dinucleotide, also showed evidence for stutter peaks in six populations; as all data was double-checked and stuttering was not likely in most populations, this evidence was not deemed to be problematic. No loci appeared to have suffered from a loss of large alleles due to favourable amplification of smaller alleles.

Four loci out of the eleven tested had more than fifty percent of alleles in one size class in all or most populations; these are Adig001, Adig004, Adig006 and Adig007. Aidg007 is the only locus in the dataset that is monomorphic, which it is in the three sites Ireland Thumb Rock, Cefas T342 (North Sea) and Devon Galicia (south England, Appendix 3). From bubble plots of allelic distribution in all data (Figure 4.2), there is clearly a similar distribution of alleles across all loci for most populations, characterised by low frequencies of many alleles. At first glance, the most disparate sample is Devon Galicia, which has a smaller number of alleles present at most loci- in particular at locus Adig009. However, this can be expected given the very small sample size of this population (N=7). The bubble plots also show that many loci appear to have rare alleles at very low frequencies, indicating a high proportion of private alleles. Therefore, the distribution of private alleles was examined in more detail and they are listed in Table 4.3. In total, 47 private alleles were detected in eighteen populations; the only populations missing private alleles are Devon Galicia (where only seven individuals were included), and the two sites from southern Brittany, Men Glenan and LT Glenan (where 34 and 29 individuals were included respectively). By locus, Adig004 had the highest number of private alleles across the entire dataset, with 14 spread between 10 populations, followed by Adig010 (eight private alleles in eight populations), and Adig008 and Adig009 that each had six private alleles (shared among four and six populations respectively). By population, Roscoff 1 contained the highest number of private alleles (eight private alleles in six loci), followed by Roscoff 2 (six private alleles from three loci, four of which were at the same locus Adig004). Despite being relatively prevalent across all data, private alleles were rare within each population and there was only one occurrence of the same private allele in more than one individual in a population (allele 233 in locus Adig004 occurred in two individuals in Roscoff 2). In contrast to the pattern of allele frequency distributions shown by most of the *Eunicella verrucosa* microsatellites, the *Alcyonium digitatum* microsatellites show more evidence of normal distributions in frequency, especially at loci Adig004, 008, 009 and 010 (Figure 4.3). This pattern is indicative of stepwise mutation as allele frequencies are related to those of

neighbouring alleles. Fst values by locus were low and varied between zero (Adig005, 010 and 011) and 0.027 (Adig006) with a mean of 0.004. There is some evidence that the latter locus is under positive selection, as is also the case for Adig004 and Adig009 (Appendix 4). Nonetheless, all loci were used in analyses for reasons outlined in Chapter 3 (3.3.1). By locus, allelic richness varied between 1.5 (Adig007/88A04) and 10.8 (Adig010/88E09) with a mean value of 5.9 (Table 4.5).

4.3.2 Heterozygosity, Summary Statistics and Allelic Richness

Differentiation between the 21 sampled populations was weak according to Analysis of Molecular Variation (AMOVA) (Table 4.4), with only 0.13% of the variation explained at this level (Fst= 0.001, P=1). Most of the variation was partitioned within individuals, at 89.4% (Fit = 0.106, P=0), and to a much lesser extent it could also be explained by variation between individuals within populations at 10.47% (Fis = 0.105, P=0). In summary, this test shows no population genetic structuring within *Alcyonium digitatum* but rather variation is defined at the individual-level both within- and across the sampled populations.

From eleven loci, seven of them showed deviations from HWE in at least one population; those loci never showing HWE deviation are Adig005, Adig007, Adig008 and Adig011 (Appendix 3). In most cases, the extent of HWE deviation was limited to a minority of populations, such as for the loci Adig001, Adig002, Adig006 and Adig009 (four, three, one and four populations respectively). The extent of HWE deviation was much higher in other loci such as Adig003 and Adig010 (observed in 18 and 14 populations respectively). Adig004 showed HWE deviation in all populations, likely driving the strong evidence of null alleles (Table 4.2). The presence of null alleles can imitate heterozygote deficiencies, and for some analyses data may be corrected to account for them (e.g., in STRUCTURE, Falush *et al.* 2007). However, these corrections may themselves introduce error and/or require prior knowledge about the null allele(s), such as if a homozygous null allele is present in cases where no amplification is achieved or if poor DNA quality may explain this result (Falush *et al.* 2007). Therefore, I did not apply such corrections to this data. Although the inclusion of a locus containing null alleles may lead to false patterns of connectivity based upon large heterozygote deficiencies, all 11 loci were included in analyses here and the lack of structure observed overall in *Alcyonium digitatum* indicates that locus Adig004 is not creating spurious structure.

A high proportion of populations, nineteen out of twenty one, have significant inbreeding coefficients at the 5% level (Table 4.6). The only exceptions to this are a site from the North Sea (Cefas Mix) and from Pembrokeshire, Wales (Skomer Tusker Rock), which are

coincidentally at the eastern and western limits of our sampled area in the UK respectively (but are not at the limits of the UK *A. digitatum* distribution).

The number of alleles combined across all loci for each population ranged between 54 and 144, and the average number of alleles per locus ranged between 5 and 31 in a given population (with an average total allele number of 118 per population and 10.8 alleles per locus). There is a linear relationship between the number of samples included from each population and the number of alleles observed, with the lowest numbers of alleles corresponding to the lower sample sizes (e.g., 54 alleles in the seven individuals sampled from the Devon Galicia site). Sample sizes of approximately 30 individuals are required to attain the average total number of alleles observed per population, although as for *Eunicella verrucosa*, there is evidence that genetic diversity was under-represented in my samples, as allelic numbers correlated with sample size failed to reach a plateau (Appendix 5). After correction for sample size, allelic richness measures varied from 4.9 (the Devon Galicia wreck) to 6.1 (Roscoff 2) with a mean of 5.7 across all populations. There was no striking pattern of geographic variation in the distribution of allelic richness as sites from different regions were mixed when ranked according to Ar (data not shown). Nonetheless, it is interesting to note that both Irish sites had among the lowest Ar values (5.5 and 5.6) for *A. digitatum* as they did for *E. verrucosa*. The lowest value found in the Devon Galicia sample is highly likely to be an artefact of a small sample (N=7). The highest allelic richness was observed in samples from Brittany (Brest 2 and Roscoff 2, 6.0 and 6.1 respectively).

4.3.3 Evidence for Genetic Bottlenecks

Evidence for genetic bottlenecks, as supported by both Wilcoxon sign-rank and sign tests, was most prevalent under the stepwise mutation model (SMM, Table 4.7). Although caveats must be considered when interpreting these data (see section 3.3.3), it is interesting to note that the Irish sample Thumb Rock shows significant likelihood of a bottleneck for *Alcyonium digitatum*, whereas samples of *Eunicella verrucosa* collected there do not. Most significant indications of a bottleneck are lost under infinite alleles (IAM) and two phase mutation models (TPM), despite an increase in the proportion of SMM allowed in TPM simulations from 10% to 30%. It is likely that the *A. digitatum* loci are a better fit to the stepwise mutation model than the *E. verrucosa* loci. Therefore, inferences of a bottleneck here may be more reliable.

4.3.4 Pairwise Population Indices and Permutation Tests

Pairwise population comparisons calculated using standard Fst values and a newer Dest statistic (Jost 2008) are given in Tables 4.8 and 4.9 respectively. Fst values are low, with a range from 0 to 0.024 (between the sites Devon Galicia and Frognor). There are few

statistically significant comparisons and there is no trend with geographical sampling location. Most differentiation is seen between Roscoff 2 in northern Brittany and ten other populations spread across the sampled area, followed by CEFAS T342 versus eight other sites. However, CEFAS Mix, also from the North Sea, is not significantly different to any other population with the exception of one site in Lundy (Hen and Chicks) and one site in Cornwall (Manacles CD). Dest values are higher than Fst, with a range from zero to a maximum value of 0.087 (Devon Galicia and Men Glenan). Dest values show more frequent significance than Fst values, and there is a higher incidence of significant values between samples between Brittany and elsewhere (i.e., the left and the right of the table). However, this pattern appears to be largely driven by Roscoff2, which is significantly different to most other sites except for Roscoff 1. This is expected given the close proximity of these sites, but they are not significantly different to disparate sites in Ireland, Dorset or Skomer. Samples collected at both Irish sites (IreTR and IreITWest) are not significantly different to almost all other sites in both Fst and Dest comparisons. Likewise, samples collected from the North Sea are not substantially different to sites from distant areas. Although the CEFAS Mix samples are significantly different to half of the other sites in the Dest table, differences are insignificant to sites as distant as Ireland Thumb Rock, Men Glenan in southern Brittany, and Skomer in southwest Wales. In summary, pairwise comparisons show somewhat chaotic genetic patterns and there is not an obvious pattern of spatial distribution of genetic differentiation at local or regional scales.

After dividing the populations into four distinct groups according to sampling location (France, Ireland, SW England and the North Sea), permutation tests failed to detect any significant variation between them for allelic richness, expected and total heterozygosity, inbreeding and overall Fst (Table 4.10). Therefore genetic spatial structure across the sampled range of *Alcyonium digitatum* appears to be very limited.

4.3.5 Principal Components Analysis

The cumulative percent of the variance explained by the first three coordinates in PCA analyses is 66.13% for Nei's distance (26.96%, 21.43%, 17.73% for axes 1, 2 and 3 respectively) and 63.27% for pairwise F_{st} (25.72%, 21.7%, 15.85% for axes 1, 2 and 3 respectively, only values for the first three coordinates are given in GenAlEx). Both plots demonstrate a complete lack of spatial clustering in the data, as samples from the southwest UK and Wales, Brittany and the North Sea are mixed with no affinity between samples based upon geographic sampling location. The most acute example showing a lack of geographic clustering is given in Figure 4.4b, where a site from western Ireland (Thumb Rock) is adjacent to a site from the North Sea (CefT342); these sites are among the most distant sites from each other in the *A. digitatum*

dataset (approx. 1708km apart, using methods outlined in Chapter 2). PCA analyses therefore support a theory of high connectivity across the sampled range of this species.

4.3.6 Isolation by Distance

The results of a Mantel correlation test for patterns of isolation by distance are shown in Figure 4.5. The results show a significantly positive yet weak correlation (R^2 = 0.0693, P = 0.041), suggesting high gene flow at large spatial scales. However, the pattern shown by the distribution in Fst variance in the scatter plot is reminiscent of disequilibrium, as opposed to a pattern of strong correlation and increased variance and mean Fst with geographic distance as would be expected under equilibrium isolation by distance scenarios (Figure 1.1). There appears to be two distinct clusters, where the range of Fst values expands beyond distances of 600km, indicating a biogeographic barrier. To test for IBD at a smaller spatial scale, the Irish and French samples were removed from the analysis and the test was repeated on the remaining thirteen populations, resulting in a significant yet extremely weak correlation (R^2 = -0.01, P=0.03710, graph not included). Distances between sites were calculated by compiling linear measurements around coastlines manually, as such they are likely to under-represent distances between sampling sites in terms of currents and therefore resultant correlations may also be inaccurate.

4.3.7 Bayesian Clustering Methods

Bayesian admixture plots, showing a lack of resolution of population clusters in *Alcyonium digitatum*, are shown in Figure 4.6. The data was tested from K=2 to K=10 and resolution was not improved at higher K values. This result supports a lack of spatial clustering at regional scales in this dataset, as noted in principal components analyses (4.3.5, above), and supports a hypothesis of high levels of connectivity between sites sampled for this study.

4.3.8 Coalescent Analyses

Bayesian estimates of the value of θ, as calculated in MIGRATE (Beerli & Felsenstein 2001) were from 0.85 (Ireland cluster), to 1.95 (the North Sea cluster) to 2.85 (both the Brittany and England clusters), which translated, in this order, into effective population sizes of 2125, 4875, and 7125 (Table 4.11). Estimated migration rates varied substantially, from lowest values of 0.43 (from the Brittany to the England cluster) and 0.5 (from the North Sea to the England cluster), to 24.2 (Brittany to Ireland) and 55.9 (England to Brittany). Although it is of note that these clusters represent crude groupings and do not reveal migration between, for example, geographically distant sites within the same cluster, some interesting patterns can be discerned. Migration between clusters was typified by highly asymmetrical patterns. From

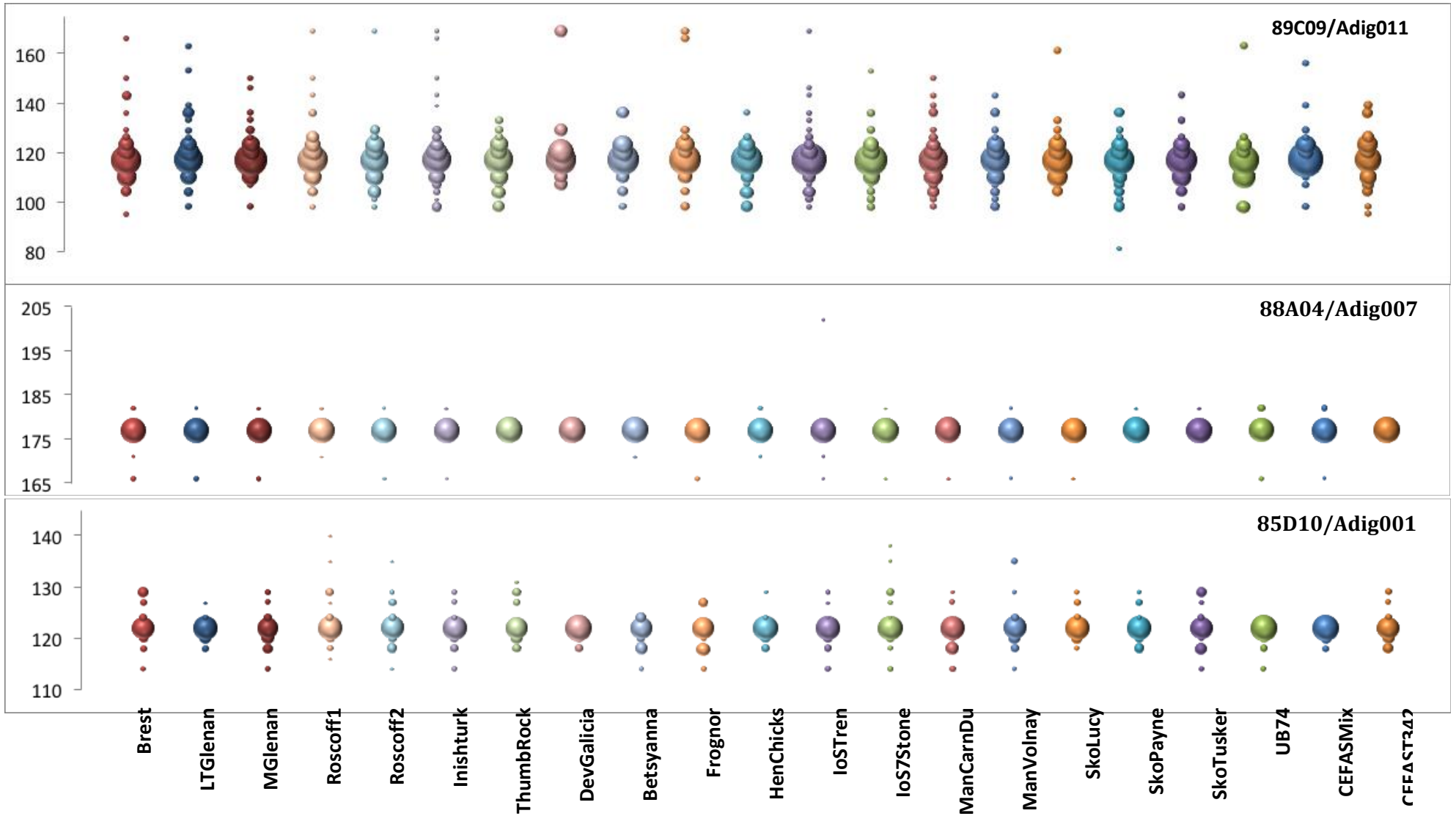
Ireland, there was little migration to the UK, Brittany, or the North Sea (1.2, 1.8 and 1.6 respectively). However, in the opposite direction, there was high migration into the Irish cluster from the UK, Brittany and the North Sea (15.2, 24.2 and 37.3 respectively), indicating that the Irish cluster is a 'sink' as opposed to a 'source' cluster (albeit surprising that populations in western Ireland are connected to the North Sea, suggesting potentially spurious results). The same can be inferred for the England cluster, migration into it was low from the other three regions (1.2, 0.4 and 0.5 from Ireland, Brittany and the North Sea respectively), yet was much higher in the opposite direction (15.2, 55.9 and 17.7 to Ireland, Brittany and the North Sea respectively). Therefore the UK also appears to be more 'sink' than 'source'. Relatively similar migration rates were calculated in both directions between the Brittany and the North Sea clusters, with apparently marginally higher migration towards Brittany (21.9 vs. 24.3). In contrast to *Eunicella verrucosa* (Chapter 3), these *Alcyonium digitatum* patterns imply that migration is much greater from the UK to Brittany than vice versa (55.9 vs. 0.4 here, cf. 8.05 vs. 24.95 for *E. verrucosa*). However, it is again of note that sampling sites are not parallel between both studies and it is therefore difficult to directly compare the two. Finally, migration appears to be much higher from the UK cluster to the North Sea than in the other direction (17.7 vs. 0.5). Interestingly, Ireland appears to have the smallest effective population size (2125), as it does for *Eunicella verrucosa*.

Table 4.2: Results from Microchecker v2.2.3 (van Oosterhout *et al.* 2004) for all loci and populations of *Alcyonium digitatum*. Columns correspond to: 1= evidence of null alleles, 2=evidence for stutter peaks, 3=evidence for large allelic dropout and 4=more than 50% of alleles belong to one size class. Shaded cells indicate positives, N represents total number of individuals tested. Coloured cells represent collection region (green = France, pink = Ireland, blue = UK)

	Clone/ Locus	85D10 Adig001				86B02 Adig002				87B11 Adig003				8.70E+03 Adig004				87H01 Adig005				87H03 Adig006			
Population	**N**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Bre2	43	Y	N	N	Y	N	N	N	Y	Y	Y	N	Y	Y	Y	N	N	N	N	N	N	Y	N	N	Y
LTGlen	29	Y	N	N	Y	N	N	N	Y	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
Mglen	34	Y	N	N	Y	N	N	N	N	N	Y	N	N	Y	N	N	N	N	N	N	N	N	N	N	Y
Ros1	41	N	N	N	Y	N	N	N	N	Y	Y	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
Ros2	41	N	N	N	Y	N	N	N	N	Y	Y	N	N	Y	N	N	N	Y	N	N	N	N	N	N	Y
IreIT	48	Y	N	N	Y	N	N	N	N	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	N
IreTR	18	N	N	N	Y	N	N	N	N	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
DevGal	7	n/a				n/a				n/a				n/a				n/a				n/a			
DorBA	24	N	N	N	Y	N	N	N	Y	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
Frog	18	N	N	N	Y	N	N	N	N	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
HC	36	N	N	N	Y	N	N	N	Y	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
Tren	42	N	N	N	Y	N	N	N	Y	Y	Y	N	Y	Y	Y	N	N	N	N	N	N	N	N	N	Y
Stone	40	N	N	N	Y	N	N	N	N	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
ManCD	33	N	N	N	Y	N	N	N	N	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
ManV	28	N	N	N	Y	N	N	N	N	N	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
Lucy	22	N	N	N	Y	N	N	N	N	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
PR	51	Y	N	N	Y	N	N	N	Y	N	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
TR	21	Y	N	N	Y	N	N	N	Y	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
UB74	19	N	N	N	Y	N	N	N	N	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
CefMiX	27	N	N	N	Y	N	N	N	N	Y	Y	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y
CefT342	33	N	N	N	Y	N	N	N	Y	Y	N	N	Y	Y	N	N	N	N	N	N	N	N	N	N	Y

Table 4.2 continued.

	Clone	88A04				88C09				8.80E+09				8.80E+10				89C09			
	Locus	Adig007				Adig008				Adig009				Adig010				Adig011			
Population	**N**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Bre2	43	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
LTGlen	29	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
Mglen	34	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
Ros1	41	N	N	N	Y	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N
Ros2	41	N	N	N	Y	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N
IreIT	48	N	N	N	Y	N	N	N	N	Y	Y	N	N	Y	N	N	N	N	N	N	N
IreTR	18	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
DevGal	7	n/a				n/a				n/a				n/a				n/a			
DorBA	24	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Frog	18	N	N	N	Y	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N
HC	36	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
Tren	42	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Y
Stone	40	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N
ManCD	33	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
ManV	28	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
Lucy	22	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
PR	51	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
TR	21	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N
UB74	19	N	N	N	Y	N	N	N	N	Y	N	N	N	Y	N	N	N	N	N	N	N
CefMiX	27	N	N	N	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	Y
CefT342	33	N	N	N	Y	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N	N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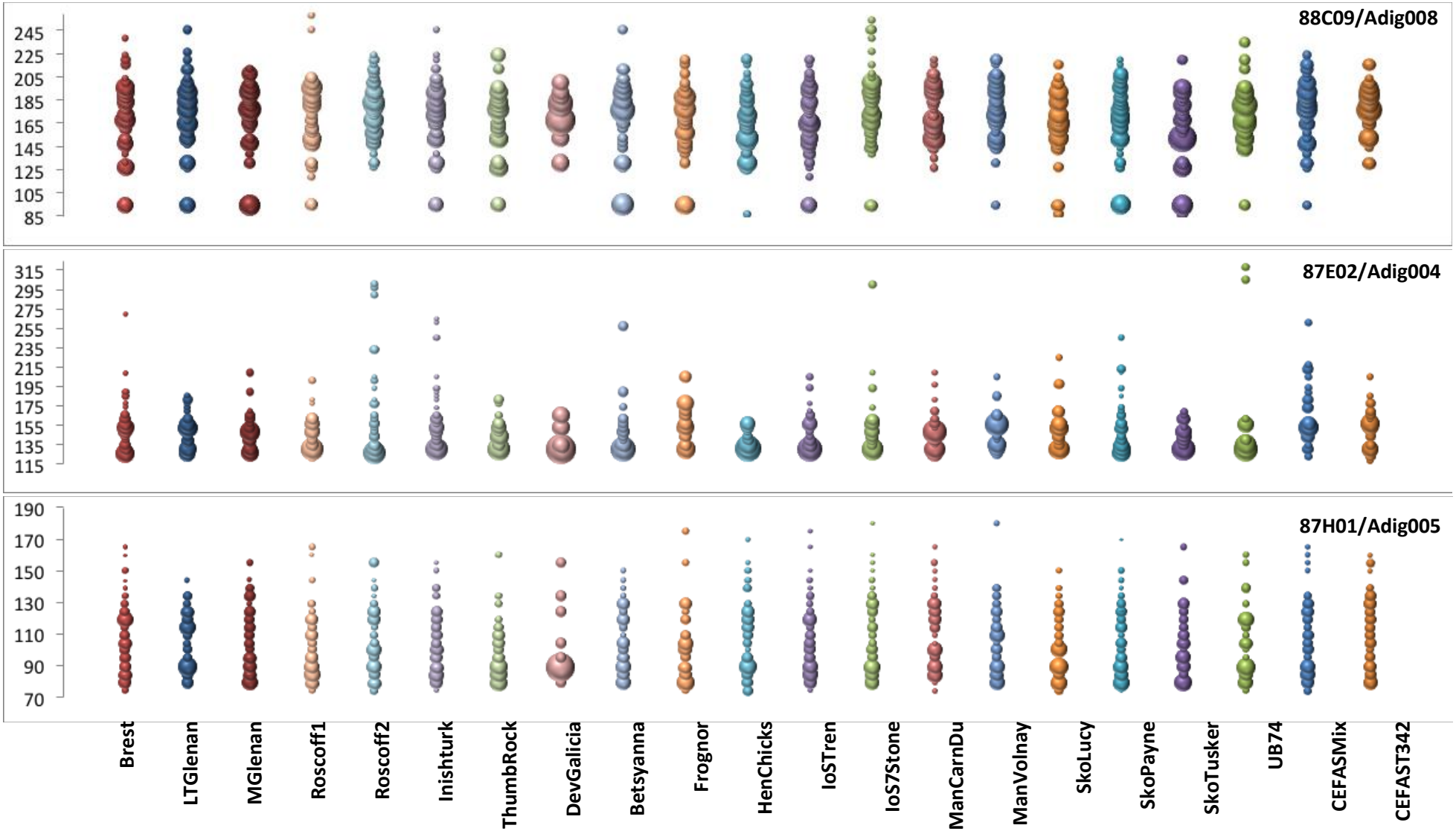
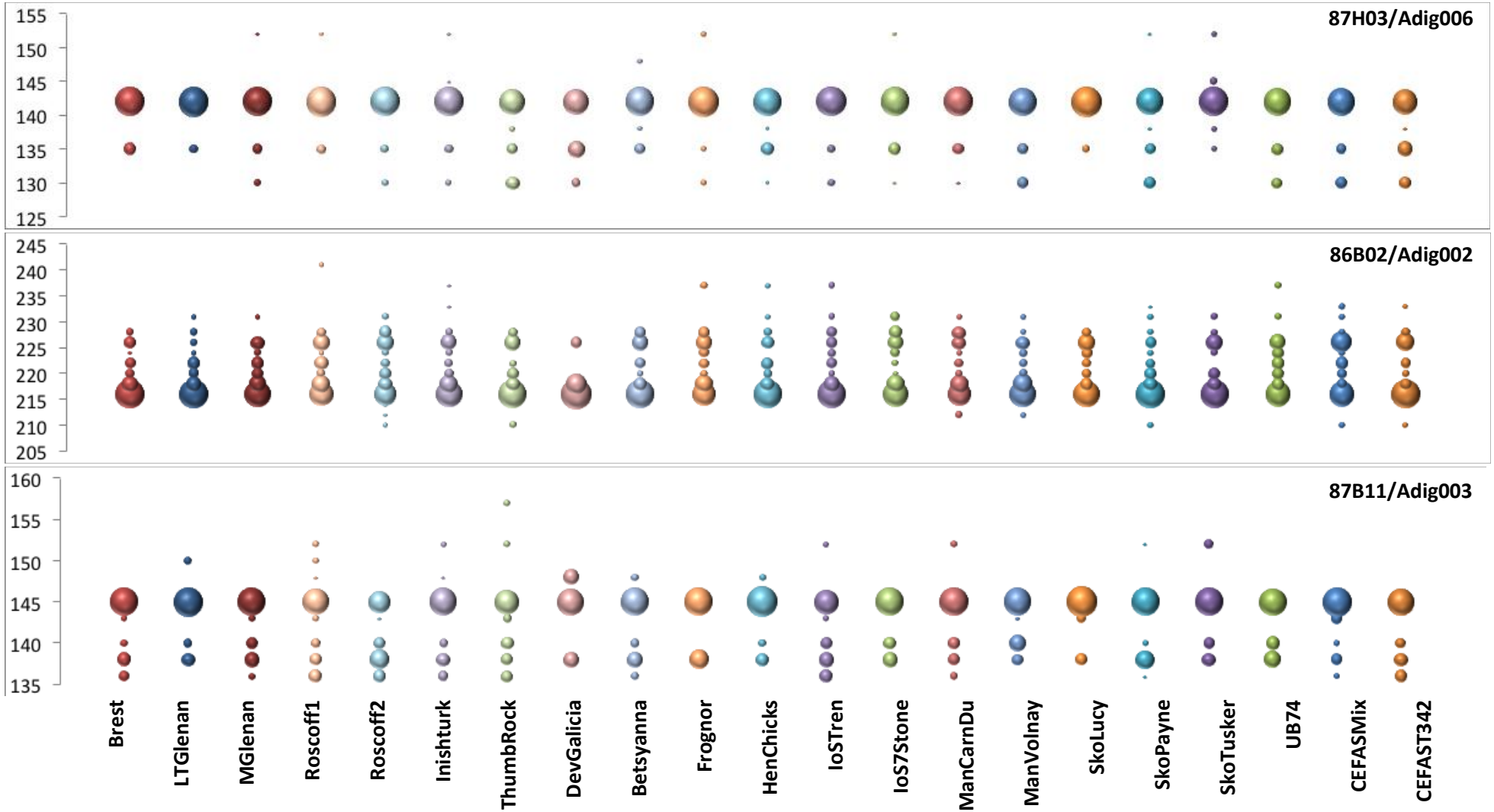
160
140
120
100
80
88A04/Adig007
205
195
185
175
165
85D10/Adig001
140
130
120
110
Brest
LTGlenan
MGlenan
Roscoff1
Roscoff2
Inishturk
ThumbRock
DevGalicia
Betsyanna
Frognor
HenChicks
IoSTren
IoS7Stone
ManCarnDu
ManVolnay
SkoLucy
SkoPayne
SkoTusker
UB74
CEFASMix


88C09/Adig008
245
225
205
185
165
145
125
105
85
87E02/Adig004
315
295
275
255
235
215
195
175
155
135
115
87H01/Adig005
190
170
150
130
110
90
70
Brest
LTGlenan
MGlenan
Roscoff1
Roscoff2
Inishturk
ThumbRock
DevGalicia
Betsyanna
Frognor
HenChicks
IoSTren
IoS7Stone
ManCarnDu
ManVolnay
SkoLucy
SkoPayne
SkoTusker
UB74
CEFASMix
CEFAST342


87H03/Adig006
155
150
145
140
135
130
125
86B02/Adig002
245
240
235
230
225
220
215
210
205
87B11/Adig003
160
155
150
145
140
135
Brest
LTGlenan
MGlenan
Roscoff1
Roscoff2
Inishturk
ThumbRock
DevGalicia
Betsyanna
Frognor
HenChicks
IoSTren
IoS7Stone
ManCarnDu
ManVolnay
SkoLucy
SkoPayne
SkoTusker
UB74
CEFASMix
CEFAST342

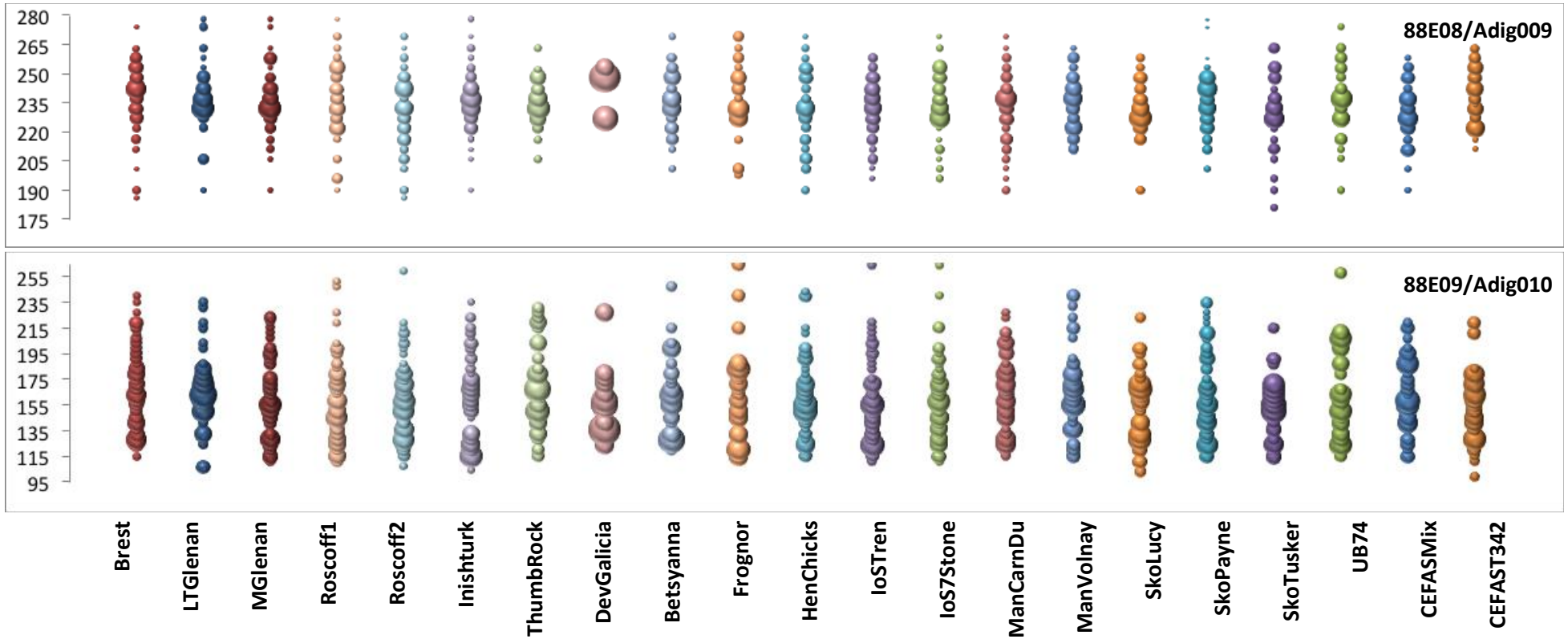
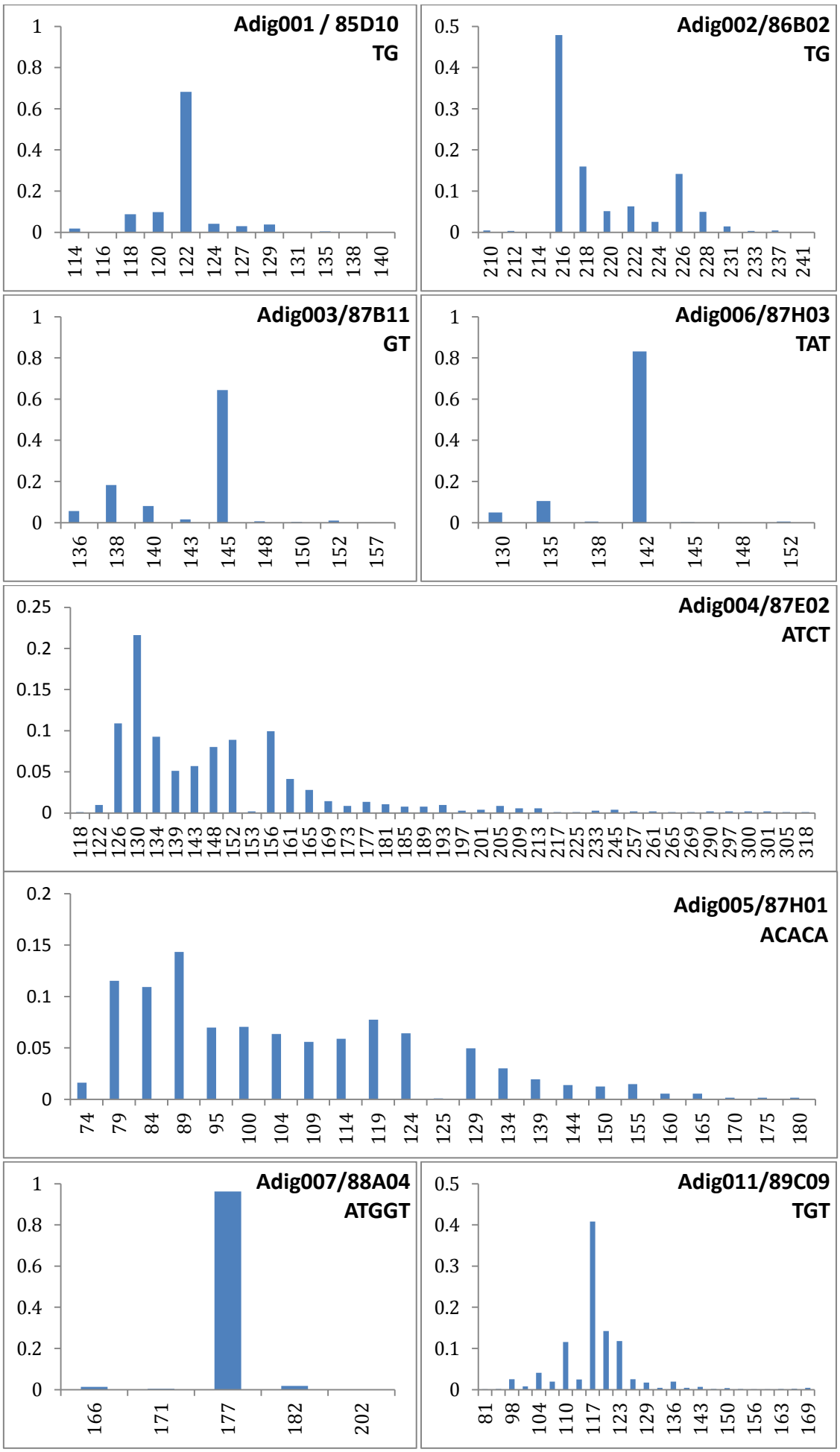


Figure 4.2: *Alcyonium digitatum* bubble plots showing distribution of alleles across all populations for all loci. Y axis represents size of alleles (bp) and X axis shows allele frequency by population in the order indicated underneath (a different colour per population). The larger the circle, the more frequent the allele by proportion within that particular population (i.e., two circles of the same size within a population would represent the presence of two alleles with a 50% frequency each).


Adig001 / 85D10
TG
1
0.8
0.6
0.4
0.2
0
114 116 118 120 122 124 127 129 131 135 138 140
Adig002/86B02
TG
0.5
0.4
0.3
0.2
0.1
0
210 212 214 216 218 220 222 224 226 228 231 233 237 241
Adig003/87B11
GT
1
0.8
0.6
0.4
0.2
0
136 138 140 143 145 148 150 152 157
Adig006/87H03
TAT
1
0.8
0.6
0.4
0.2
0
130 135 138 142 145 148 152
Adig004/87E02
ATCT
0.25
0.2
0.15
0.1
0.05
0
118 122 126 130 134 139 143 148 152 153 156 161 165 169 173 177 181 185 189 193 197 201 205 209 213 217 225 233 245 257 261 265 269 290 297 300 301 305 318
Adig005/87H01
ACACA
0.2
0.15
0.1
0.05
0
74 79 84 89 95 100 104 109 114 119 124 125 129 134 139 144 150 155 160 165 170 175 180
Adig007/88A04
ATGGT
1
0.8
0.6
0.4
0.2
0
166 171 177 182 202
Adig011/89C09
TGT
0.5
0.4
0.3
0.2
0.1
0
81 98 104 110 117 123 129 136 143 150 156 163 169

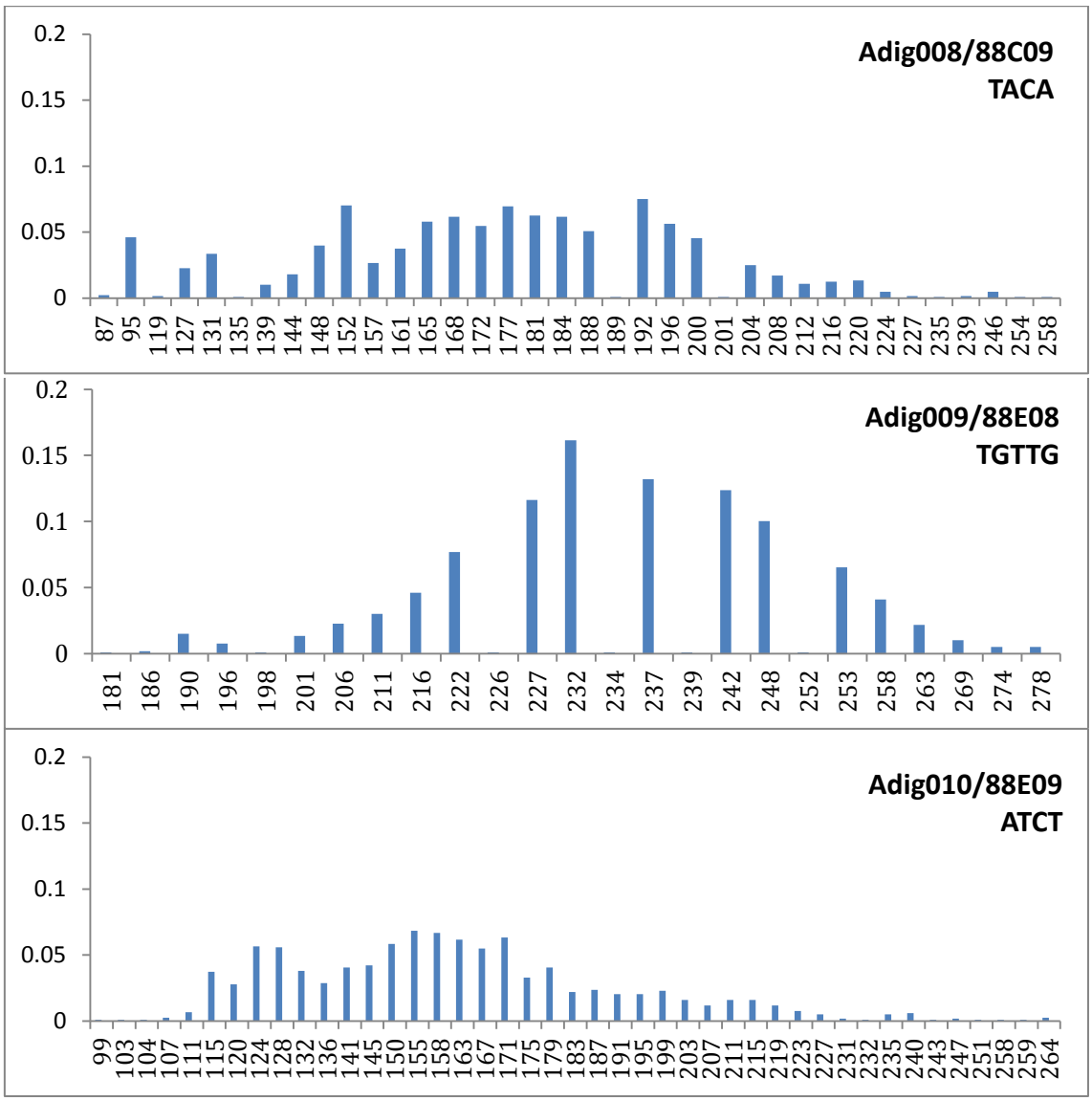


Figure 4.3: Total allele frequencies shown by locus across all *Alcyonium digitatum* data (N=655), calculated in Microsatellite Analyser (MSA) v.4.05 (Dieringer and Schlötterer 2003). All alleles detected at each locus are shown on the X axis and frequency indicated by the Y axis (note Y axis scale varies; cumulative frequencies =1). Repeat motif for each locus is given, to illustrate prevalence of imperfect repeats and aberrant allele sizes compared to expectations based upon models of microsatellite evolution (e.g., infinite alleles and stepwise mutation models).

Table 4.3: Summary of private alleles in *Alcyonium digitatum* as calculated in GenAlEx v6.5b3 (Peakall and Smouse 2012). No distinction is made between heterozygotes and homozygotes in allele frequencies and therefore number of individuals containing the allele was counted manually as indicated. Coloured cells represent collection region (green = France, pink = Ireland, blue = UK)

Population	Locus	Clone	Allele	Freq	# Indivs with allele
Bre2	Adig004	87E02.	269	0.013	1
Bre2	Adig009	88E08.	239	0.012	1
Ros1	Adig001	85D10.	116	0.013	1
Ros1	Adig001	85D10.	140	0.013	1
Ros1	Adig002	86B02.	241	0.013	1
Ros1	Adig004	87E02.	153	0.030	1
Ros1	Adig008	88C09.	189	0.012	1
Ros1	Adig008	88C09.	201	0.012	1
Ros1	Adig008	88C09.	258	0.012	1
Ros1	Adig010	88E09.	251	0.013	1
Ros2	Adig002	86B02.	214	0.013	1
Ros2	Adig004	87E02.	233	0.045	2
Ros2	Adig004	87E02.	290	0.030	1
Ros2	Adig004	87E02.	297	0.030	1
Ros2	Adig004	87E02.	301	0.030	1
Ros2	Adig010	88E09.	259	0.013	1
IreIT	Adig004	87E02.	265	0.012	1
IreIT	Adig009	88E08.	226	0.011	1
IreIT	Adig010	88E09.	104	0.011	1
IreTR	Adig001	85D10.	131	0.028	1
IreTR	Adig003	87B11.	157	0.028	1
IreTR	Adig009	88E08.	252	0.029	1
DorBA	Adig004	87E02.	257	0.059	1
DorBA	Adig006	87H03.	148	0.021	1
Frog	Adig009	88E08.	198	0.033	1
HC	Adig010	88E09.	243	0.015	1
Tren	Adig007	88A04.	202	0.012	1
Stone	Adig001	85D10.	138	0.013	1
Stone	Adig004	87E02.	300	0.033	1
Stone	Adig008	88C09.	254	0.014	1
ManCD	Adig008	88C09.	135	0.015	1
ManV2	Adig010	88E09.	232	0.019	1
Lucy	Adig004	87E02.	225	0.028	1
Lucy	Adig010	88E09.	103	0.024	1
Lucy	Adig011	89C09.	161	0.023	1
PR	Adig005	87H01.	125	0.010	1
PR	Adig011	89C09.	81	0.010	1
TR	Adig009	88E08.	181	0.031	1
UB74	Adig004	87E02.	305	0.036	1
UB74	Adig004	87E02.	318	0.036	1
UB74	Adig008	88C09.	235	0.029	1
UB74	Adig010	88E09.	258	0.031	1
CefMiX	Adig004	87E02.	217	0.031	1
CefMiX	Adig011	89C09.	156	0.019	1
CefT342	Adig004	87E02.	118	0.025	1
CefT342	Adig009	88E08.	234	0.016	1
CefT342	Adig010	88E09.	99	0.018	1

Table 4.4: Analysis of Molecular Variance (AMOVA) test of *Alcyonium digitatum* to determine hierarchical population structure (tested in Arlequin v3.5.1.2, Excoffier 2010). Data was not split into sub-groups following preliminary results from STRUCTURE indicating little differentiation.

One Group:							
Source of variation	**Percentage of Variation**	**d.f.**	**Fstat**	**Fvalue**	**P-value**	**Sum of squares**	**Variance components**
Among populations	0.13	20	F_{ST}	0.00128	1	77.945	0.00421 Va
Among individuals within populations	10.47	634	Fis	0.10481	0	2305.557	0.34498 Vb
Within individuals	89.4	655	Fit	0.10595	0	1930	2.94656 Vc
Total		**1309**				**4313.502**	**3.29575**

Table 4.5: *Alcyonium digitatum* heterozygosity summary statistics by locus. *H*o observed heterozygosity, *H*s heterozygosity, Fst values, Ar allelic richness. Calculated in MSAnalyzer v4.05 (Dieringer and Schlötterer 2003).

	Ho	Hs	Fst	Ar
Adig001/85D10	0.403	0.504	0.007	4.02
Adig002/86B02	0.656	0.710	0.005	5.15
Adig003/87B11	0.240	0.533	0.010	3.23
Adig004/87E02	0.347	0.886	0.012	7.65
Adig005/87H01	0.894	0.914	0.000	8.87
Adig006/87H03	0.305	0.298	0.027	2.49
Adig007/88A04	0.071	0.070	0.000	1.47
Adig008/88C09	0.955	0.949	0.001	10.35
Adig009/88E08	0.792	0.892	0.006	4.74
Adig010/88E09	0.740	0.959	-0.002	10.77
Adig011/89C09	0.781	0.783	-0.002	6.17
Overall	**0.562**	**0.682**	**0.004**	**5.901**

Table 4.6: Summary statistics by population for all *Alcyonium digitatum* samples in this study. Coloured cells represent collection region (green = France, pink = Ireland, blue = UK). *N* number of individuals included from each population, *k* (total) all alleles in all loci totalled, *k* (mean) average number of alleles per locus, *Ar* average allelic richness (calculated in MSAnalyzer v4.05, Dieringer and Schlötterer 2003), *Ho* observed heterozygosity, *Hs* heterozygosity within populations, *G*is inbreeding coefficient, *F*is inbdreeding coeffiecient, P value of F*is* value(Rand FIS>=Obs FIS). * values calculated in Arlequin v. 3.5.1.2 (Excoffier 2010), all other values derived from Genodive v2.0b23 (Meirmans 2013). Populations in **bold type** had 10/11 usable loci as calculated in Arlequin, in all others 11 loci were useable. Standard errors of F-statistics in Genodive are obtained via jack-knifing over loci and 95% confidence intervals through bootstrapping (data not shown). For a detailed presentation of summary statistics by locus for all populations, see Appendix 3.

Populations	N	k* (total)	k* (mean)	Ar	Ho	Hs	Gis	FIS*	P-value*
Brest2	43	141	12.8	5.96	0.58	0.69	0.16	**0.11**	**0**
LT_Glen	29	118	10.7	5.73	0.54	0.65	0.17	**0.10**	**0**
MenGlen	34	125	11.4	5.84	0.59	0.70	0.16	**0.11**	**0**
Ros1	41	137	12.5	5.94	0.55	0.69	0.21	**0.15**	**0**
Ros2	41	143	13.0	6.07	0.57	0.71	0.21	**0.16**	**0**
IreITWest	48	144	13.1	5.56	0.56	0.68	0.18	**0.15**	**0**
IreTR	18	102	9.3	5.52	0.63	0.72	0.12	**0.09**	**0**
DevGal	7	54	4.9	4.91	0.5	0.64	0.21	**0.14**	**0.05**
DorBA	24	107	9.7	5.61	0.57	0.68	0.17	**0.08**	**0.01**
Frog	18	102	9.3	5.85	0.51	0.69	0.26	**0.15**	**0**
HenChicks	36	121	11.0	5.55	0.53	0.65	0.18	**0.10**	**0**
IoSTren	42	140	12.7	5.93	0.57	0.67	0.15	**0.07**	**0**
IoS_Stones	40	132	12.0	5.83	0.55	0.67	0.18	**0.10**	**0**
ManCD	33	130	11.8	5.87	0.58	0.68	0.15	**0.09**	**0**
ManV	28	117	10.6	5.91	0.61	0.70	0.14	**0.05**	**0.04**
SkoLUCY	22	100	9.1	5.77	0.53	0.64	0.17	**0.08**	**0.02**
SkoPR	51	138	12.5	5.70	0.59	0.68	0.13	0.08	**0**
SkoTR	21	104	9.5	5.80	0.57	0.69	0.17	0.04	0.12
UB74	19	97	8.8	5.61	0.55	0.69	0.20	**0.13**	**0**
CefasMix	27	122	11.1	5.84	0.55	0.67	0.18	0.05	0.06
CefasT342	33	114	10.4	5.93	0.57	0.72	0.21	**0.12**	**0**

Table 4.7: Tests for evidence of genetic bottlenecks according to the software BOTTLENECK v1.2.02 (Cornuet and Luikart 1996) under the infinite alleles (IAM), two phase model (TPM) and stepwise mutation model (SMM) assuming mutation-drift equilibrium. He E expected number of loci with heterozygosity excess, E/D numbers of loci with heterozygosity excess / heterozygosity deficiency. Shaded cells represent significant P values at the 5% level (i.e., showing evidence of a bottleneck) under Sign or Wilcoxon sign ranked tests.. Coloured cells represent collection region (green = France, pink = Ireland, blue = UK).

Population	IAM				TPM				SMM			
	He E	E/D	Sign Test	Wilcoxon Test	He E	E/D	Sign Test	Wilcoxon Test	He E	E/D	Sign Test	Wilcoxon Test
Brest 2	6.4	6/5	0.516	0.413	6.4	6/5	0.510	0.520	6.4	4/7	0.120	0.067
LT Glen	6.3	5/6	0.303	1.000	6.4	4/7	0.117	0.102	6.5	2/9	0.007	0.005
Men Glen	6.5	7/4	0.514	0.365	6.5	5/6	0.272	0.465	6.5	3/8	0.036	0.021
Ros 1	6.5	6/5	0.497	0.638	6.6	6/5	0.472	0.320	6.5	4/7	0.112	0.054
Ros 2	6.4	7/4	0.491	0.278	6.6	5/6	0.240	0.413	6.6	7/4	0.105	0.054
Ire InishTurk	6.5	6/5	0.485	1.000	6.6	3/8	0.030	0.102	6.6	3/8	0.030	0.007
IR ThumbRock	6.0	7/3	0.392	0.105	6.1	5/5	0.350	0.432	6.0	9/1	0.002	0.014
Dev Gal	6.1	7/3	0.405	0.846	6.4	6/4	0.528	1.000	5.9	4/6	0.185	0.275
Dor BA	6.4	8/3	0.255	0.520	6.5	4/7	0.104	0.240	6.4	3/8	0.037	0.007
Frognor	6.3	7/4	0.464	0.577	6.4	6/5	0.509	0.831	6.3	5/6	0.299	0.365
Hen Chicks	6.4	6/5	0.509	0.577	6.6	5/6	0.238	0.206	6.5	3/8	0.034	0.054
IoS Tren	6.4	6/5	0.482	0.765	6.8	5/6	0.214	0.206	6.5	2/9	0.007	0.009
IoS Stone	6.4	7/4	0.480	0.700	6.5	7/4	0.511	0.700	6.6	4/7	0.104	0.083
Man CD	6.4	5/6	0.297	0.700	6.4	3/8	0.037	0.054	6.4	2/9	0.008	0.003
ManV2	6.5	7/4	0.495	0.465	6.6	5/6	0.254	0.520	6.6	4/7	0.095	0.206
Skomer Lucy	6.3	6/5	0.541	0.638	6.3	5/6	0.301	0.320	6.3	4/7	0.130	0.067
Skomer PR	6.4	5/6	0.279	0.160	6.3	5/6	0.310	0.413	6.4	2/9	0.008	0.016
Skomer TR	6.5	5/6	0.274	0.831	6.6	4/7	0.101	0.206	6.4	3/8	0.038	0.016
UB74	6.4	9/2	0.097	0.240	6.7	5/6	0.237	0.765	6.6	3/8	0.030	0.067
CEFAS Mix	6.4	5/6	0.288	0.831	6.6	4/7	0.099	0.278	6.7	4/7	0.092	0.021
CEFAS T342	5.9	8/2	0.156	0.005	6.0	8/2	0.162	0.232	5.9	4/6	0.184	0.625

Table 4.8: Matrix of pairwise population Fst values of *Alcyonium digitatum*, as calculated in GenAlEx. Significantly different values following correction for multiple tests using the false discovery method (Benjamini and Hochberg 1995) are highlighted, and negative values are treated as zeros. For a more detailed description of population sampling locations and numbers sampled see Table 4.1.

	Brest2	LT_Glen	MenGlen	Ros1	Ros2	IreITWest	IreTR	DevGal	DorBA	Frog	HenChicks	IoSTren	IoS_Sstones	ManCD	ManVtake2	SkoLUCY	SkoPR	SkoTR	UB74	CefasMix
Brest2																				
LT_Glen	0.004																			
MenGlen	0.001	0.005																		
Ros1	0.003	0.008	0.002																	
Ros2	**0.008**	**0.014**	**0.008**	0.005																
IreITWest	0.003	-0.001	0.001	-0.001	**0.010**															
IreTR	0.007	0.004	0.001	0.002	0.010	0.002														
DevGal	0.007	0.007	0.016	0.001	0.010	-0.003	-0.012													
DorBA	-0.002	0.000	-0.004	-0.001	0.009	-0.004	-0.002	0.003												
Frog	0.001	0.009	-0.001	0.002	-0.001	-0.002	0.000	0.024	0.000											
HenChicks	0.006	0.002	**0.008**	0.005	**0.017**	0.001	0.004	-0.004	-0.002	0.010										
IoSTren	0.002	0.001	0.004	-0.003	0.005	-0.003	0.002	-0.014	0.002	0.008	-0.002									
IoSSstones	0.004	0.005	0.003	0.000	**0.010**	-0.001	0.004	-0.007	0.003	0.003	0.002	0.002								
ManCD	**0.008**	**0.009**	0.005	0.004	**0.012**	0.003	0.011	0.011	0.005	0.007	**0.011**	0.005	0.000							
ManVtake2	0.002	0.004	0.000	0.004	**0.011**	0.001	-0.004	0.002	0.003	0.007	0.007	0.004	0.004	0.008						
SkoLUCY	-0.007	-0.005	-0.003	-0.006	-0.001	-0.011	-0.009	0.025	-0.007	-0.006	-0.003	-0.010	-0.013	-0.002	-0.006					
SkoPR	0.004	0.004	0.004	**0.009**	**0.010**	0.002	0.001	-0.005	-0.001	0.002	0.004	-0.003	0.003	**0.014**	0.002	-0.002				
SkoTR	-0.003	0.008	-0.007	-0.006	-0.001	-0.007	-0.014	0.017	-0.009	-0.004	0.001	-0.008	-0.008	-0.002	0.001	0.003	-0.001			
UB74	0.005	0.007	0.006	0.003	0.008	-0.002	-0.003	-0.013	0.004	0.009	0.002	-0.001	0.003	0.006	0.003	-0.011	0.001	-0.005		
CefasMix	0.004	0.008	-0.003	0.003	0.008	-0.003	-0.008	0.008	0.003	0.008	**0.010**	0.001	-0.001	**0.009**	0.005	-0.008	0.003	0.008	0.005	
CefasT342	0.001	**0.010**	0.003	0.006	**0.010**	0.007	-0.007	-0.001	0.005	**0.013**	**0.012**	**0.017**	**0.013**	**0.016**	0.001	0.001	0.003	0.001	**0.011**	0.009

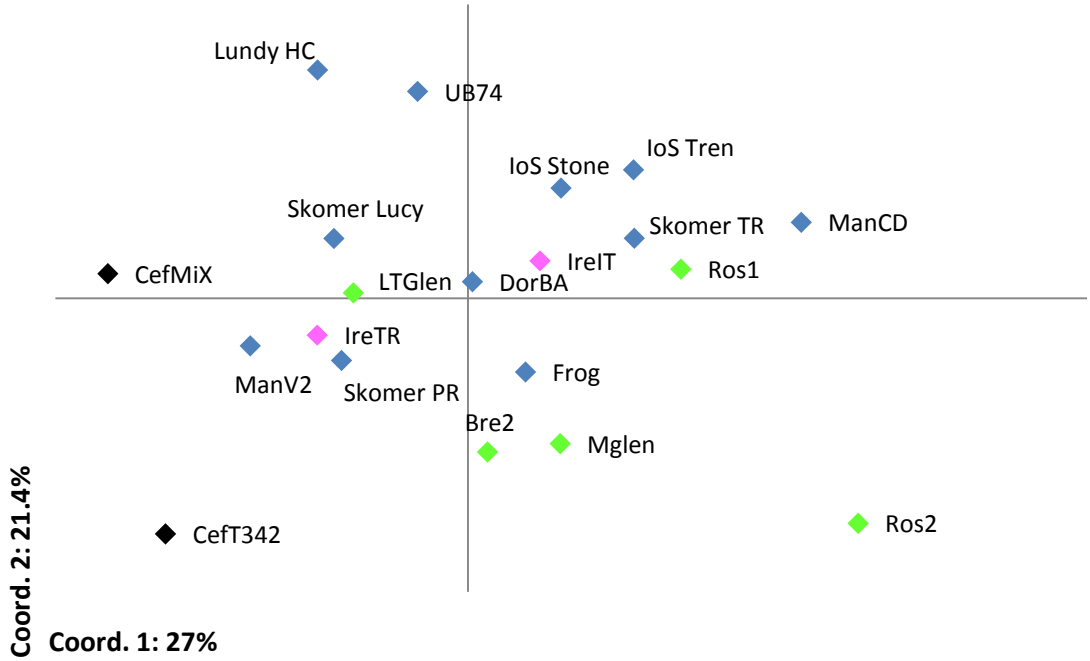
Table 4.9: Matrix of Dest (Jost 2008) values of *Alcyonium digitatum,* as calculated in GenAlEx (99 permutations). Highlighted cells indicate significant comparisons based upon P values at the 5% level. For a more detailed description of population sampling locations and numbers sampled see Table 4.1

	Brest2	LT_Glen	MenGlen	Ros1	Ros2	IrelTWest	IreTR	DevGal	DorBA	Frog	HenChicks	IoS_Tren	IoS_Sstones	ManCD	ManVtake2	SkoLUCY	SkoPR	SkoTR	UB74	CefasMix
Brest2																				
LT_Glen	0.005																			
MenGlen	-0.003	0.007																		
Ros1	0.005	**0.015**	0.003																	
Ros2	**0.020**	**0.033**	**0.018**	0.010																
IrelTWest	0.006	0.000	0.000	-0.007	**0.018**															
IreTR	0.015	0.011	0.002	0.003	0.020	-0.002														
DevGal	**0.065**	**0.050**	**0.087**	**0.052**	**0.083**	**0.046**	0.029													
DorBA	0.005	0.002	-0.003	-0.002	0.022	-0.007	-0.007	0.032												
Frog	0.009	0.010	0.003	0.006	0.006	0.000	0.001	0.051	-0.005											
HenChicks	**0.018**	0.004	**0.024**	0.010	**0.048**	0.007	0.012	0.013	-0.003	0.013										
IoSTren	**0.014**	0.013	**0.014**	-0.002	**0.017**	0.001	0.006	0.031	0.000	0.012	0.004									
IoS_Sstones	**0.010**	0.010	0.004	-0.003	**0.023**	0.001	0.009	0.026	0.001	0.001	0.005	0.001								
ManCD	**0.018**	**0.021**	0.008	0.003	**0.026**	0.005	0.022	**0.070**	0.010	0.020	**0.025**	**0.016**	-0.001							
ManVtake2	0.008	0.009	0.008	0.008	**0.028**	0.004	-0.004	**0.056**	0.008	0.013	**0.012**	0.012	0.007	**0.020**						
SkoLUCY	0.004	-0.007	0.010	0.008	**0.029**	0.000	0.008	0.043	0.001	-0.009	0.000	0.013	-0.003	**0.016**	0.007					
SkoPR	0.006	0.010	0.007	**0.021**	**0.023**	0.005	0.002	0.035	0.004	0.008	0.009	0.007	**0.010**	**0.030**	0.008	0.012				
SkoTR	0.000	0.009	-0.010	-0.008	0.016	-0.007	-0.013	0.034	-0.015	-0.010	-0.004	0.001	-0.013	-0.001	0.012	-0.004	0.002			
UB74	**0.019**	0.016	**0.025**	0.005	**0.024**	0.002	-0.002	0.023	0.003	0.011	0.000	-0.004	0.001	0.017	0.003	0.006	0.007	0.001		
CefasMix	**0.026**	**0.021**	0.017	**0.022**	**0.050**	**0.015**	0.016	**0.056**	0.019	0.013	**0.022**	**0.029**	0.010	**0.035**	0.017	-0.003	**0.022**	**0.025**	0.018	
CefasT342	0.008	**0.025**	0.015	0.015	**0.028**	**0.021**	-0.010	0.050	0.007	0.017	**0.026**	**0.033**	**0.023**	**0.036**	-0.002	0.024	0.011	0.018	0.019	0.021

Table 4.10: Permutation tests as carried out in FSTAT v2.9.3.2 (Goudet 2002). P values represent a two-sided P-value obtained after 1000 permutations. The data set was divided into four putative clusters as follows: **France**= Britt_Brest2, Britt_LTGlen, Britt_MenGlen, Britt_Roscoff1, Britt_Roscoff2; **Ireland**= Ireland_IT, Ireland_TR; **SW England**= Devon_Galicia, Dorset_BetsyAnna, Dorset_Frognor, IslesofScilly_HenChicks, IslesofScilly_Trenemene, IslesofScilly_7Stones, Cornwall_ManCD, Cornwall_ManV, Skomer_Lucy, Skomer_PayneRock, Skomer_TuskerRock; **N Sea**= Cefas_MIX, Cefas_T342.

Region	Ar	Ho	Hs	Fis	Fst
France	3.704	0.567	0.692	0.181	0.005
Ireland	3.714	0.579	0.689	0.160	-0.001
SW England	3.580	0.563	0.675	0.166	0.003
N Sea	3.706	0.563	0.699	0.194	0.007
P-value	0.468	0.855	0.579	0.486	0.542

a) *A. digitatum* PCA of covariance of Nei's unbiased genetic distance



b) *A. digitatum* PCA of Pairwaise F_{st}

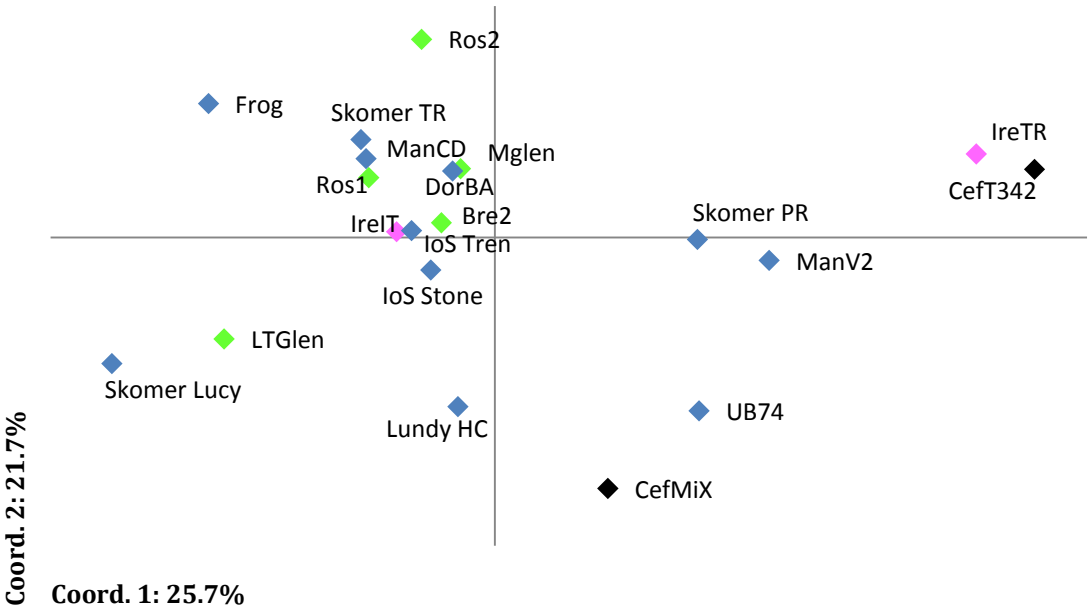


Figure 4.4: Principal coordinates analyses (PCA) of: a) covariance matrices based upon Nei's unbiased genetic distance and b) pairwise Fst for *Alcyonium digitatum*. Populations are colour coded as follows: blue = UK sites, green = Brittany, pink = Ireland and black = North Sea. The first two coordinates only are plotted here; percentage variation explained by the first three axes is 66.13% for Nei's distance (26.96%, 21.43%, 17.73% for axes 1, 2 and 3 respectively) and 63.27% for pairwise F_{st} (25.72%, 21.7%,15.85% for axes 1, 2 and 3 respectively). PCA analyses conducted in GenAlEx v6.5b.3 (Peakall and Smouse 2012).

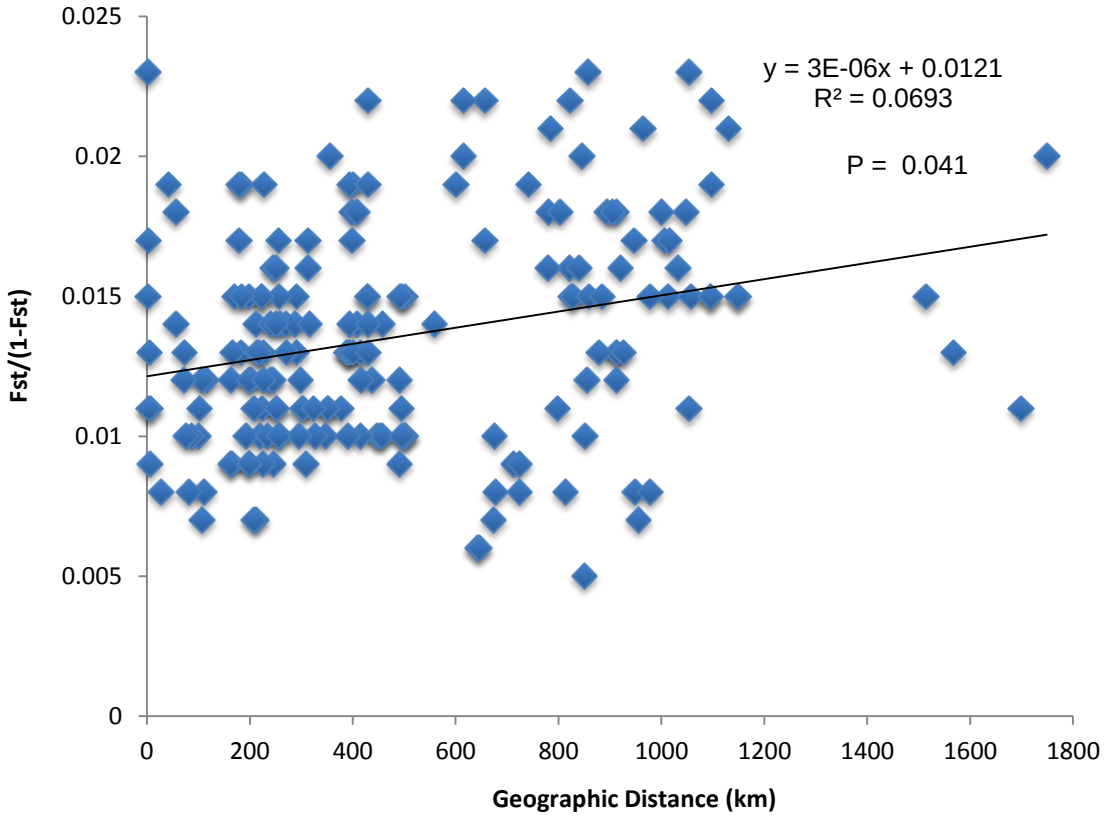


Figure 4.5: Mantel correlation test to determine Isolation by Distance (IBD) in *Alcyonium digitatum*, using all sampled data and all loci. Geographical distance explained almost 7% of the variation in genetic difference across all samples. P value corresponds to a one tailed P value (Pr correlation > observed correlation) as calculated in Genepop v4.2 under the null hypothesis of independence of geographic and genetic distance (Raymond and Rousset 1995, Rousset 2008). Regression slope is fit using Spearman rank correlation coefficient (1000 permutations).

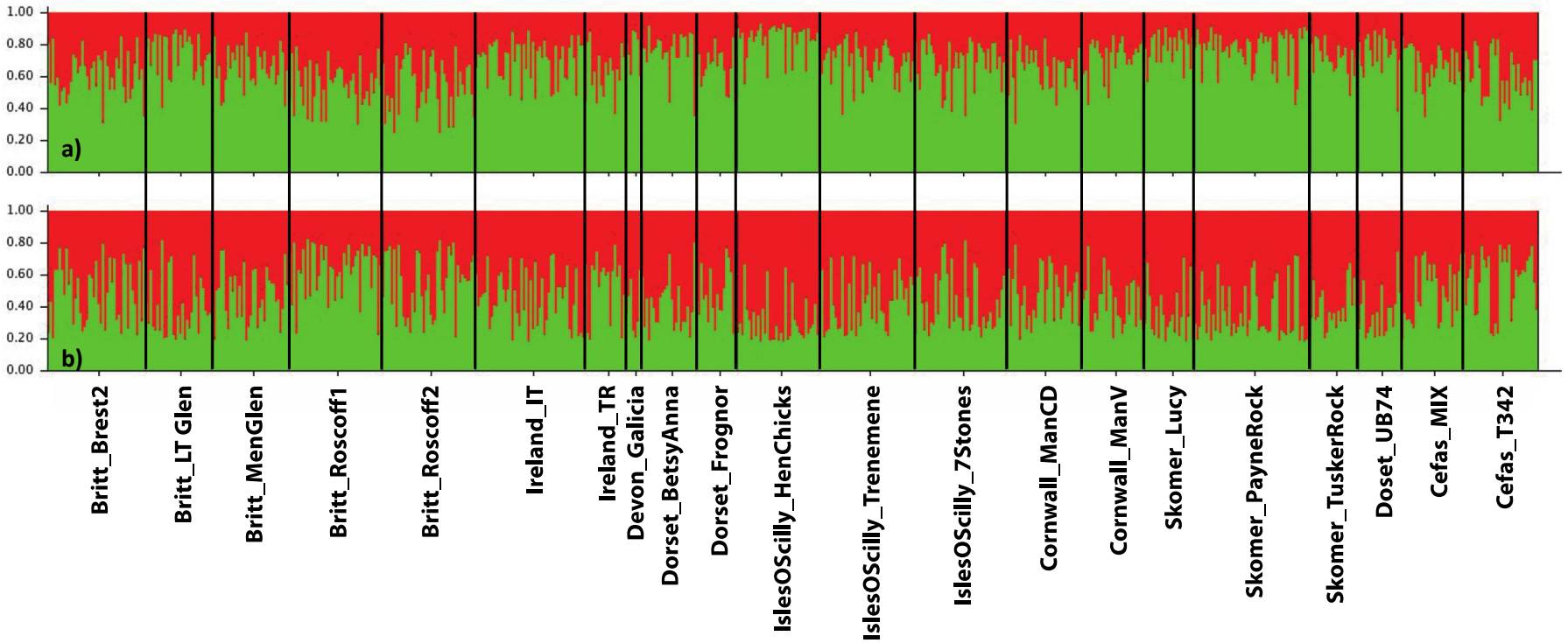


Figure 4.6: Bar plots of admixture proportions from STRUCTURE analysis, showing lack of genetic structure in *Alcyonium digitatum*. Y-axis represents the average probability of membership of individuals based upon 11 loci, X-axis represents individuals (grouped into populations). **a)** clustering pattern across all data (N=655, 21 populations), showing K=2, with the following settings: 10k burnin, 10^6 MCMC, using sampling location as a prior (LOCPRIOR option), correlated allele frequencies, and admixed populations, with each run replicated twice; **b)** data and settings as previous, but without the application of an admixture model. In all cases convergence was verified by graphing MCMC progress *a posteriori*. Populations in both figures are arranged geographically in the regional order Brittany / Ireland / southwest UK / UK North Sea, see Table 4.1 for details.

Table 4.11: Gene flow estimates among the clusters of *Alcyonium digitatum* populations suggested by STRUCTURE analyses, based on a Bayesian likelihood inference calculated using the software MIGRATE (Beerli & Felsenstein 2001). Theta (θ) values are shaded in bold on the diagonal and were used to calculate Ne assuming a mutation rate of $\mu=10^{-4}$ per locus per generation ($Ne = \theta / 4\mu$). Rows and columns represent source and sink populations, respectively, and all populations (N=21) were grouped according to four regional clusters as indicated.

Cluster Group	"Brittany" (1)	"Ireland" (2)	"UK" (3)	"North Sea" (4)	Ne
"Brittany" (1)	**2.85**	1.77	55.90	24.30	7125
"Ireland" (2)	24.17	**0.85**	15.17	37.30	2125
"UK" (3)	0.43	1.23	**2.85**	0.50	7125
"North Sea" (4)	21.90	1.63	17.70	**1.95**	4875

4.4 Discussion

4.4.1. Genetic Structure in *Alcyonium digitatum*

Alcyonium digitatum appears to be panmictic in the areas sampled for this study, implying high levels of connectivity at large spatial scales and a lack of genetic structuring at local to regional scales. Although comparable microsatellite data are limited in this genus, the pattern we found contrasts with *A. rudyi* in the northwestern USA, which shows strong genetic structuring concordant with philopatric settlement, low dispersal, and locally-defined structure (based upon allozymes, McFadden 1997), and allegedly distinct populations in the UK and France in *Alcyonium digitatum* (d'Hondt and d'Hondt 1992). Allozyme spatial autocorrelation tests in clonal *Alcyonium* sp sampled in the same area (NW USA) demonstrate significant small scale structure at scales less than 40cm, but not at larger spatial scales (McFadden and Aydin 1996). In the latter studies, *Alcyonium* sp and *A. rudyi* have low dispersal abilities and a high extent of clonality, whereas *Alcyonium digitatum* shows strong evidence of sexual reproduction, as evidenced in this study by low incidence of duplicate genotypes (11 out of 666 in a dataset allowing for failed amplifications at two loci). A lack of asexual reproduction or fission has also been recorded in other *A. acaule* (Garrabou 1999), which is an externally-brooding species (McFadden *et al.* 2001). McFadden (1999) examined allozyme variation in *Alcyonium* spp in the North East Atlantic and found prevalence of one genotype around the Ireland and the Isle of Man in *A. hibernicum*, indicating little genetic structuring among them which she suggested matched expectations from asexual reproduction. This species is thought to reproduce parthenogenetically as opposed to sexually, although genetic confirmation for asexual reproduction in this species is pending (McFadden 1999).

Multivariate spatial clustering analyses (PCA) did not discriminate any region in our study, further supporting evidence for high admixture at large spatial scales; the most extreme example of this being that samples collected from the most distant sites, the North Sea and western Ireland, were adjacent in Fst-based PCA (Fig. 4.3b). The PCA analysis supports lack of clustering in *A. digitatum* as also noted in Structure analyses. Mantel tests showed a weak but significant positive correlation between genetic and geographic distances across the whole sampled area, suggesting weak isolation by distance, although at local scale the correlation is lost. This corresponds to genetic structure in a clonal Pacific *Alcyonium* sp. that also did not show isolation by distance pattern at small spatial scale (McFadden and Aydin 1996), although in the same area as this study IBD correlations have been found in other sessile taxa (e.g., *Asterina gibbosa*, Baus *et al.* 2005).

Despite a lack of broad-scale genetic structure, most samples of *A. digitatum* are likely subject to high rates of inbreeding, as 19 out of 21 sampled populations had significant Fis coefficients, indicating an overall heterozygote deficiency (Table 4.6). Low heterozygosity has been previously suggested for *A. digitatum* from unpublished allozyme data, although interestingly, those samples are possibly from the NE Atlantic as in this study (McFadden *et al.* 2001). Complete homozygosity of allozyme gentotypes was reported in *A. hibernicum* from Ireland and the Isle of Man (McFadden 1999) suggesting that heterozygote deficiencies which indicate inbreeding are common in this genus in the British Isles.

High levels of inbreeding are more likely to be observed in smaller isolated populations, where it can lead to decreased fitness and an increased risk of extinction (Wright *et al.* 2008); in terms of conservation this scenario would be of concern. However, the sampled areas in this study are within the central portion of the NE Atlantic range of *A. digitatum*, which extends from Portugal to Iceland (Hartnoll 1975). Due to the ubiquitous distribution of *A. digitatum* within this range, none of the sites I included can be considered marginal; likewise none of them are likely to be isolated from adjacent *A. digitatum* populations, supported here by the high extent of insignificant pairwise Fst and Dest values between neighbouring sites (e.g., Payne's Rock, Tusker Rock and The Lucy in Skomer). However, this assertion is made without considering local hydrography which could of course result in local isolation.

Additionally, I did not detect any evidence that any of the populations are suffering a bottleneck, genetic evidence for which can be detected by localised depression of allelic richness (as opposed to a decrease in heterozygosity, Broquet *et al*. 2010). Although I did detect fewer alleles in some populations, this appears to be an artefact of sampling as opposed to, for example, that particular population being marginal or isolated; a strong positive relationship was obtained between sample size and the number of alleles detected, and the effective number of effective alleles also correlates positively with sample size ('*K* effective' in Table 4.6). In this dataset, to obtain the average total number of alleles encountered in a populations from all loci detected (118), 31 individuals need to be sampled as based on the empirical data (Appendix 5). This number corresponds to sample sizes of 25-30 individuals recommended for other taxa using microsatellites (Hale et al 2012). Nonetheless, a strong positive correlation between individuals sampled and the total number of alleles obtained does not appear to have reached a plateau (Appendix 5), indicating that the full extent of genetic diversity in *A. digitatum* in our sampled area may be under-represented in this dataset and that implications for bottleneck detections must be assessed with caution.

Patterns showing little structure between populations alongside high rates of heterozygote deficiencies have been noted elsewhere in octocorals. Baco and Shank (2005) examined connectivity between *Corallium lauuense* populations on Hawai'ian seamounts and detected low hierarchical structure, low Fst values and high rates of inbreeding, as I did in *A. digitatum*. They suggest that *C. lauuense* populations are therefore likely to be self-seeding with occasional long distance recruitment, with rates of migration high enough to overcome diversifying effects of genetic drift.

4.4.2 Correlating the Biology of *Alcyonium digitatum* with Connectivity

Alcyonium digitatum is unusual in that it spawns in winter, which is rare in octocorals (Hickson 1895). Spawning in winter is well characterised for temperate fish, such as North Sea plaice (*Pleuronectes platessa*), a broadcast spawner with a spawning season between February and March (Hunter *et al.* 2003), Atlantic cod (*Gadus morhua*), which form spawning aggregations in late winter/early spring (Moller 1968) and Atlantic herring (*Clupea harengus*, Evans and Geffen 1998). In marine invertebrates, winter spawning is best documented in the southern hemisphere or polar regions, for example the squid *Moroteuthis ingens* spawns in austral winter between June and July off New Zealand (Jackson 2001), and echinoderm gastrulae and bipinnariae larvae were most abundant in Antarctic plankton trawls in winter (Stanwell-Smith *et al.* 1999). Spawning during the winter months appears to be rare in European temperate marine invertebrates and for *A. digitatum* these represent the months when its food source of phytoplankton and zooplankton are least abundant (Hartnoll 1975). However, it is thought that *A. digitatum* allocates all food to gametogenesis until September and then undergoes a quiescent period for several months until spawning between December and January (Hartnoll 1975). As larvae are lecithotrophic in *Alcyonium* and not reliant upon planktonic abundance during their dispersive phase, winter spawning could prove advantageous in that juvenile colonies would already be settled, metamorphosed and ready to feed prior to the occurrence of spring plankton blooms (Hartnoll 1975). Reduced feeding activity, or 'dormancy', has also been observed in Mediterranean *A. acaule*, which shows the highest rate of contracted polyps during the summer, which is when a decrease in storms and water motion correlates to low food abundance (although it is unclear if this occurs after or during the summer spawning period; Garrabou *et al.* 1999). *Alcyonium glomeratum*, which shares part of the range of *A. digitatum* in the south and western British Isles, is also a broadcast spawner (McFadden *et al*. 2001), although timing of spawning for this species is unknown. However, congenerics in the more southerly part of the species range release (or brood) gametes in summer, including *A. acuale* which surface-broods mature gametes in June-July and *A. coralloides*, an internal brooder, that releases larvae between May and July (Fiorelli *et al.* 2012). In general, in UK waters evidence for winter

spawning by marine invertebrates appears to be scarce, but is inferred in the bivalve *Nucula nitidosa* and the widely-distributed hydroid *Abietinaria abietina* (marlin.ac.uk). Although data remain scarce generally, the release of gametes in winter is highly unusual in a shallow water octocoral, and contrasts with spring and summer release typically observed in other tropical and temperate octocorals (e.g., *Plexaura flexuosa*, Pakes and Woollacott 2008). Autumnal peaks in spawning effort have been reported for some deeper (500m) octocorals in the North West Atlantic (September to December in *Drifa* sp., Sun *et al.* 2010) which the authors attributed to maximal chlorophyll concentrations in the water as opposed to rising temperature- a correlation supported by a second peak in planulation and chlorophyll concentration following the spring plankton bloom in March. Deep-sea scleractinian species in the North East Atlantic also show spawning patterns that correspond with phytodetrital food fall, although in this case in July (*Lophelia pertusa*, Waller *et al.* 2005). In summary, reproductive patterns in octocorals, especially in temperate boreal representatives, warrant further investigation.

The seasonality of spawning is likely to have significant implications for connectivity in my sampled area (Figure 4.1), particularly the UK, due to an increase in the strength and frequency of strong south-westerly winds in winter corresponding to an increase in Atlantic atmospheric depressions at this time (metoffice.gov.uk/climate/uk/sw). These winds could theoretically drive larvae further from their spawning site, especially if they are abundant in surface waters where water from wind generated currents flows fastest (Roberts *et al.* 2009). *Alcyonium digitatum* produces neutrally buoyant eggs (Hartnoll 1975) and therefore their exposure to wind currents probably relates to the depth of the parent colony (in addition to larval behaviour and mortality rates, both of which are poorly documented in *A. digitatum*). Frequent changes in wind direction are more likely to lead to local larval retention, as has been suggested in the invasive mussel *Mytilus galloprovincialis* in South Africa (McQuaid and Phillips 2000). This effect is likely to have a stronger disruptive effect upon the dispersal of longer-lived larvae, in that the overall effect of further dispersal caused by wind forcing will be mitigated by repeated import/export of larvae from the spawning site (Lefebvre *et al.* 2003). *A. digitatum* appears to have long-lived larvae (Mathews 1917). However, in winter in the south west UK, it seems that there is little wind variability (as seen in the Plymouth Mount Batten wind rose averages from Jan 1991-Dec 2000, metoffice.gov.uk), and it is therefore plausible that higher levels of connectivity related to farther larval dispersal due to a winter-spawning strategy could be expected, at least in the south west UK. Hydrography and modelling components are excluded from this research and I am unable to determine likely dispersal routes by *A. digitatum* larvae in other unsampled parts of its range. However, hydrodynamic lagrangian modelling attempts, including the

effect of wind-driven currents, have been carried out within part of my sampled range on the brittle star *Ophiothrix fragilis* (Lefebvre *et al.* 2003). This study, in which larval dispersal was modelled based upon a 26 day life span, suggests that wind currents can promote both local retention and distant dispersal of larvae, depending upon the release site, and suggests that the English Channel acts "partly as a metapopultion with a flux of migrants varying among populations and in relation to wind forcing" with regard to this species. Interestingly, in this paper one of our sampled areas was considered- Roscoff, and it is suggested that few larvae released from there could reach other areas in the Normanno-Breton Gulf or the central English Channel. In our study, despite little differentiation overall, Roscoff2 was the site that had the most differentiation to other areas (e.g., Table 4.6) lending weight to this observation (although of note - wind speeds in this case were based upon actual wind speeds averaged over the summer months for 29 years and may thus actually underestimate hibernal wind forcing in *A. digitatum*). In summary, wind is likely to play a significant role in the dispersal of *A. digitatum*, although the extent of this would vary according to local meteorological trends; estimates (albeit somewhat crude) suggest that in some cases, larval dispersal distances may be doubled in the UK by wind (Roberts *et al.* 2009). Therefore spawning in wintertime could be a significant driving factor in the high levels of connectivity found here in *A. digitatum.*

Alcyonium species produce lecithotrophic larvae (McFadden *et al.* 2001), an instinctively advantageous trait if food is not abundant during the dispersive period of larvae as in *A. digitatum*. However, a planktonic lecithotrophic stage coupled with extended larval longevity appears paradoxical. Yolk-fed lecithotrophs are thought to have a shorter dispersal capacity than planktotrophs, a pattern usually inferred from less pronounced population structure in farther dispersal by larvae of species exhibiting the latter mode. This has been demonstrated genetically in, for example, corals (lecithotroph *Balanophyllia elegans* vs. planktotroph *Paracyathus stearnsii*; Hellberg 1996), bryozoans (lecithotroph *Celleporella hyalina* and planktotroph *Electra pilosa*; Goldson *et al.* 2001) and a sponge (lecithotroph *Scopalina lophyropoda*, Blanquer and Uriz 2010). Lecithotrophic larvae are more energetically costly to produce than planktotrophs, resulting in a trade-off whereby fecundity is reduced and benthic development time increased, but mortality inherent in the planktonic phase is lowered (Krug 1998, Ellingson and Krug 2006). In rare cases, some species of marine invertebrates maximise dispersal and survivorship with the occurrence of both modes of feeding, such as the opisthobranch mollusc *Alderia modesta*, in which lecithotrophic and planktotrophic gametes may be produced by the same individual (Krug 1998). Poecilogony (i.e., alternative larval forms occurring from the same organism) is also known for gastropods (*Alderia* spp.) that can alternate between lecithotrophy and planktotrophy seasonally (Ellingson and Krug

2006), and sipunculans (*Phascolosoma agassizii*) that have different lecithotrophic and planktotropic forms (Schulze *et al.* 2012).

Although I found little populations structure in *A. digitatum*, I did observe high levels of inbreeding. It is difficult to marry the genetic data with life history traits in this species; a long pelagic larval duration *in situ* (Mathews 1917) coupled with winter spawning is likely to increase connectivity, as supported here by little population structure, even between the North Sea and western Ireland. On the other hand, lecithotrophy usually corresponds to shorter dispersal distances (as discussed above), a theory which could be supported in our study by high levels of inbreeding within most of the sampled sites. Perhaps this paradox, of short dispersal inferred by lecithotrophic larvae coupled with a long dispersal suggested by a long pelagic larval duration, could be explained by the possibility that lecithotrophic larvae survive for longer (and could therefore foster increased connectivity) at lower sea surface temperatures (SSTs) as suggested for chitons in the Pacific Northwest (Kelly and Eernisse 2007). It is well documented that dispersal potential correlates positively with reduced SSTs (e.g, in the ascidian *Styela plicata*, Gwendolyn *et al.* 2010), so this hypothesis at least seems plausible.

4.4.3 Conservation and Management Implications for *Alcyonium digitatum*

Although widespread and abundant around UK coasts, *Alcyonium digitatum* is locally vulnerable in some areas, primarily due to fishing activity. For example, some populations of *A. digitatum* have been damaged by benthic scallop dredging for *Pecten maximus* and *Aequipecten opercularis* (the king and queen scallop respectively), such as in Lyme Bay, Devon (Hinz *et al.* 2011), whereas others are likely to be detrimentally affected by trawling for benthic fish including *Solea solea* (sole), such as in Anglesey, Wales (Kaiser *et al.* 1998). The overall extent of anthropogenic disturbance and its long-term effect upon *A. digitatum* has not been adequately studied, as data concerning its recovery potential are scarce or inconclusive; Kaiser *et al.* (1998) were unable to quantify changes in the biomass of *A. digitatum* pre- and immediately post-trawl. However, they assert that it is likely to be affected in the long term by intense and repeated fishing activity given the significant proportion of the biomass that they represent. Hinz *et al.* (2011) recorded a 67% reduction in abundance after trawling activity compared to control sites and also observed that surviving colonies were reduced in size. Although this research is limited in terms of geographical scope, it is highly unlikely that benthic trawling using heavy mobile fishing gear will be anything BUT detrimental for *A. digitatum* populations (and indeed most other epibenthic and sessile marine invertebrate fauna). Furthermore, the reduced colony size noted by Hinz *et al.* (2011) also implies that remaining colonies may not be sexually mature as this is not attained until 2-3 years of age (Hartnoll 1975).

Deep-sea octocoral species have incurred damage from both longline and bottom trawling activity, and are particularly vulnerable given their inferred longevity of more than 300 years in some cases (Watling and Auster 2005). Recruitment rates and growth rates of *Alcyonium digitatum* are unknown, but a relatively long time taken to colonise artificial substrate (a year, compared to a few months several other invertebrates such as tube worms, barnacles, urchins, scallops, anemones) suggests that recruitment is infrequent in at least some parts of its range (Hiscock *et al.* 2010). In addition, a common lifespan of over 20 years is likely and individual colonies are known to have persisted for almost thirty years (marlin.co.uk). For a taxonomic comparison, the congeneric *A. acaule* was found to grow very slowly in the northwestern Mediterranean and larger colonies were thought to be several decades old (Garrabou 1999). When extrapolated to *A. digitatum* for temperate waters, it is highly likely that trawling activity will locally reduce the numbers of sexually mature colonies and extensive rates of inbreeding seen in this study could correlate with trawling damage; all three of our sampled populations in Lyme Bay had significant inbreeding coefficients. Although all three samples happen to be from shipwrecks (Devon Galicia, Frognor and U-boat 74), and as such are highly unlikely to be trawled populations, these data nonetheless imply that local source populations may also be suffering from high rates of inbreeding, as detected across the south west UK.

Chapter 5: General Discussion

5.1 Using Molecular Markers to Infer Connectivity

Molecular methods are invaluable in understanding marine ecosystem function as they can infer interactions at several levels including measurement of gene flow and connectivity, assessment of population structure and parentage, phylogenetic relationships and biogeography (Feral 2002). The new microsatellite panels developed for this study are comparable to panels developed from other species in terms of loci numbers and are a reliable method to identify population structure in my target study species, in addition to other congenerics. A need for high resolution markers such as microsatellites has been recognized in the genus *Eunicella* by Gori *et al.* (2012), who could not discriminate between depth-related morphotypes in *E. singularis* or between different *Eunicella* species in their study. Consequently, these panels will be useful across this genus due to their cross-species amplifiability. It would be interesting to use them to assess phenotypic variation between congeneric morphotypes as suggested by Gori *et al.* (2012), although in this study, white and pink morphs were not discernible, and neither were orange and white morphs of *Alcyonium digitatum*. During the microsatellite development stage, a high rate of attrition resulted in low yields of useable loci from the total number tested (approximately 20%, Chapter 2, Table 2.5). Reasons for low returns in octocoral (and anthozoan) microsatellite isolation remain unclear, although microsatellites are thought to be rare in cnidarian genomes (e.g., Liu *et al.* 2005, see Chapter 2). Development of alternate markers is challenging in octocorals. Anthozoan mitochondria are very stable evolutionarily, exhibit little variation and evolve approximately 10-20 times more slowly than rates inferred for vertebrate mitochondria (Shearer *et al.* 2002). Reasons for this may include elements such as homing endonuclease genes ('selfish DNA') that have been found in some actinarians (anemones) and which are thought to stabilise the mitochondrial genome (Goddard *et al.* 2006). In octocorals, mitochondria are atypical with reference to other Anthozoa with anomalies including slow evolution (McFadden *et al.* 2010), alternate mitochondrial gene orders between families (Brugler and France 2008), and the presence of rare mismatch repair homologs that may suppress mitochondrial mutation rates (Bilewitch and Degnan 2011). Therefore mtDNA markers are of limited use for population-level analyses in octocorals, as has been demonstrated by several studies including Calderon *et al.*'s (2006) research on genetic structure of four Mediterranean gorgonian species, where CO1 variation was so low, relationships between geographically distant populations could not be deduced. In *Eunicella* spp, CO1, internal spacer ITS regions and mutation suppression homolog msh1 genes have failed to resolve species level relationships (Calderon *et al.* 2006, Gori *et*

al. 2012). However, RFLPs generated from CO1 PCR amplicons may have use in the species-level identification of scleractinian coral larvae (Shearer and Coffroth 2006).

Despite such obstacles, some previous research on octocorals has combined DNA sequence data with microsatellite data or used an assortment of mitochondrial and nuclear markers to infer population structure or phylogeography which may offer better resolution if combined. For example, Concepcion *et al.* (2010) used nuclear signal recognition particle subunit 54kDa (SRP54) and mitochondrial NADH dehydrogenase subunits 2 and 6 (ND2 and ND6) to track the spread of *Carijoa riisei* between the Atlantic / Caribbean and Pacific, with SRP54 being considered the most promising marker for resolving closely related lineages (see also Concepcion *et al.* 2008). Herrera *et al.* (2012) used a combination of mitochondrial genes (including NADH subunits 2, 3 and 6, CO1 and msh) and nuclear (ITS) markers to examine phylogeography of the deep sea bubblegum coral *Paragorgia arborea.* In threatened Mediterranean *Corallium rubrum* populations, sequences from the nuclear elongation factor 1 gene (EF1) have been combined with microsatellite data to infer population structure (Aurelle *et al.* 2011). EF1 corroborated microsatellite data but with less resolution (although sample sizes were different between the two datasets). In my study, a lack of suitable population-level DNA sequence markers resulted in reliance purely upon microsatellites to examine *E. verrucosa* and *A. digitatum* genetic connectivity. A lack of congruence and lowered resolution between sequence data and microsatellites in other taxa (e.g, *Corallium rubrum*, Costantini *et al.* 2007b) and less resolution offered by sequence data in octocorals in particular means that I do not feel that my study was data deficient by only including microsatellites. Although more promising population level nuclear markers are emerging for scleractinians (e.g, β-tubulin, Nunes *et al.* 2009), microsatellites are likely to be the population marker of choice for octocorals for the foreseeable future.

The limitations imposed by microsatellites, in my opinion, stem from inadequate appreciation of their limited ability to resolve certain questions in population genetics, which by extension can lead to misinterpretation of data inferred by them. As outlined in Chapter 2, microsatellites are hypothetically neutral, and as such they are not under selective constraint and accrue mutations via genetic drift. Their high variability is perfect for population genetics. However, limited selection pressure on microsatellites could also result in the loss of genetic variation in them faster than at more conserved loci that may be associated with fitness. Therefore, when using microsatellites, the assumption that reduced heterozygosity (indicated by significant inbreeding coefficients) equals an associated fitness loss is presumptuous (reviewed in Reed and Frankham 2003). In other words, although in some cases reduced heterozygosity has been shown empirically to correlate with

inbreeding depression (e.g., Wright *et al.* 2008, Slate *et al.* 2000), it cannot be taken for granted. As neutral markers, microsatellites are therefore not as useful in determining divergence patterns based upon selective traits as the actual genes under selection associated with such traits themselves, for example to increased salinity following retraction of ice sheets at the end of the last glacial maximum (Coyer *et al.* 2003). Markers used in population genetics also typically represent only a small portion of the genome; ideally, estimates of genetic variability would include quantification of adaptive traits, variation at critical functional loci and variation at non-coding and coding regions of the genome (reviewed in Ljungqvist *et al.* 2010). One locus in my *Eunicella verrucosa* panel showed evidence for positive selection (albeit based upon models of evolution that have not been quantified in this species). With further scrutiny, it appeared that this locus is an important driver in the slight distinctions found between disparate sites, and indicates that there is some selective pressure at this locus on Irish populations, even if the identity and function cannot be characterized at this stage. In this study, fourteen microsatellite loci were used; Nei (1978) suggests that in order to gain a reliable genome-wide approximation of heterozygosity, fifty loci or more should be used, especially with small sample sizes. In the future, more genomic resources for octocorals could facilitate more comprehensive mining for useful markers.

An additional issue with microsatellites lies in their polymorphism. There is some suggestion that size homoplasy and high marker heterozygosity may result in a downward bias of estimates of population differentiation and thus limit utility of Fst and similar measures (Hellberg 2009). As such alternative metrics such as Dest ('Jost's D', Jost 2008) have been suggested, which calculates actual between-population differentiation independently of within-popultion heterozygosity (Casado-Amueza *et al.* 2010). In this study, Fst and Dest indices show an overall similar pattern of spatial differentiation at regional scales with more sensitive resolution at some local scales in the Dest data. As the latter analysis was restricted to eleven out of fourteen loci, it is likely that differences between Fst and Dest calculations may be more pronounced if all loci could be assessed with the latter. However, overall the two datasets prove useful in that they generally support each other, as in other studies concerning marine invertebrate genetic connectivity (e.g., Casado-Amezua *et al.* 2012, White *et al.* 2010).

The extent of polymorphism and hence allelic richness was higher in *A.digitatum* than *E. verrucosa* (interestingly, the low polymorphism reported for *E. verrucosa* here is the lowest of any octocoral, with the exception of congeneric *Eunicella singularis*, Table 2.5). A negative correlation has previously been demonstrated between Fst values and locus polymorphism (here measured by allelic richness and heterozygosity) in walleye pollock (O'Reilly *et al.* 2005) and sockeye salmon

(Olsen *et al.* 2004). The latter authors found significantly lower Fst estimates for highly polymorphic microsatellites (defined as Hs > 0.84) compared to moderately polymorphic microsatellites and allozymes (Hs < 0.6); therefore in this study confidence in Fst values may be drawn from the relatively low average heterozygosity value found across my markers. However, Olsen *et al.* (2004) also suggest that markers with variable heterozygosities be analyzed separately and O'Reilly *et al.* (2005) suggest that other measures that take allelic identity (size) as opposed to frequency measures (Fst) into account be used. Such measures include Slatkin's Rst (Slatkin 1995), which measure a between-population component of variance that accounts for allelic size. This approach was not taken in this study; Rst is based upon a stepwise-mutation model (O'Reilly *et al.* 2005), which may not represent the mutation model of my loci, and *E. verrucosa* clearly have highly imperfect repeats as seen from the allele sizes indicated in Figure 3.3).

In summary, although microsatellites are the marker of choice in conservation genetics, they are limited for inferring whole genome variation. Currently, genomic data remain scarce in octocorals, although mitochondrial genomes have recently been used as a measure of divergence between higher level cnidarian lineages (Kayal *et al.* 2013). Thus increased resources may lead to more markers and hence more coverage of other areas of the genome in the future.

5.2 Connectivity Patterns in the North East Atlantic

The North East Atlantic region is divided into several provinces according to borders defined by Spalding *et al.* (2007); Lusitanian (including the South European Atlantic Shelf, Saharan Upwelling, and the Azores, Canaries and Madeira Islands ecoregions), the Temperate North Atlantic (i.e. Boreal, including the south and West Iceland, Faroe Plateau, Southern Norway, Northern Norway and Finnmark, Baltic Sea, North Sea and Celtic Sea ecoregions) and the Mediterranean, West African Transition, and Gulf of Guinea. The ranges of *Eunicella verrucosa* and *Alcyonium digitatum* therefore span several provinces. In this area, connectivity of diverse marine invertebrates has been assessed, including the crab *Carcinas maenas,* which extends from Mauritania to the North Sea and Norway (including Norway and the Faro Islands, Roman and Palumbi 2004), the spiny spider crab *Maja brachydactyla,* which extends from Senegal to Norway (Sotelo *et al.* 2008), the edible urchin *Paracentrotus lividus,* which is Atlanto-Mediterranean with a limited British distribution to Scotland and rare occurrences in the south west of England and with high abundance in western Ireland (Duran *et al.* 2004c, marlin.ac.uk), and the common shrimp *Crangon crangon*, which extends from the Black Sea and Mediterranean up though western Europe to the North and Baltic seas and Iceland

(Luttikhuizen *et al.* 2008). It has been suggested that the general pattern for genetic subdivision in marine taxa in the North East Atlantic is delineated by the Mediterranean, western and northern European areas (Roman and Palumbi 2004). Although this is an oversimplified view, evidenced by affinity between Irish and Spanish samples in some studies (Sotelo *et al.* 2008), admixture between western and northern Europe (Luttikhuizen *et al.* 2008) or genetic breaks observed between the Mediterranean and NE Atlantic (Lowe *et al.* 2011), it seems that *Eunicella verrucosa* supports this theory. In this study, strong divergence between southern Europe and the British Iles, slight differentiation between England and Brittany, and strong divergence between western Ireland and everywhere else in the sampled area was observed, highlighting regional scale variation.

Alcyonium digitatum was sampled in a more northerly area and appears to be highly admixed in this region; therefore I cannot yet support or refute Palumbi and Roman's (2004) pattern for this species without sampling further afield. In my study, little divergence was seen in *Alcyonium digitatum* between Brittany, the UK and the North Sea, such a pattern has also been reported for cuttlefish (Wolfram *et al.* 2006). It is clear that the two octocorals studied here differ in their UK and Ireland connectivity patterns; distant North Sea and western Ireland samples showed little divergence in *A. digitatum*, whereas Ireland samples are more distinct for *E. verrucosa*. Muths *et al.* (2009) examined connectivity of brittle stars between Galicia, Brittany, the English Channel and the Irish Sea (and one population in the Mediterranean) and found that *O. fragilis* is a distinct lineage around the British Isles though to Norway, that the *O. f. echinata* variety is divergent between the Iberian/Mediterranean and Irish Sea / English channel populations and that *O.f. pentaphyllum* has no clear structure in the British Isles. McFadden (1999) found no genetic difference in *Alcyonium hibernicum* in Ireland and the Isle of Man. In summary, little divergence around the British Isles is seen here for two octocorals species, and has been documented previously for other marine invertebrates.

Genetic patterns in each species may be explained by historical range expansions from southern refugia. During the Pleistocene, (1.8 million-12,000 year ago), Europe was subject to a series of ice ages (glacials), the most severe of which was 18,000 years ago and is known as the last glacial maximum (LGM, Luttikhuizen *et al.* 2008). Glaciers and sea ice extended as far south as southern Britain and France and essentially restricted the range of terrestrial and marine fauna to southern Europe, from where it expanded and retracted to coincide with glacial and interglacial periods. The Mediterranean and Atlantic-Iberian coasts were not under ice and therefore have a continuous marine history, since the opening of the Gibraltar Strait 5 million years ago (Duran *et al.* 2004c),

although the Mediterranean and Atlantic basins were separated during glacial periods which may explain genetic divergence in some taxa between the two (Baus *et al.* 2005). Northern Atlantic areas are younger and boreal-temperate communities around the British Isles are characterized by an assemblage of species that returned from southern temperate regions or that survived in northern glacial refugia, such as those in South West Ireland and North West Scotland (Luttikhuizen *et al.* 2008, Jolly *et al.* 2006). Genetic signatures reflecting range expansions and retractions are detectable and have been utilized to suggest migration patterns or locations of refugia for several Lusitanian and Boreal marine species. For example, Coyer *et al.* (2003) proposed that high microsatellite allelic diversity in Brittany compared to elsewhere in the NE Atlantic and Nova Scotia implies that this area was a refuge during the last LGM or has been recolonized since for the seaweed *Fucus serratus*. This theory is plausible given that, although water was 125m below present sea levels during the last LGM, the Hurd Deep, a depression in the English Channel, persisted as a marine lake and could sustain *F. serratus* (Hoarau *et al.* 2007). The research of Coyer *et al.* (2003) was expanded by Hoarau *et al.* (2007), who used mitochondrial DNA to determine that three refugia were likely for *Fucus serratus*, evidenced by high levels of haplotypic diversity and endemism; the Hurd Deep , South West Ireland and North West Iberia. They suggest that the Irish refugium was the colonization source for northern Scotland into Scandanavia, manifested by the prevalence of a single haplotype at those sites, and that Iberian samples represent a 'remnant refugium' at the southerly limits of the range of *F. serratus*. Support for a refugium in North West France has recently been strengthened using microsatellite and CO1 data of salmon (*Salmo salar*), which along with the Iberian refuge, is thought to have been the source for colonisation initially into the South West UK and subsequently Ireland (Finnegan *et al.* 2013). Although there is some uncertainty over the extant of glacier and permafrost coverage in Britain and Ireland, it is likely that most of the northern part of the current range of *Eunicella verrucosa* was close to the southerly limit of the ice sheet (Hoarau *et al.* 2007). Therefore the current range of *E. verrucsosa* might not have expanded substantially as the ice retreated.

However, a lack of sampling at range limits for both species and little genetic structure within either species make it difficult infer range expansion pathways in this study. Ireland is clearly divergent in *E. verrucosa*, and has potentially lowered effective population sizes and allelic richness there for both species. Sampling at closer intervals between Portugal and Brittany may elucidate potential source areas for these populations. The range of *A. digitatum* extends much further north and re-colonization in the UK may have followed a northerly or southerly route (e.g., if it persisted in northern refugia highlighted in Luttikhuizen *et al.* 2008). High haplotypic diversity but low

nucleotide diversity has inferred a range expansion during the last Pleistocene in spiny spider crabs (*Maja brachydactyla*) using mitochondrial DNA (Sotelo *et al.* 2008).

5.3 Inferring Connectivity from Life-History Strategies

This research has demonstrated that estimating connectivity patterns based upon reproductive traits, when they are unknown (e.g., *Eunicella verrucosa*) or even relatively well understood (e.g., *Alcyonium digitatum*) is highly unreliable. There is a scarcity of data on reproductive biology for many marine invertebrates of conservation interest, so reproductive traits from taxonomically related species or unrelated species with similar reproductive traits are used as a proxy to infer pelagic larval duration and hence connectivity (e.g., Jones and Carpenter 2009). This is a risky strategy, given that some genera exhibit exceptionally diverse reproductive behaviours (especially corals and octocorals). For example, the octocoral *Corallium secundum* is a spawner, whereas *C. rubrum* is a brooder; management plans for the threatened *C. lauuense* based upon growth rates and reproductive patterns in *C. secundum* may therefore be flawed (Baco and Shank 2005). Some species exhibit temporal and spatial plasticity in timing of reproduction, for example the cuttlefish *Sepia officinalis* has a different reproduction window in the Mediterranean compared to the Bay of Biscay; in warmer waters they are able to reproduce year round as opposed to just a few weeks in spring and summer in colder water (Wolfram *et al.* 2006). *Alcyonium* is also a reproductively varied genus and has brooding and broadcasting representatives; *A. coralliodes* and *A. acaule* brood larvae, yet *A. digitatum, A. glomeratum* and *A. palmatum* are broadcasters (McFadden *et al.* 2001). In some cases, reproductive traits can alternate even within the same species. In addition, some taxa also vary their reproductive mode temporally and spatially, including the coral *Pocillopora damicornis*, which broods and broadcast spawns both within the same location and between different locations (Fautin 2002). For example, *P. damicornis* is a broadcast spawner in the Tropical Eastern Pacific but broods parthenogenetic larvae elsewhere in the Indo-West Pacific (Combosch and Vollmer 2011). Interestingly and contrary to other research, these authors found higher population structure in broadcast spawning populations than in brooders, indicating that population structure in corals may sometimes be independent of reproductive strategies. Furthermore, pelagic larval duration, itself inferred from (sometimes plastic) life history traits and almost always lacking empirical measurement, does not always correlate positively with distance (reviewed in Selkoe and Toonen 2011).

Eunicella verrucosa and *Alcyonium digitatum* are thought to disperse less than 1km and more than 10km respectively from a parent colony (marlin.ac.uk). Genetic data in this study suggest that dispersal is vast in both species and at scales of hundreds of kilometres, evidenced by lack of structure at this scale in the South West UK. In summary, these data suggest that dispersal estimates being used as a proxy for connectivity in the UK MPA network design guidelines are highly likely to be inaccurate (Jones and Carpenter 2009, Roberts *et al.* 2009) and further highlight the critical need to incorporate genetic connectivity data in reserve design and management.

5.4 Conservation Implications

Globally, unprecedented rates of biodiversity loss, declining fish stocks, habitat degradation and detrimental impacts of climate change (e.g., Hall-Spencer and Moore 2000, Martin *et al.* 2008, Robinson *et al.* 2008) have led to international efforts to protect marine ecosystems. Marine reserves have proven their value and efficiency globally. Benefits of them usually manifest in the augmentation of biomass and abundance of target fish (Tetreult and Ambrose 2007), protection of coral reefs (Mumby *et al.* 2007, Harborne *et al.* 2008), and coincident indirect benefits such as improved ecosystem services and economical value (Roncin *et al.* 2008) or increased ecosystem resilience led by a reduction of disease within protected areas (Raymundo *et al.* 2009). Spillover effects and enhancement of adjacent populations is sometimes a beneficial 'side-effect' of an MPA (Goni *et al.* 2008), although this benefit is uncertain and requires, for example, suitable habitat (Forcada *et al.* 2009). The science behind MPA design is complicated and a lack of data concerning, for example, availability of suitable habitats, local hydrodynamics and connectivity of species of interest may impede their success (reviewed in Sale *et al.* 2005). As such, connectivity is recognised as a key ecological criterion in the design of MPAs (e.g., Foley *et al.* 2010), although distinctions between genetic and demographic connectivity are rarely made.

In Europe, each member state is required to implement 'coherent and representative networks' of Marine Protected Areas by 2020 as a requirement of the Maine Strategy Framework Directive (MSFD), and the sixteen signatories of the OSPAR Commission have pledged to halt further degradation and biodiversity loss in the OSPAR maritime area by 2020 (Jones and Carpenter 2009, ospar.com). At a national level, legislation to protect the marine environment and to moves to develop a network of marine protected areas began under the 2009 Marine and Coastal Access Act, which in England was directed by four regional groups that suggested candidate sites for protection to the UK Government though the Marine Conservation Zone (MCZ) project (jncc.defra.gov.uk). This

culminated in 127 sites around Great Britain being put forward to the UK Government for protection. *Eunicella verrucosa* is one of seven Cnidaria targeted by the network, *A. digitatum* is not. From my data, it appears that genetic connectivity, at least for some sessile benthic invertebrates, needs consideration at European levels and would likely fall within the remit of OSPAR. At present, neither species is of consideration in this legislation.

In this study, although not within the scope of England's MCZ project, the marginality seen in Irish *Eunicella verrucosa* populations could be levied as a case for their protection at large spatial scales. Marginal populations usually contain rare alleles (three private alleles were found here), they may recruit slowly, and may be genetically divergent due to isolation, all of which imply vulnerability and reduced resilience (Sanderson 1996). Compared to the overall range of *E. verrucosa* (Angola to western Ireland), the extent of it in the UK is very small, and divergence from Portugal and Brittany also highlights the genetic uniqueness of British populations. Coupled with its status as an IUCN red-listed octocoral, a UK BAP Priority species, and with its unofficial role as a 'poster child' for UK marine biodiversity and conservation efforts, an argument could be made for the protection of *E. verrucosa* across its range as a connected metapopulation. This approach may be strengthened by the high rates of local inbreeding noted in the UK, including populations in Plymouth Sound, the Manacles, The Isles of Scilly and Lyme Bay, but NOT at Lundy, currently the only designated MCZ where populations are not apparently genetically isolated.

Alcyonium digitatum is ubiquitous, has no protective status, is not peripheral to its global range in the UK and appears to have large-scale genetic homogeneity and high genetic diversity. Therefore, the rationale for designating MPAs for protection of this species is, at first glance, unjustifiable. However, inbreeding depression seen at almost all sites from the North Sea to western Ireland highlights the possibility that this species is not freely able to exchange genetic material between populations, and that these areas may be isolated with high rates of self-seeding (as suggested for this pattern in the octocoral *Corallium lauuense*, Baco and Shank 2005). Reduced heterozygosity and impaired sexual reproduction is known to result from trawling damage (e.g., Henry and Kenchington 2004). Reduced numbers of colonies and smaller sizes have already been observed in areas of Lyme Bay subject to trawling activity (Hinz *et al.* 2011); significant inbreeding coefficients detected in all three of my samples collected from there and across the region highlight vulnerability of this species despite its prevalence. Therefore, genetic patterns observed here in *A. digitatum* may be used as a proxy to highlight the occurrence of inbred and damaged sessile populations in areas in need of protection for other targeted species.

At the time of writing this thesis, 127 marine conservation zones (MCZs) had been proposed to the UK Government Department for Food, the Environment and Rural Affairs (Defra) by the four regional groups tasked with recommending sites for designation, of which only 22 inshore sites were agreed (see Chapter One). Of these, two were sampled for both species in this study, the Manacles (Cornwall) and the Isles of Scilly (www.naturalengland.org.uk/ourwork/marine/mpa/mcz). High levels of inbreeding in both *E. verrucosa* and also in *A. digitatum* in these areas (Table 3.6 and 4.6) highlight the vulnerability of populations there and therefore support designation of these MCZs, should a goal of the network be to conserve genetic diversity (as it is of the IUCN). However, highly significant inbreeding coefficients were also detected at other populations in areas not put forward for protection, indicating that many populations of *E. verrucosa* remain vulnerable, at least in terms of fitness loss. The first UK MCZ to be designated was the Island of Lundy in the Bristol Channel. *Eunicella verrucosa* from there does not appear to be subject to inbreeding and a lack of distinction of this population in PCA and STRUCTURE analyses indicate that it is not an isolated population, despite the geographic isolation of the site.

Designation of an MCZ network in the UK has been primarily stake-holder driven (Defra Final Recommendations Report, 2013a). The UK Marine and Coastal Access Act of 2009 obliges Defra to review achievements of MCZs individually and as part of the network every six years. Flexibility towards the network design regarding addition, alteration in sizing or indeed addition or removal of sites to and from the network is unclear, but at present, from the genetic connectivity data generated here, it appears that localized ad-hoc stakeholder-driven reserves are neither useful nor relevant to *Eunicella verrucosa*, which requires conservation efforts to be focused across the whole UK metapopulation given the disparate spread of inbred populations (Table 3.6). In Ireland, divergent and inbred populations could be used to advocate protection of *E. verrucosa* in this part of its range, and the same could be applied to southern Portugal. During the MCZ network design process, *E. verrucosa* was recorded in only four MPAs nationally (designated under existing European legislation, Jackson *et al.* 2008), and its habitat ('*Eunicella verrucosa* and *Pentapora foliacea* on wave-exposed circalittoral bedrock', p62, Jackson *et al.* 2008) in less than five MPAs. This suggests that as well as connectivity, the criteria of representivity and replication are also not met for *E. verrucosa.* As far as discernible, new MCZs are extensions of existing MPAs and no new sites have been designated specifically to protect *E. verrucosa.* In summary, although the implementation of the MCZ network is in its final stages, the designation of 22 inshore sites in the current planned MCZ network is likely to fall short of its conservation objectives with regard to *Eunicella verrucosa* (and by extension other sessile invertebrates). Empirical data concerning connectivity of *Eunicella verrucosa* was not included in the draft guidelines (as this study is the first to obtain it), although

connectivity based upon potential dispersal distances inferred from its status as a 'low disperser' might have been (Roberts *et al.* 2009, Jones and Carpenter 2009). Whitsand and Looe Bay in Cornwall is one of the proposed MCZ sites and is highlighted as an important site for *E. verrucosa* (Defra 2013b). Unfortunately this study did not include samples from here, although samples from outside the proposed MCZ boundary were collected from Hand Deeps and Plymouth Sound; all three showed significant evidence for inbreeding (Hand Deeps, Plymouth Mewstone Ledges and Plymouth Breakwater Fort). These data may strengthen the requirement to protect *E. verrucosa* in this area.

Summary

This study highlights the utility of using molecular data to infer genetic connectivity in two important benthic species in the British Isles, *Eunicella verrucosa* and *Alcyonium digitatum*, and provides a solid grounding of their overall patterns of population subdivisions across parts of their respective range in the NE Atlantic. The most pertinent results are that both species appear to have a high degree of connectivity, that *E. verrucosa* is differentiated regionally, that *A. digitatum* shows very little sub-structure and that Irish populations exhibit some degree of distinction. In terms of demographic patterns, higher connectivity in *A. digitatum* may be driven by its habit of spawning in winter, and thus having larvae that disperse further than those of *E. verrucosa.* To that assertion, my hypothesis that less population subdivision would be apparent in this species was correct. Both species have a high rate of inbreeding which is challenging to explain without the application of hydrodynamic models, furthermore, the effects of inbreeding on fitness have not yet been demonstrated for either species – both of which would be interesting topics for future study.

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Appendix 1 *Eunicella verrucosa* primer note

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TECHNICAL NOTE

Isolation and characterization of fourteen microsatellite loci from the endangered octocoral *Eunicella verrucosa* (Pallas 1766)

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Abstract We isolated 165 unique microsatellite sequences from the threatened and IUCN red-listed octocoral *Eunicella verrucosa* (common name: pink sea fan). Two enriched genomic libraries were created from a single individual collected in Lyme Bay, Dorset, England. Following testing of 76 markers, 14 loci were then selected, multiplexed and characterised in 44 individuals sampled at the Mewstone Ledges, Plymouth Sound, Devon, England. The number of alleles per locus ranged from 2 to 10 and expected heterozygosities from 0.05 to 0.82. The presence of null alleles was suggested for four loci, with three showing significant deviations from Hardy–Weinberg equilibrium. No linkage disequilibrium was detected. Cross-species utility was assessed in *Eunicella singularis* (n = 20), *E. cavolinii* (n = 12) and *Leptogorgia sarmentosa* (n = 3) collected from the Mediterranean and southern Portugal. At least ten loci were polymorphic in each *Eunicella* species and five in *L. sarmentosa*. These markers should prove useful in exploring population structure in these as yet poorly studied animals.

Keywords Gorgoniidae · IUCN red list · Microsatellite · Octocoral · Pink sea fan · Simple tandem repeat (STR)

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The octocoral *Eunicella verrucosa* Pallas 1766 (common name: pink sea fan) is a focal taxon of several marine conservation strategies currently being implemented in the UK, and is one of seven cnidarians targeted for protection by the forthcoming UK Marine and Coastal Access Act. In the eastern Atlantic its range is vast, stretching from Angola to western Ireland, where it is typically found at depths between 10 and 200 m. In Britain its distribution is limited to southwest England and south Wales (Grasshof 1992), where it has been detrimentally affected by fishing activity—as such it is classed as a 'vulnerable' species on the IUCN red list.

All microsatellite markers were developed from the same individual sea fan (EverETR1) collected in September 2008 from East Tennents Reef, Lyme Bay, Dorset, England (N50°39′09″, W02°52′44″), in accordance with collection licence conditions. Genomic DNA was extracted from a 3 cm long clipping using a QIAGEN Plant Mini Kit following the manufacturer's protocols. With the exception of loci *Ever001* and *Ever009*, microsatellites were isolated at the Evolutionary Genetics Core Facility of Cornell University (Library 1) following a modified protocol of Hamilton et al. (1999). Briefly, DNA was digested with *Bsa*AI and *Hinc*II, fragments ligated to double-stranded SNX linker, denatured and enriched for repeats by hybridization to 3′-biotinylated di-, tri- and tetranucleotide repeating oligonucleotide probes, after which repeat-containing fragments were captured on streptavidin-coated magnetic beads. The polymerase chain reaction (PCR) was used to make the fragments double-stranded using the SNX forward primer, then purified and digested with *Nhe*I prior to cloning into *Xba*I-digested, dephosphorylated pUC19. Colonies were grown on ampicillin-containing Luria–Bertani agar plates, and replicated onto nylon membranes prior to probing with the radiolabelled oligonucleotides used in

Table 1 Characterisation of fourteen *Eunicella verrucosa* microsatellite loci

Locus	Clone name and EMBL accession number	Repeat motif	Fluoro-label	Primer sequences (5′–3′)	T_m (°C)[a]	Obs. allele size range (bp)	N	K	H_O	H_E	Est. null allele freq.	P_{HW}
Ever001	PSFShef01C02	$(AATG)_{17}$	HEX	F:ACTGCAACTGTTCATCGTCAG	59.0	237–269	43	9	0.51	0.66	0.13	0.01*
	HF913256			R:AAACTAGCCGGTCTATAACTCTCG	59.4							
Ever002	PSFExe15_tet1	$(TTAC)_6$	HEX	F:ATGTTGAGCTGCGTCCTTCGC	67.1	105–117	43	4	0.33	0.36	0.02	0.10
	HF913257			R:GTACAATCGAGTGGGTGTGC	59.0							
Ever003	PSFExe21_tet16	$(TTGA)_8$	HEX	F:TCTCGCAGAACTATCGCCG	62.6	170–182	44	4	0.16	0.19	0.17	0.15
	HF913258			R:AGTTATCAGTGTTCATGACTCG	54.5							
Ever004	PSFExe33_tri22	$(CAA)_{12}$	6FAM	F:CAACAATGAAAGCGGCAACAGC	67.0	148–183	44	9	0.68	0.66	−0.05	0.78
	HF913259			R:CATCTTCGACACCTTCATCC	58.1							
Ever005	PSFExe41_tet67	$(GATT)_6$	6FAM	F:GCAACTTGGTTTTAATAAACG	54.4	230–235	44	2	0.05	0.05	−0.00	1.00
	HF913260			R:GACGATGATGTTAAGAGCGGG	61.9							
Ever006	PSFExe49_tri38	$(GTT)_{22}$	6FAM	F:GCCGTTGGTGGTATCTATGG	60.2	353–394	44	9	0.66	0.82	0.11	0.02
	HF913261			R:GTTGTTTTAGAGCGACAGCAGC	61.5							
Ever007	PSFExe10_di_17	$(GA)_{10}$	6FAM	F:GGTAACAAACTTAGCACAGC	52.8	226–242	42	5	0.55	0.58	0.03	0.51
	HF913262			R:GCTAATAATGAGCCAATCACCC	59.8							
Ever008	PSFExe48_tri33	$(GAT)_6$	6FAM	F:CATTGTCCCTGTATCGATGG	58.8	151–154	38	2	0.61	0.50	−0.10	0.95
	HF913263			R:ATTTTCGTTTTTCGGGATCCC	63.3							
Ever009	PSFShef06E10	$(AATC)_8$	6FAM	F:ATACAAGTTCTGGTGGCATGG	59.9	92–131	43	9	0.44	0.66	0.20	0.00*
	HF913264			R:CCCTCCTGTAAATCAGCATATTG	59.9							
Ever010	PSFExe17_tet3	$(TATC)_{13}$	6FAM	F:GCATAATGACTCTGTCAATGTC	54.8	240–253	44	4	0.14	0.13	−0.03	1.00
	HF913265			R:CTTCTCATAGACGGTTTTATACAC	54.2							
Ever011	PSFExe34_tet28	$(TCAA)_7$	VIC	F:GGGCCACAAATTTATCAGCAGC	64.9	144–153	44	3	0.46	0.42	−0.05	0.82
	HF913266			R:CTTGGAATAAAGCCAAAAATGC	60.0							
Ever012	PSFExe47_tri32	$(GAT)_6$	6FAM	F:AAACGTAGGCACCAAGATGG	60.0	204–210	35	3	0	0.11	0.77	0.00*
	HF913267			R:TGGCTGCGAGGTATTATCTG	59.0							
Ever013	PSFExe50_tri45	$(CAA)_{14}$	PET	F:CAAAAACGACAACAGCAACGG	63.8	127–164	44	10	0.75	0.79	0.02	0.37
	HF913268			R:CATCGTCCTAATTGTTGGTGG	60.2							
Ever014	PSFExe24_tri1	$(GTT)_4$	6FAM	F:GCTTGTAGTGGTTGCCGTCG	64.0	317–357	44	5	0.73	0.70	−0.02	0.64
	HF913269			R:CGAACATTCTCACAGTTGATTGGC	65.1							

T_m, primer melting temperature, *Observed allele size range (bp)* within the amplified individuals, N number of individuals amplified and successfully genotyped (of 44 tested), K number of alleles, H_O observed heterozygosity, H_E expected heterozygosity, *Estimated null allele frequency* (calculated using CERVUS v3.0.3, Kalinowski et al. 2007), P_{HW} probability for Hardy–Weinberg equilibrium calculated using GENEPOP v.4.2 software (Raymond and Rousset 1995; Rousset 2008)

* Indicates significant values ($p < 0.05$) after correcting for multiple tests using false discovery rate (Benjamini and Hochberg 1995)

[a] A touchdown PCR program was used (see above for details)

Table 2 Cross-species utility of *Eunicella verrucosa* microsatellite loci in three other octocoral (Gorgoniidae) species

SPECIES	Locus	Ever001	Ever002	Ever003	Ever004	Ever005	Ever006	Ever007	Ever008	Ever009	Ever010	Ever011	Ever012	Ever013	Ever014
Eunicella verrucosa		273	116	173	171	235	354	245	154	115	247	150	211	144	327
Expected size 'EverETR1' (bp)															
Eunicella cavolinii (Koch 1887)	N tested:	12	12	12	12	12	12	12	12	12	12	12	12	12	12
	N amp.:	4	0	2	12	1	8	10	11	12	0	5	0	0	9
	Size (bp):	222–241	–	174/189	146–175	235	312–459	226–242	144–151	90–111	–	144–149	–	–	313–357
	K:	3	–	2	8	1	9	4	3	4	–	2	0	0	5
Eunicella singularis (Esper 1791)	N tested:	20	20	20	20	20	20	20	20	20	20	20	20	20	20
	N amp.:	20	20	20	20	20	4	20	20	15[a]	17	8	8[a]	0	19
	Size (bp):	237–262	109–117	174–191	160–168	235–353	236–355	226	151	100–144	253–285	113–143	204	–	317–333
	K:	5	3	4	3	3	3	1	1	7	8	3	1	0	3
Leptogorgia sarmentosa (Esper 1789)	N tested:	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	N amp.:	2	2	2	2	1	1	3	3	–[b]	1	0	0	0	1
	Size (bp):	237–274	109–117	174	160–167	235	367	226	151	–	253–273	–	–	–	327–370
	K:	3	3[c]	1	2	1	1	1	1	–	2	0	0	0	2

N tested number of individuals for which PCR was attempted for each species and locus, *N amp*. number of individuals that amplified (as observed on an ABI 3730 Sequencer), *Size (bp)* observed allele size range (base pairs) across successfully amplified loci (presence of heterozygotes indicated by hyphenated size range, e.g., 222–241; homozygotes indicated by forward slash, e.g., 174/189 indicates alleles were 174 or 189, and no heterozygotes were detected); *K* number of alleles observed. Samples of *Eunicella cavolinii* were collected from the Algarve region of southern Portugal (N37°02′16″, W08°21′22″). Samples of *Eunicella singularis* and *Leptogorgia sarmentosa* were collected from the Mediterranean near Banyuls-sur-Mer, France (N42°28′43″, E03°09′34″ or N42°28′55″, E03°08′14″)

[a] *Ever009* and *Ever012* were amplified together in the same multiplex, and were labelled with the same fluoro-label (6FAM). However, in some individuals of *E. singularis*, more than two alleles were present within the expected size range of one of the loci, with only one (or no) alleles present in the other, indicating overlapping allele size ranges in these loci in this species. Thus, these two loci should not be used in the same multiplex in *E. singularis*

[b] Unscoreable due to the presence of multiple peaks

[c] Three products were amplified in one individual at this locus

enrichment (details available upon request). Positive clones were sequenced using a universal M13 forward primer flanking the cloning site with BigDye Terminators (Applied Biosystems) and using an ABI 3730 Sequencer. Primer sets were designed using OligoCalc (Kibbe 2007).

Loci *Ever001* and *Ever009* were isolated from an enriched genomic DNA library (Library 2) developed at the Natural Environment Research Council (NERC) Biomolecular Analysis Facility (NBAF), University of Sheffield, following a modified protocol of Armour et al. (1994) and Glenn and Schable (2005), from the same individual (EverETR1) as used for Library 1. In brief, DNA was digested by *Mbo*I (Promega), and double-stranded linkers (*Sau*-L-A annealed to *Sau*-L-B; Royle et al. 1992) were ligated to the sea fan DNA fragments, followed by size selection (250–750 bp) and then enrichment for the following di– and tetranucleotide motifs and their complements: $(GT)_n$, $(CT)_n$, $(GTAA)_n$, $(CTAA)_n$, $(TTTC)_n$ and $(GATA)_n$, which had been bound to magnetic beads. Using *Sau*-L-A as a primer, enriched DNA was amplified by PCR and cloned using a TOPO TA Cloning Kit (Invitrogen), according to the manufacturer's instructions. Bi-directional Sanger sequencing of clones (using ABI BigDye v3.1) allowed assembly of consensus sequences for most loci; primer sets were designed using the software PRIMER3 (Rozen and Skaletsky 2000).

Sequences from both libraries were checked for duplication and linker and vector contamination using nBLAST software. No duplicates were found and vector and linker sequence were removed.

In total, 51 unique microsatellite sequences were isolated from Library 1 (Cornell; EMBL accession numbers: HF913257–HF913263, HF913265–HF913269, HF913383–HF913412, HF913414–HF913422) and 114 unique microsatellite sequences were isolated from Library 2 (Sheffield; EMBL accession numbers: HF913256, HF913264, HF913270–HF913356, HF913358–HF913382). In total, primer sets were designed for 76 microsatellites. Primers were labelled with either 6FAM or HEX fluorescent dyes (Applied Biosystems) and tested as duplexes in 49 individuals from the Mewstone Ledges, Plymouth Sound, Devon, England (N50°18′20″, W04°06′28″). For PCR, 1 µl of sea fan DNA (10 ng/µl) was air dried in PCR plates and amplified in a 2-µl PCR reaction containing 0.2 µM of each primer and 1 µl QIAGEN Multiplex PCR Master Mix (Kenta et al. 2008). PCR reactions were performed using a DNA Engine Tetrad 2 thermal cycler (MJ Research, Bio-Rad, Hemel Hempstead, UK) and conducted with the following touchdown profile (due to variation in the range of melting temperatures between primers; Table 1): 95 °C for 15 min, then 15 cycles of 94 °C for 30 s, 65 °C for 90 s, 72 °C for 60 s, decreasing by 1 °C each cycle, followed by 27 cycles of 94 °C for 30 s, 50 °C for 90 s, 72 °C for 90 s, and finally 72 °C for 10 min. PCR products were genotyped on an ABI 3730 48-well capillary DNA Analyser (Applied Biosystems) and alleles were scored using the GENEMAPPER v.3.7 software (Applied Biosystems). From these, fourteen polymorphic loci that amplified reliably and were easy to score were selected for subsequent analysis.

Observed and expected heterozygosities were calculated using CERVUS v3.0.3 (Kalinowski et al. 2007), after which tests for deviations from Hardy–Weinberg expectations were calculated in GENEPOP v.4.2 (Raymond and Rousset 1995; Rousset 2008). After correcting for multiple tests using the false discovery rate method (Benjamini and Hochberg 1995), three loci showed significant departures from HWE: *Ever001*, *Ever009* and *Ever012*. These loci also demonstrated high estimated null allele frequencies, as did *Ever006*, calculated in CERVUS v3.0.3 (Kalinowski et al. 2007) and Microchecker v.2.2.3 (Van Oosterhout et al. 2004).

All loci were also tested in three other octocoral species (Family Gorgoniidae): *Eunicella cavolinii* (N = 12), *Eunicella singularis* (N = 20) and *Leptogorgia sarmentosa* (N = 3, Table 2). Several loci were polymorphic, especially in the *Eunicella* species (12 in *E. singularis* and nine in *E. cavolinii* of 14 tested), highlighting the potential use of these genetic markers in these little studied and vulnerable marine taxa.

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Appendix 2. *Alcyonium digitatum* primer note

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TECHNICAL NOTE

Isolation and characterization of 11 microsatellite loci from the ubiquitous temperate octocoral *Alcyonium digitatum* (Linnaeus, 1758)

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Abstract We isolated 116 unique microsatellite sequences from an enriched genomic library generated from a single colony of the temperate octocoral *Alcyonium digitatum* sampled off The Lizard, Cornwall, southwest England. Primer pairs were designed for 54 loci and tested in four individuals from a neighbouring site. Subsequently, 11 polymorphic loci were selected and characterized in 42 individuals sampled from the Isles of Scilly, southwest England. All loci were polymorphic in the Scilly population, with 3–27 alleles per locus and expected heterozygosities of 0.07–0.96. Two loci deviated from Hardy–Weinberg equilibrium possibly due to the presence of null alleles. No loci showed significant evidence of linkage disequilibrium following correction for multiple tests. Cross-amplification was assessed in *Alcyonium coralloides*, *Alcyonium acaule* and *Alcyonium palmatum* collected from the French Mediterranean; six loci amplified successfully in at least one other species. These markers should prove useful for future conservation studies of this ubiquitous animal.

Keywords Alcyoniidae · Gorgonian · Microsatellite · Octocoral · Population genetics · Simple sequence repeats (SSRs)

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Alcyonium digitatum (Linnaeus, 1758), commonly termed 'dead man's fingers', has a vast boreal and Lusitanian range and has been recorded from the western Atlantic (east coast of Canada) to the northeast (NE) Atlantic, from Portugal to Norway and Iceland (Hartnoll 1975; Hauksson 2000). In the British Isles it is the most prevalent octocoral, where it dominates rocky sublittoral substrata to at least 50 metres deep all around the coastline. However, while congeners elsewhere in the NE Atlantic have been more extensively studied (e.g., McFadden 1999) biology and population structure of *A. digitatum* across most of its range, including the British Isles, remains poorly understood. We therefore isolated novel microsatellite markers from *A. digitatum* to facilitate exploration of patterns of genetic connectivity of this species in the NE Atlantic, and to investigate how ubiquitous sessile taxa might be represented in a network of marine protected areas currently under consideration around the UK coast.

Microsatellite sequences were isolated from a single individual *A. digitatum* colony collected from the Volnay wreck, Porthoustock, Cornwall (N 50°04′22.3″, W 04°59′48.1″), in March 2009. Genomic DNA was extracted using the Wizard-SV Genomic DNA Purification System (Promega) according to the manufacturer's protocol for animal tissues. The library was constructed using modified methods of Armour et al. (1994) and Glenn and Schable (2005). In brief, *Mbo*I digested *A. digitatum* DNA was annealed to *Sau*-L-A+*Sau*-L-B linkers (Royle et al. 1992), size selected (250–750 bp) and then enriched for the following di- and tetra-nucleotide motifs separately: $(GT)_n$, $(CT)_n$, $(GTAA)_n$, $(CTAA)_n$, $(TTTC)_n$ and $(GATA)_n$ which had been bound to magnetic beads. Using *Sau*-L-A as a primer, the repeat-enriched DNA was amplified by PCR to make it double-stranded and cloned using a TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions.

Table 1 Characterisation of eleven *A. digitatum* microsatellite loci

Locus	Clone name and EMBL accession number	Repeat motif	Fluoro label	Primer sequences (5′–3′)	T_m (°C)	Expected/ observed size (bp)	Obs. allele size (bp)	*K*	*N*	H_O	H_E	Est. null allele freq.	P_{HW}
Adig001	DMF85D10	$(TG)_8$	HEX	F: ACATACTCGGCTCATACTCGTG	59.3	124/	114–129	7	42	0.36	0.41	+0.09	0.10
	HF677589			R: CTCGTGCTCACAGACAAACAC	59.5	118, 122							
Adig002	DMF86B02	$(TG)_{12}$	HEX	F: GACTGGAGATTATGTTTTCATCG	57.8	219/	216–237	9	42	0.69	0.68	−0.00	0.40
	HF677590			R: ACGCATTCAGTTTTCTCTAACC	57.6	212, 220							
Adig003	DMF87B11	$(GT)_9$	HEX	F: TTTCAATTGTGCTACTGTTTGG	57.9	146/	136–152	6	41	0.34	0.67	+0.30	0.00*
	HF677591			R: TTGACGTTTCTAATTGCAATACC	58.3	145							
Adig004	DMF87E02	$(ATCT)_8$	6FAM	F: GACCTATGACGCATGCTCTG	59.4	129/	126–205	13	32	0.28	0.85	+0.50	0.00*
	HF677592			R: CGGATGCGAATTCTTTATCTTAC	59.2	126							
Adig005	DMF87H01	$(ACACA)_9$	6FAM	F: ATGACACGATAAACCCAAACG	59.7	116/	74–175	18	42	0.88	0.92	+0.02	0.52
	HF677593			R: ATTTGTTGTGTTGTGCTGTGC	59.7	89, 114							
Adig006	DMF87H03	$(TAT)_8$	HEX	F: CTTATTTGTAAAGCGCCTTGAAC	59.4	142/	130–142	3	42	0.21	0.20	−0.05	1.00
	HF677594			R: AAGCACGCTTCAAGACAACTC	59.4	142							
Adig007	DMF88A04	$(ATGGT)_5$	6FAM	F: GTGGTGTGGTAGTGGTGTGG	59.8	176/	166–202	4	41	0.07	0.07	−0.01	1.00
	HF677595			R: GCGTGTTCGGACTACATAGC	58.4	177, 182							
Adig008	DMF88C09	$(TACA)_{22}$	HEX	F: TTGTGGAAATCAACTATTTCTGTTG	59.5	179/	95–220	24	42	0.98	0.95	−0.02	0.65
	HF677596			R: TCTGTGACTGGTTCATATTGTGG	59.9	161, 177							
Adig009	DMF88E08	$(TGTTG)_{17}$	6FAM	F: GTGTTTCATCACCCTTGCAG	59.1	249/	196–258	13	39	0.82	0.90	+0.04	0.33
	HF677597			R: CCGACGATTGTTATAAAGATTAAATG	59.3	242, 253							
Adig010	DMF88E09	$(ATCT)_{16}$	6FAM	F: CAGTTTCTACTGCAATGGTTATTC	57.1	122/	111–264	27	30	0.87	0.96	+0.04	0.20
	HF677598			R: ATTAGGGAAACAAGCTTCGAC	57.5	120, 167							
Adig011	DMF89C09	$(TGT)_{11}$	HEX	F: GGTCGGGTTAGACGTGGAG	60.5	118/	98–169	16	41	0.76	0.70	−0.06	0.64
	HF677599			R: GTCACTCGAACTCGGCATTC	60.8	101, 120							

T_m primer melting temperature (calculated using Primer3), *Expected/observed* allele sizes expected, based on the individual cloned and sequenced, and genotyping of the individual colony from which the library was created (id: ManV1), *K* number of alleles, *N* number of individuals successfully amplified and genotyped (of 42 tested), H_O Observed heterozygosity, H_E Expected heterozygosity, *estimated null allele frequency* (calculated using CERVUS v3.0.3, Kalinowski et al. 2007), P_{HW} probability for HWE test calculated using GENEPOP v.4.2 software (Raymond and Rousset 1995; Rousset 2008)

* significant values after correcting for multiple tests using the false discovery rate correction (Benjamini and Hochberg 1995)

Table 2 Cross-species amplification of *A. digitatum* microsatellite loci in three other *Alcyonium* species

Species	Locus	Adig001	Adig002	Adig003	Adig004	Adig005	Adig006	Adig007	Adig008	Adig009	Adig010	Adig011
A. digitatum expected size ManV1 (bp)		124	219	146	129	116	142	176	179	122	249	118
A. coralloides Pallas, 1766	N tested	10	10	10	10	10	10	10	10	10	10	10
	N amp	8	1	0	2	2	1	0	3	0	0	0
	Size (bp)	120–124	226	–	126/130	84/95	238	–	157–192	–	–	–
	K	3	1	–	2	2	1	–	4	–	–	–
A. acaule Marion, 1878	N tested	11	11	11	11	11	11	10	11	11	11	10
	N amp	8	7	0	1	4	2	0	3	0	0	0
	Size (bp)	108–129	226	–	241–245	79/84	135	–	177/200	–	–	–
	K	4	1	–	2	2	1	–	2	–	–	–
A. palmatum Pallas, 1766	N tested	1	1	1	1	1	1	1	1	1	1	1
	N amp	1	0	0	0	1	0	0	0	0	0	0
	Size (bp)	122	–	–	–	104	–	–	–	–	–	–
	K	1	–	–	–	1	–	–	–	–	–	–

Expected size ManV1 expected allele size (bp) based on that of the individual (id: *ManV1*) cloned and sequenced during the library preparation, *N tested* number of individuals for which PCR was attempted for that species and locus, N_{amp} number of individuals that amplified (as observed on an ABI3730 Sequencer), *Size* observed allele size range (bp) across successfully amplified loci (presence of heterozygotes indicated by hyphenated size range, e.g., 120–124; homozygotes indicated by forward slash, e.g., 177/200 indicates that alleles were 177 or 200 bp, but with no heterozygotes detected), *K* number of alleles observed. Samples were collected from the French Mediterranean near Banyuls-sur-Mer (N 42°28′42.5″, E 03°09′33.5″ or N 42°28′54.5″, E 03°08′14.0″, with the exception of the single *A. palmatum* individual, which originated from an aquarium at the Observatoire Océanologique de Banyuls)

Bi-directional Sanger sequencing of clones (using ABI BigDye v3.1) allowed assembly of consensus sequences for most sequences and primer sets were designed using the software PRIMER3 (Rozen and Skaletsky 2000). In total, 116 unique *A. digitatum* sequences were isolated (EMBL accession numbers HF677589–HF677704), from which 54 primer sets were designed and tested in four individuals collected from a single site (Manacles reef, The Lizard, Cornwall; N 50°02′43.9″, W 05°02′45.4″) near (~1 km) to the location of the individual used to develop the library. Polymorphic loci that were easy to score and could be amplified reliably were genotyped in 42 individuals collected at Trenemene Reef, Isles of Scilly (N 49°52′10.0″, W 06°23′09.9″). PCR products were genotyped on an ABI 3730 48-well capillary DNA Analyser (Applied Biosystems Inc.) and alleles were scored using the GENEMAPPER v3.7 software (Applied Biosystems Inc.). From these, the most promising polymorphic loci were selected and arranged into multiplexes using the software Multiplex Manager 1 (Holleley and Geerts 2009). Duplexes (6FAM and HEX) or single-plex PCRs were performed (rather than multiplexes) due to the large allele size ranges of some loci. For PCR, 1 µl of template DNA (10 ng/µl) was air dried in the wells of PCR plates and amplified in a 2 µl PCR reaction containing 0.2 µM of each primer and 1 µl Multiplex PCR Master Mix (QIAGEN; Kenta et al. 2008). PCR reactions were performed using a DNA Engine Tetrad-2 thermal cycler (MJ Research, BioRad, UK) and conducted with the following profile: 95 °C for 15 min, followed by 44 cycles of 94 °C for 30 s, 58 °C for 90 s, 72 °C for 90 s extension and a final extension of 60 °C for 30 min. In total, 11 loci amplified reliably (Table 1). When amplified in the 42 individuals collected from the Isles of Scilly the number of alleles per locus ranged from 3 to 27 and expected heterozygosities from 0.07 to 0.96 (Table 1). Observed and expected heterozygosities were calculated using CERVUS v3.0.3 (Kalinowski et al. 2007). We also checked for the presence of duplicate genotypes with this program using the 'Identity Analysis' option (with the minimum number of loci needed for a match set at 1, fuzzy matching disallowed); none were detected. Deviations from Hardy–Weinberg equilibrium (HWE) and tests for linkage disequilibrium were calculated in GENEPOP v.4.2 software (Raymond and Rousset 1995; Rousset 2008). After correcting for multiple tests using the false discovery rate (FDR) method (Benjamini and Hochberg 1995), two loci displayed departure from HWE: *Adig*003 and *Adig*004 (5 % cut-off value). These loci also had high estimated null allele frequency estimates (>0.1; calculated using CERVUS v3.0.3; Kalinowski et al. 2007). There was no evidence of significant linkage disequilibrium between any pair of loci following an FDR correction for multiple tests (Benjamini and Hochberg 1995).

All eleven loci were tested in three other *Alcyonium* spp. (*A. coralloides* N = 10, *Alcyonium acaule* N = 11, *Alcyonium palmatum* N = 1, Table 2). Six loci amplified in at least one of the three other *Alcyonium* species tested (Table 2), suggesting potential cross-species utility of these markers.

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Appendix 3. Summary statistics for all loci in all populations.

		Brest3		N=43		DevBF		N=40	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.400	0.423	0.129	8	0.552	0.614	0.516	9
Exe15.	Ever002	0.233	0.279	0.353	3	0.200	0.320	0.023	3
Exe21.	Ever003	0.209	0.196	1.000	4	0.200	0.184	1.000	3
Exe33.	Ever004	0.651	0.632	0.571	7	0.650	0.719	0.157	7
Exe41.	Ever005	monomorphic: no test			1	0.075	0.074	1.000	3
Exe49.	Ever006	0.714	0.807	0.399	12	0.658	0.824	0.038	8
Exe10.	Ever007	0.667	0.717	0.620	9	0.513	0.645	0.343	5
Exe48.	Ever008	0.439	0.419	1.000	2	0.525	0.491	0.749	2
06E10.	Ever009	**0.442**	**0.681**	**0.001**	9	0.550	0.687	0.141	10
Exe17.	Ever010	0.238	0.221	1.000	5	0.128	0.122	1.000	2
Exe34.	Ever011	0.349	0.344	0.554	3	0.250	0.269	0.621	4
Exe47.	Ever012	monomorphic: no test			1	monomorphic: no test			1
Exe50.	Ever013	0.605	0.698	0.192	9	0.632	0.695	0.216	10
Exe24.	Ever014	0.605	0.689	0.073	5	0.641	0.615	0.136	3
		EvARM		**N=27**		**Faro1**		**N=41**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.370	0.363	0.732	6	0.500	0.471	0.862	8
Exe15.	Ever002	**0.040**	**0.184**	**0.007**	2	0.162	0.237	0.104	2
Exe21.	Ever003	0.074	0.073	1.000	3	0.073	0.072	1.000	3
Exe33.	Ever004	0.481	0.618	0.071	6	0.732	0.709	0.687	8
Exe41.	Ever005	monomorphic: no test			1	monomorphic: no test			1
Exe49.	Ever006	0.815	0.859	0.398	11	0.711	0.828	0.033	10
Exe10.	Ever007	0.778	0.724	0.207	6	0.561	0.677	0.248	7
Exe48.	Ever008	0.333	0.465	0.205	2	0.293	0.438	0.065	2
06E10.	Ever009	**0.346**	**0.769**	**0.000**	8	**0.475**	**0.756**	**0.000**	8
Exe17.	Ever010	0.148	0.142	1.000	3	0.154	0.148	1.000	4
Exe34.	Ever011	0.185	0.331	0.044	2	0.390	0.360	1.000	3
Exe47.	Ever012	0.154	0.151	1.000	3	0.115	0.113	1.000	4
Exe50.	Ever013	**0.615**	**0.729**	**0.010**	6	0.500	0.609	0.384	8
Exe24.	Ever014	0.741	0.705	0.543	6	0.737	0.687	0.087	7
		Faro2		**N=43**		**Faro3**		**N=42**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.488	0.431	1	7	0.526	0.552	0.258	7
Exe15.	Ever002	0.073	0.116	0.1206	2	0.128	0.245	0.015	2
Exe21.	Ever003	0.140	0.155	0.2326	3	0.167	0.199	0.214	3
Exe33.	Ever004	0.628	0.647	0.3417	8	0.833	0.791	0.918	8
Exe41.	Ever005	monomorphic: no test			1	0.048	0.047	1.000	3
Exe49.	Ever006	0.884	0.865	0.7692	10	0.750	0.861	0.416	11
Exe10.	Ever007	0.651	0.685	0.628	8	0.683	0.715	0.018	9
Exe48.	Ever008	0.442	0.417	1	2	0.341	0.419	0.268	2
06E10.	Ever009	**0.512**	**0.748**	**0.0016**	7	**0.463**	**0.701**	**0.000**	10
Exe17.	Ever010	0.071	0.115	0.1166	4	0.167	0.160	1.000	5
Exe34.	Ever011	0.349	0.350	0.8172	3	0.333	0.378	0.625	3
Exe47.	Ever012	0.042	0.042	-	2	0.000	0.073	0.019	2
Exe50.	Ever013	0.512	0.684	0.1035	9	0.364	0.569	0.017	6
Exe24.	Ever014	0.674	0.652	0.9551	6	0.533	0.655	0.026	6
		Faro4		**N=35**		**Faro5**		**N=44**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.536	0.535	0.458	10	0.364	0.410	0.168	6
Exe15.	Ever002	**0.063**	**0.347**	**0.000**	2	**0.049**	**0.137**	**0.007**	2
Exe21.	Ever003	0.143	0.137	1.000	4	0.250	0.267	0.453	4
Exe33.	Ever004	0.657	0.677	0.121	8	0.659	0.700	0.814	8
Exe41.	Ever005	0.029	0.029	1.000	2	monomorphic: no test			1
Exe49.	Ever006	0.848	0.859	0.317	10	0.791	0.864	0.445	9
Exe10.	Ever007	0.686	0.675	0.902	7	0.558	0.661	0.170	6
Exe48.	Ever008	0.571	0.414	0.033	2	0.409	0.379	0.705	2
06E10.	Ever009	**0.412**	**0.703**	**0.001**	7	**0.455**	**0.782**	**0.000**	9
Exe17.	Ever010	0.200	0.189	1.000	4	0.159	0.152	1.000	5
Exe34.	Ever011	0.286	0.330	0.650	3	0.341	0.326	1.000	1
Exe47.	Ever012	**0.000**	**0.221**	**0.001**	3	monomorphic: no test			3
Exe50.	Ever013	**0.424**	**0.646**	**0.012**	9	**0.535**	**0.715**	**0.004**	7
Exe24.	Ever014	0.600	0.662	0.853	6	0.636	0.678	0.368	7

Clone	Locus	DevHandsDeep		N=36		IoSHath		N=30	
		Ho	*He*	*P* value	*K*	*Ho*	*He*	*P* value	*K*
01C02.	Ever001	0.800	0.748	0.723	8	0.630	0.581	0.818	8
Exe15.	Ever002	0.371	0.442	0.284	4	0.379	0.379	0.605	3
Exe21.	Ever003	0.194	0.183	1.000	3	0.200	0.274	0.044	4
Exe33.	Ever004	0.528	0.697	0.052	9	0.700	0.702	0.576	9
Exe41.	Ever005	0.028	0.028	1.000	2	monomorphic: no test			1
Exe49.	Ever006	0.824	0.817	0.413	7	0.586	0.817	0.042	9
Exe10.	Ever007	0.743	0.711	0.116	7	0.586	0.659	0.378	6
Exe48.	Ever008	0.457	0.437	1.000	2	0.500	0.440	0.674	2
06E10.	Ever009	**0.500**	**0.752**	**0.001**	9	**0.321**	**0.550**	**0.002**	7
Exe17.	Ever010	0.139	0.133	1.000	3	0.067	0.066	1.000	2
Exe34.	Ever011	0.250	0.251	0.516	3	0.300	0.267	1.000	3
Exe47.	Ever012	0.042	0.120	0.065	2	0.056	0.160	0.028	3
Exe50.	Ever013	**0.515**	**0.806**	**0.000**	10	monomorphic: no test			1
Exe24.	Ever014	0.694	0.674	0.303	4	**0.364**	**0.656**	**0.003**	4
		IoS_LR		**N=22**		**IoSnnw**		**N=23**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.773	0.736	0.378	7	0.609	0.558	0.897	7
Exe15.	Ever002	0.400	0.381	0.687	3	0.130	0.127	1.000	3
Exe21.	Ever003	0.136	0.132	1.000	3	0.217	0.270	0.411	3
Exe33.	Ever004	0.682	0.724	0.247	6	0.652	0.636	0.874	7
Exe41.	Ever005	monomorphic: no test			1	0.043	0.043	1.000	2
Exe49.	Ever006	0.545	0.801	0.010	7	0.652	0.829	0.013	7
Exe10.	Ever007	0.810	0.642	0.650	5	0.652	0.666	0.866	7
Exe48.	Ever008	0.429	0.438	1.000	2	0.565	0.476	0.652	2
06E10.	Ever009	0.455	0.462	0.790	7	0.455	0.647	0.061	7
Exe17.	Ever010	0.136	0.174	0.046	4	0.087	0.127	0.067	3
Exe34.	Ever011	0.227	0.210	1.000	3	0.261	0.232	1.000	2
Exe47.	Ever012	monomorphic: no test			1	monomorphic: no test			1
Exe50.	Ever013	0.864	0.855	0.219	8	0.600	0.577	0.782	6
Exe24.	Ever014	0.545	0.673	0.522	4	0.739	0.680	0.753	4
		Ire_BlackRock		**N=29**		**Ire_ThumbRock**		**N=48**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.690	0.690	0.349	10	0.681	0.677	0.577	11
Exe15.	Ever002	0.069	0.068	1.000	2	0.125	0.154	0.271	2
Exe21.	Ever003	0.241	0.224	1.000	4	0.167	0.175	0.166	4
Exe33.	Ever004	0.724	0.767	0.550	7	0.667	0.721	0.399	9
Exe41.	Ever005	monomorphic: no test			1	monomorphic: no test			1
Exe49.	Ever006	0.897	0.826	0.886	10	0.638	0.830	0.044	11
Exe10.	Ever007	0.692	0.683	0.153	5	0.638	0.746	0.076	7
Exe48.	Ever008	0.321	0.431	0.204	2	0.489	0.483	1.000	2
06E10.	Ever009	0.286	0.473	0.026	6	**0.188**	**0.567**	**0.000**	6
Exe17.	Ever010	0.034	0.034	1.000	2	0.064	0.063	1.000	3
Exe34.	Ever011	0.310	0.279	1.000	3	0.125	0.120	1.000	3
Exe47.	Ever012	monomorphic: no test			1	**0.034**	**0.226**	**0.000**	4
Exe50.	Ever013	0.211	0.193	1.000	2	monomorphic: no test			1
Exe24.	Ever014	0.655	0.673	0.841	4	0.795	0.759	0.551	7
		JTEten		**N=7**		**LTGlen**		**N=40**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	monomorphic: no test			1	0.400	0.437	0.277	8
Exe15.	Ever002	0.143	0.495	0.105	2	0.175	0.248	0.118	4
Exe21.	Ever003	0.143	0.143	1.000	2	0.100	0.097	1.000	3
Exe33.	Ever004	0.714	0.758	1.000	5	0.744	0.767	0.516	9
Exe41.	Ever005	monomorphic: no test			1	monomorphic: no test			1
Exe49.	Ever006	0.833	0.848	0.563	5	**0.528**	**0.775**	**0.003**	11
Exe10.	Ever007	0.857	0.824	0.823	5	0.758	0.723	0.970	7
Exe48.	Ever008	0.286	0.440	0.440	2	0.500	0.464	0.730	2
06E10.	Ever009	0.571	0.791	0.605	5	**0.300**	**0.528**	**0.002**	6
Exe17.	Ever010	monomorphic: no test			1	0.300	0.276	1.000	6
Exe34.	Ever011	0.429	0.385	1.000	3	0.300	0.265	1.000	3
Exe47.	Ever012	monomorphic: no test			1	0.000	0.073	0.019	2
Exe50.	Ever013	0.667	0.682	1.000	4	0.444	0.709	0.026	5
Exe24.	Ever014	0.714	0.703	0.807	3	0.750	0.693	0.960	4

Clone	Locus	Lundy		N=22		LymeHW		N=9	
		Ho	*He*	*P* value	*K*	*Ho*	*He*	*P* value	*K*
01C02.	Ever001	0.824	0.775	0.502	9	0.667	0.621	0.494	6
Exe15.	Ever002	0.300	0.345	0.584	4	0.222	0.209	1.000	2
Exe21.	Ever003	0.045	0.045	1.000	2	0.222	0.216	1.000	3
Exe33.	Ever004	0.682	0.702	0.688	7	0.778	0.843	0.565	6
Exe41.	Ever005	monomorphic: no test			1	monomorphic: no test			1
Exe49.	Ever006	**0.476**	**0.827**	**0.001**	7	0.889	0.837	0.121	7
Exe10.	Ever007	0.727	0.635	0.718	6	0.875	0.808	0.359	6
Exe48.	Ever008	0.500	0.485	1.000	2	0.500	0.500	1.000	2
06E10.	Ever009	**0.381**	**0.722**	**0.004**	8	**0.250**	**0.825**	**0.003**	6
Exe17.	Ever010	0.182	0.246	0.325	3	0.222	0.216	1.000	3
Exe34.	Ever011	0.273	0.249	1.000	3	0.000	0.366	0.011	2
Exe47.	Ever012	monomorphic: no test			1	monomorphic: no test			1
Exe50.	Ever013	0.571	0.672	0.384	8	0.625	0.817	0.215	7
Exe24.	Ever014	0.818	0.730	0.541	4	0.556	0.503	0.349	3
		ManMo		**N=30**		**ManRR**		**N=43**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.500	0.464	1.000	3	0.741	0.681	0.738	9
Exe15.	Ever002	0.300	0.406	0.084	3	0.349	0.408	0.354	5
Exe21.	Ever003	0.167	0.158	1.000	3	0.140	0.154	0.232	3
Exe33.	Ever004	0.700	0.708	0.154	8	0.605	0.601	0.961	9
Exe41.	Ever005	0.033	0.033	1.000	2	monomorphic: no test			1
Exe49.	Ever006	0.577	0.804	0.013	9	**0.628**	**0.796**	**0.008**	12
Exe10.	Ever007	0.600	0.667	0.714	7	0.780	0.655	0.756	6
Exe48.	Ever008	0.433	0.440	1.000	2	0.400	0.475	0.333	2
06E10.	Ever009	**0.379**	**0.655**	**0.000**	8	**0.410**	**0.784**	**0.000**	10
Exe17.	Ever010	0.107	0.138	0.112	4	0.143	0.136	1.000	3
Exe34.	Ever011	0.233	0.362	0.023	3	0.419	0.350	0.431	3
Exe47.	Ever012	monomorphic: no test			1	0.033	0.098	0.016	3
Exe50.	Ever013	**0.318**	**0.559**	**0.001**	6	0.605	0.693	0.073	12
Exe24.	Ever014	0.586	0.702	0.020	4	0.744	0.709	0.919	5
		ManV		**N=24**		**Marseilles (EvMai)**		**N=13**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.778	0.739	0.812	6	0.667	0.580	0.372	5
Exe15.	Ever002	0.167	0.297	0.030	3	0.154	0.369	0.076	2
Exe21.	Ever003	0.167	0.156	1.000	2	monomorphic: no test			1
Exe33.	Ever004	0.609	0.635	0.570	7	0.462	0.462	0.520	5
Exe41.	Ever005	monomorphic: no test			1	monomorphic: no test			1
Exe49.	Ever006	0.750	0.796	0.417	7	0.583	0.757	0.466	5
Exe10.	Ever007	0.818	0.662	0.821	6	0.900	0.563	0.045	3
Exe48.	Ever008	0.391	0.476	0.411	2	0.462	0.443	1.000	2
06E10.	Ever009	0.619	0.779	0.092	7	**0.000**	**0.394**	**0.000**	3
Exe17.	Ever010	0.087	0.086	1.000	3	0.231	0.218	1.000	3
Exe34.	Ever011	0.333	0.356	0.088	3	0.385	0.542	0.320	3
Exe47.	Ever012	0.063	0.063	1.000	2	monomorphic: no test			1
Exe50.	Ever013	**0.286**	**0.436**	**0.003**	6	0.250	0.236	1.000	3
Exe24.	Ever014	0.750	0.648	0.674	4	0.615	0.717	0.221	4
		MenGlen		**N=43**		**nrPad**	**N=7**		
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.486	0.499	0.436	10	1.000	0.742	0.860	5
Exe15.	Ever002	**0.140**	**0.299**	**0.001**	4	0.500	0.409	1.000	2
Exe21.	Ever003	0.163	0.155	1.000	3	0.286	0.264	1.000	2
Exe33.	Ever004	0.767	0.677	0.863	8	1.000	0.725	0.606	5
Exe41.	Ever005	monomorphic: no test			1	monomorphic: no test			1
Exe49.	Ever006	0.810	0.810	0.113	10	0.857	0.835	0.274	6
Exe10.	Ever007	0.744	0.731	0.448	7	0.429	0.670	0.328	3
Exe48.	Ever008	0.452	0.471	1.000	2	0.571	0.440	1.000	2
06E10.	Ever009	0.349	0.448	0.122	9	**0.286**	**0.615**	**0.030**	3
Exe17.	Ever010	0.209	0.199	1.000	7	monomorphic: no test			1
Exe34.	Ever011	0.302	0.291	1.000	2	0.429	0.363	1.000	2
Exe47.	Ever012	monomorphic: no test			1	monomorphic: no test			1
Exe50.	Ever013	**0.485**	**0.737**	**0.002**	9	0.833	0.879	0.335	6
Exe24.	Ever014	0.571	0.627	0.140	4	0.857	0.758	0.245	4

		PlyMew		N=44		Ros2		N=36	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.512	0.661	0.072	9	0.548	0.512	0.949	9
Exe15.	Ever002	0.326	0.358	0.403	4	0.278	0.408	0.010	3
Exe21.	Ever003	0.159	0.192	0.038	4	0.194	0.205	0.182	4
Exe33.	Ever004	0.682	0.636	0.614	9	0.667	0.751	0.178	9
Exe41.	Ever005	0.045	0.045	1.000	2	monomorphic: no test			1
Exe49.	Ever006	0.659	0.821	0.044	9	0.765	0.828	0.082	12
Exe10.	Ever007	0.548	0.578	0.511	5	0.750	0.752	0.060	7
Exe48.	Ever008	0.605	0.498	0.205	2	0.382	0.433	0.685	2
06E10.	Ever009	**0.442**	**0.660**	**0.000**	9	**0.429**	**0.689**	**0.000**	10
Exe17.	Ever010	0.136	0.131	1.000	4	0.167	0.158	1.000	4
Exe34.	Ever011	0.455	0.417	1.000	3	0.278	0.372	0.039	3
Exe47.	Ever012	**0.000**	**0.111**	**0.000**	3	0.000	0.082	0.021	2
Exe50.	Ever013	0.750	0.788	0.587	10	0.735	0.737	0.145	8
Exe24.	Ever014	0.727	0.704	0.827	5	0.639	0.663	0.883	5
		Ros1		**N=40**		**Sawtooth**		**N=12**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.600	0.556	0.789	9	0.727	0.710	0.752	6
Exe15.	Ever002	0.237	0.295	0.405	4	0.167	0.159	1.000	2
Exe21.	Ever003	0.150	0.187	0.155	3	0.167	0.159	1.000	2
Exe33.	Ever004	0.750	0.769	0.581	8	0.500	0.656	0.144	5
Exe41.	Ever005	monomorphic: no test			1	0.083	0.083	1.00	2
Exe49.	Ever006	0.775	0.822	0.507	10	0.636	0.823	0.224	6
Exe10.	Ever007	0.718	0.641	0.072	5	0.667	0.659	0.768	4
Exe48.	Ever008	0.474	0.472	1.000	2	0.417	0.431	1.000	2
06E10.	Ever009	**0.308**	**0.641**	**0.000**	8	**0.167**	**0.601**	**0.000**	6
Exe17.	Ever010	0.050	0.050	1.000	3	0.167	0.163	1.000	3
Exe34.	Ever011	0.250	0.313	0.055	3	0.083	0.083	1.000	2
Exe47.	Ever012	0.042	0.042	1.000	2	monomorphic: no test			1
Exe50.	Ever013	0.611	0.697	0.060	11	**0.250**	**0.612**	**0.000**	5
Exe24.	Ever014	0.725	0.683	0.993	5	0.583	0.656	0.198	5
		Skomer		**N=39**		**WestTen**		**N=43**	
Clone	**Locus**	***Ho***	***He***	***P* value**	***K***	***Ho***	***He***	***P* value**	***K***
01C02.	Ever001	0.686	0.630	0.620	8	0.821	0.773	0.497	9
Exe15.	Ever002	0.263	0.378	0.093	3	0.256	0.338	0.129	4
Exe21.	Ever003	0.128	0.122	1.00	2	0.163	0.196	0.242	4
Exe33.	Ever004	0.641	0.641	0.628	7	0.651	0.707	0.752	10
Exe41.	Ever005	monomorphic: no test			1	0.023	0.068	0.036	2
Exe49.	Ever006	0.757	0.813	0.382	9	**0.610**	**0.818**	**0.001**	10
Exe10.	Ever007	0.811	0.700	0.279	7	0.780	0.712	0.881	7
Exe48.	Ever008	0.538	0.495	0.744	2	0.512	0.495	1.000	2
06E10.	Ever009	**0.487**	**0.713**	**0.001**	9	0.581	0.690	0.184	8
Exe17.	Ever010	0.256	0.295	0.207	5	0.214	0.200	1.000	5
Exe34.	Ever011	0.333	0.351	0.721	3	0.214	0.220	0.456	4
Exe47.	Ever012	monomorphic: no test			1	monomorphic: no test			1
Exe50.	Ever013	0.684	0.762	0.023	11	0.651	0.750	0.026	10
Exe24.	Ever014	0.769	0.687	0.423	4	0.674	0.673	0.616	4

	POP:	Brest 2	N=43			CefMiX	N=27		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	**0.43902**	**0.62993**	**0.00192**	7	0.17391	0.30531	0.06204	3
86B02	Adig002	0.54762	0.6354	0.20699	7	0.8	0.75755	0.83287	10
87B11	Adig003	**0.28947**	**0.48667**	**0.00018**	5	**0.10526**	**0.44523**	**0.00002**	5
87E02	Adig004	**0.4359**	**0.90743**	**0**	17	**0.4375**	**0.9254**	**0**	15
87H01	Adig005	0.92857	0.92484	0.54386	18	0.96296	0.93711	0.83878	17
87H03	Adig006	0.14286	0.24785	0.02253	2	0.44	0.37469	1	3
88A04	Adig007	0.13953	0.1554	0.13816	4	0.14815	0.14186	1	3
88C09	Adig008	0.97674	0.95321	0.719	23	1	0.954	1	22
88E08	Adig009	0.85714	0.90247	0.16279	16	0.84	0.89551	0.07708	12
88E09	Adig010	0.87179	0.96537	0.041	28	**0.54167**	**0.95213**	**0**	21
89C09	Adig011	0.7907	0.7844	0.69147	14	0.62963	0.65968	0.35831	11
		CefT342		N=33		Dgal	N=7		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	0.51613	0.6092	0.1148	6	0.28571	0.27473	1	3
86B02	Adig002	**0.51515**	**0.65455**	**0.01111**	8	0.14286	0.53846	0.02121	3
87B11	Adig003	**0.27586**	**0.5729**	**0.00008**	4	0.4	0.62222	0.23632	3
87E02	Adig004	**0.35**	**0.91923**	**0**	15	0.33333	0.72727	0.03716	4
87H01	Adig005	0.87879	0.938	0.0602	16	0.71429	0.76923	0.57203	8
87H03	Adig006	0.625	0.5501	0.26602	4	0.42857	0.53846	1	3
88A04	Adig007	monomorphic: no test			1	monomorphic: no test			1
88C09	Adig008	0.84848	0.931	0.03639	17	1	0.94505	1	10
88E08	Adig009	0.78125	0.89782	0.19159	12	0.66667	0.73333	1	3
88E09	Adig010	**0.60714**	**0.94805**	**0**	19	0.85714	0.94505	0.42343	10
89C09	Adig011	0.875	0.85665	0.2879	12	0.71429	0.76923	0.55713	6
		DorBA	N=24			Frog	N=18		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	0.52174	0.59034	0.24808	5	0.46667	0.5931	0.26246	5
86B02	Adig002	0.66667	0.64716	0.6736	6	0.72222	0.79048	0.88747	8
87B11	Adig003	**0.29167**	**0.54344**	**0.0041**	5	**0.125**	**0.44355**	**0.00747**	2
87E02	Adig004	**0.23529**	**0.86809**	**0**	12	**0.07692**	**0.90769**	**0**	9
87H01	Adig005	0.875	0.92642	0.10093	15	0.88889	0.91746	0.3948	13
87H03	Adig006	0.20833	0.26418	0.39405	4	0.16667	0.1619	1	4
88A04	Adig007	0.04167	0.04167	1	2	0.05556	0.05556	1	2
88C09	Adig008	0.86957	0.94493	0.09677	19	1	0.9619	1	20
88E08	Adig009	0.9	0.89872	0.40186	12	**0.66667**	**0.90115**	**0.00979**	12
88E09	Adig010	0.85	0.94487	0.11365	19	**0.57143**	**0.96296**	**0**	16
89C09	Adig011	0.75	0.75089	0.4312	8	0.83333	0.77937	0.97708	11
		HC	N=36			IrelT	N=48		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	0.40625	0.40079	0.77038	5	0.35417	0.47303	0.0318	7
86B02	Adig002	0.58824	0.65716	0.26893	8	0.6875	0.72303	0.91635	9
87B11	Adig003	**0.16129**	**0.36118**	**0.00016**	4	**0.14583**	**0.53333**	**0**	6
87E02	Adig004	**0.17241**	**0.79915**	**0**	8	**0.43902**	**0.89672**	**0**	19
87H01	Adig005	0.91429	0.92547	0.53731	17	0.9375	0.92215	0.94783	15
87H03	Adig006	0.30556	0.30908	1	4	0.25532	0.23473	1	5
88A04	Adig007	0.09375	0.09177	1	3	0.0625	0.06162	1	3
88C09	Adig008	0.94286	0.95114	0.11253	22	0.97917	0.94781	0.43502	22
88E08	Adig009	0.83871	0.91803	0.13215	15	**0.77778**	**0.8819**	**0.0105**	16
88E09	Adig010	**0.66667**	**0.95198**	**0.00022**	25	**0.74468**	**0.95447**	**0**	26
89C09	Adig011	0.75	0.77191	0.23166	10	0.75	0.80482	0.59326	16

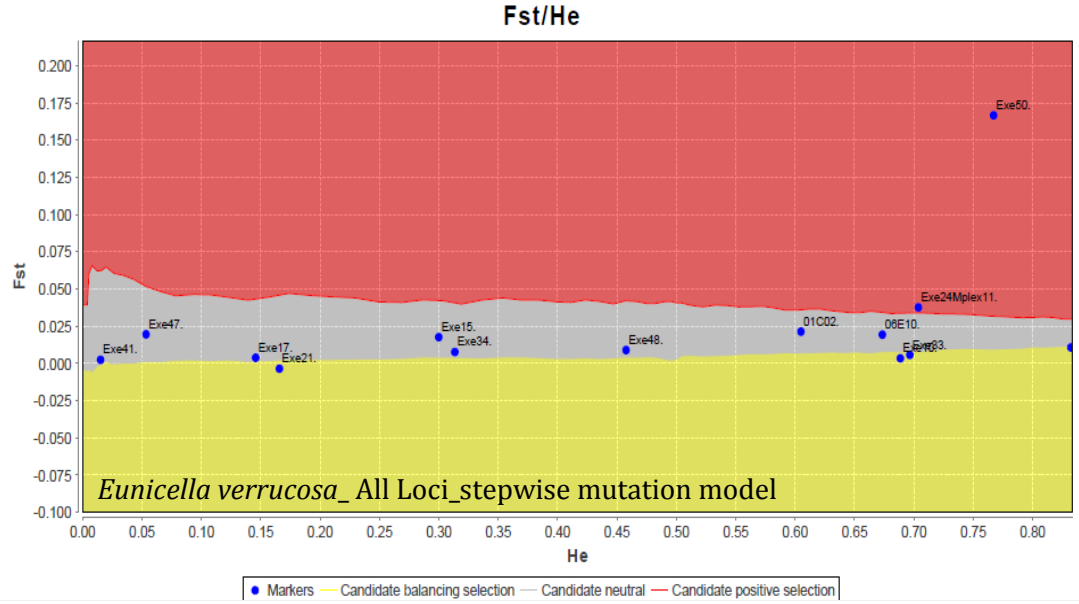
		IreTR	N=18			LTGlen	N=29		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	0.61111	0.60635	0.76037	6	0.34483	0.42952	0.18738	5
86B02	Adig002	0.77778	0.71111	0.49703	7	0.58621	0.64489	0.38097	8
87B11	Adig003	0.16667	0.69206	0	7	0.24	0.4302	0.00104	4
87E02	Adig004	0.5	0.86895	0	10	0.44	0.89388	0	13
87H01	Adig005	0.88889	0.90159	0.40215	12	0.93103	0.90018	0.96004	13
87H03	Adig006	0.66667	0.51905	0.60364	4	0.14815	0.13976	1	2
88A04	Adig007	monomorphic: no test			1	0.10345	0.10103	1	3
88C09	Adig008	1	0.95556	1	16	0.88462	0.95626	0.36349	22
88E08	Adig009	0.76471	0.86631	0.12253	10	0.76	0.87184	0.18384	13
88E09	Adig010	0.76471	0.95544	0.03984	19	0.73077	0.94193	0.01144	21
89C09	Adig011	0.83333	0.82857	0.39244	10	0.75862	0.81246	0.33295	14
		Lucy	N=22			ManCD	N=33		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	0.33333	0.47503	0.02677	6	0.34375	0.51587	0.03372	6
86B02	Adig002	0.7	0.72949	0.58943	7	0.80645	0.78583	0.01294	9
87B11	Adig003	0.11765	0.31373	0.00881	3	0.28125	0.50446	0.00004	5
87E02	Adig004	0.44444	0.8873	0.0001	10	0.34615	0.8356	0	13
87H01	Adig005	0.95455	0.90486	0.31714	14	0.9697	0.91935	0.13786	18
87H03	Adig006	0.1	0.09744	1	2	0.21212	0.24289	0.46754	3
88A04	Adig007	0.04545	0.04545	1	2	0.03125	0.03125	1	2
88C09	Adig008	0.86364	0.95032	0.31217	18	1	0.94406	0.98249	21
88E08	Adig009	0.71429	0.88889	0.18067	10	0.87097	0.90217	0.36327	16
88E09	Adig010	0.66667	0.95122	0.00198	18	0.71875	0.95833	0	23
89C09	Adig011	0.90909	0.7833	0.87017	10	0.8125	0.82242	0.71299	14
		ManV2	N=28			Mglen	N=34		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	0.48	0.59265	0.09183	7	0.52941	0.67647	0.35881	7
86B02	Adig002	0.7037	0.71908	0.59126	9	0.61765	0.7331	0.32194	7
87B11	Adig003	0.36	0.51592	0.03112	4	0.3	0.54068	0.00019	5
87E02	Adig004	0.25	0.85769	0	10	0.46667	0.89774	0	14
87H01	Adig005	0.88462	0.92232	0.67202	14	0.85294	0.92845	0.05713	15
87H03	Adig006	0.46154	0.38914	0.76899	3	0.3125	0.28125	1	4
88A04	Adig007	0.07143	0.07078	1	3	0.12121	0.11748	1	3
88C09	Adig008	1	0.95779	0.93203	21	0.88235	0.94381	0.57598	19
88E08	Adig009	0.85185	0.90007	0.0367	11	0.8125	0.87897	0.07277	15
88E09	Adig010	0.76923	0.95701	0.00196	22	0.83333	0.96441	0.01906	24
89C09	Adig011	0.82143	0.82013	0.84716	13	0.75758	0.73613	0.54491	12
		PR	N=51			Ros1	N=41		
Clone	Locus	Hobs	He	Pvalue	Na	Hobs	He	Pvalue	Na
85D10	Adig001	0.35294	0.47098	0.00195	6	0.47368	0.53544	0.236	9
86B02	Adig002	0.69388	0.65622	0.88673	10	0.71053	0.77228	0.06998	8
87B11	Adig003	0.36364	0.48302	0.21004	5	0.16216	0.63088	0	8
87E02	Adig004	0.37209	0.90698	0	17	0.27273	0.88298	0	14
87H01	Adig005	0.90196	0.91672	0.14824	18	0.92308	0.91708	0.25225	15
87H03	Adig006	0.41176	0.43526	0.01505	5	0.18421	0.17158	1	3
88A04	Adig007	0.03922	0.03883	1	2	0.07317	0.07197	1	3
88C09	Adig008	0.93878	0.95077	0.38152	23	1	0.95092	0.67547	23
88E08	Adig009	0.79167	0.88355	0.24746	13	0.8	0.91582	0.00397	15
88E09	Adig010	0.8	0.95758	0.00136	26	0.71795	0.96037	0	26
89C09	Adig011	0.84	0.79919	0.85863	13	0.73171	0.80608	0.61332	13

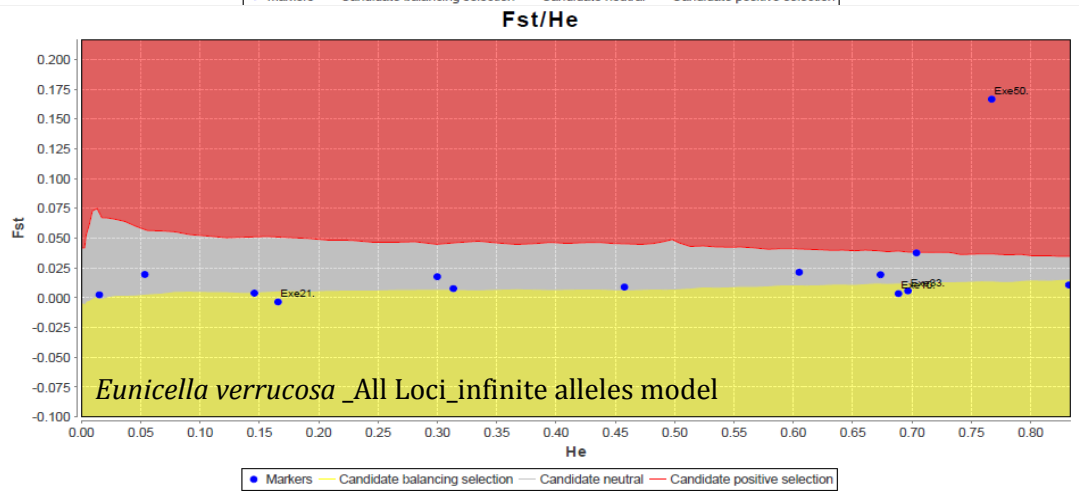
Clone	Locus	Ros2 Hobs	N=41 He	Pvalue	Na	Stone Hobs	N=40 He	Pvalue	Na
85D10	Adig001	0.4	0.57373	0.00257	8	0.46154	0.44123	0.58299	9
86B02	Adig002	0.825	0.81551	0.90079	11	0.7	0.76108	0.00175	8
87B11	Adig003	0.35	0.69335	0	5	0.26316	0.48702	0.00015	3
87E02	Adig004	0.30303	0.89883	0	19	0.23333	0.88475	0	12
87H01	Adig005	0.825	0.92595	0.03202	16	0.875	0.92215	0.68268	18
87H03	Adig006	0.2	0.18703	1	3	0.28205	0.28904	1	4
88A04	Adig007	0.07317	0.07197	1	3	0.05	0.04968	1	3
88C09	Adig008	1	0.95	0.85457	24	0.94595	0.95446	0.96207	24
88E08	Adig009	0.79487	0.90809	0.03055	16	0.71053	0.88175	0.0211	14
88E09	Adig010	0.7	0.9538	0	26	0.87879	0.96037	0.1177	25
89C09	Adig011	0.75	0.82247	0.11455	12	0.68421	0.75088	0.27192	12

Clone	Locus	TR Hobs	N=21 He	Pvalue	Na	Tren Hobs	N=42 He	Pvalue	Na
85D10	Adig001	0.36842	0.60882	0.00054	7	0.35714	0.41136	0.0962	7
86B02	Adig002	0.55556	0.68413	0.08066	7	0.69048	0.67814	0.40872	9
87B11	Adig003	0.13333	0.53103	0.00043	4	0.34146	0.66847	0	6
87E02	Adig004	0.52941	0.86809	0.00478	10	0.28125	0.84573	0	13
87H01	Adig005	0.94737	0.92319	0.90279	13	0.88095	0.92054	0.52076	18
87H03	Adig006	0.26316	0.24751	1	5	0.21429	0.19937	1	3
88A04	Adig007	0.04762	0.04762	1	2	0.07317	0.07227	1	4
88C09	Adig008	1	0.93974	0.76785	16	0.97619	0.95439	0.62006	24
88E08	Adig009	0.875	0.91532	0.25242	14	0.82051	0.90609	0.34013	13
88E09	Adig010	0.75	0.93974	0	16	0.86667	0.95876	0.31014	27
89C09	Adig011	0.80952	0.7921	0.66502	10	0.7561	0.7016	0.62485	16

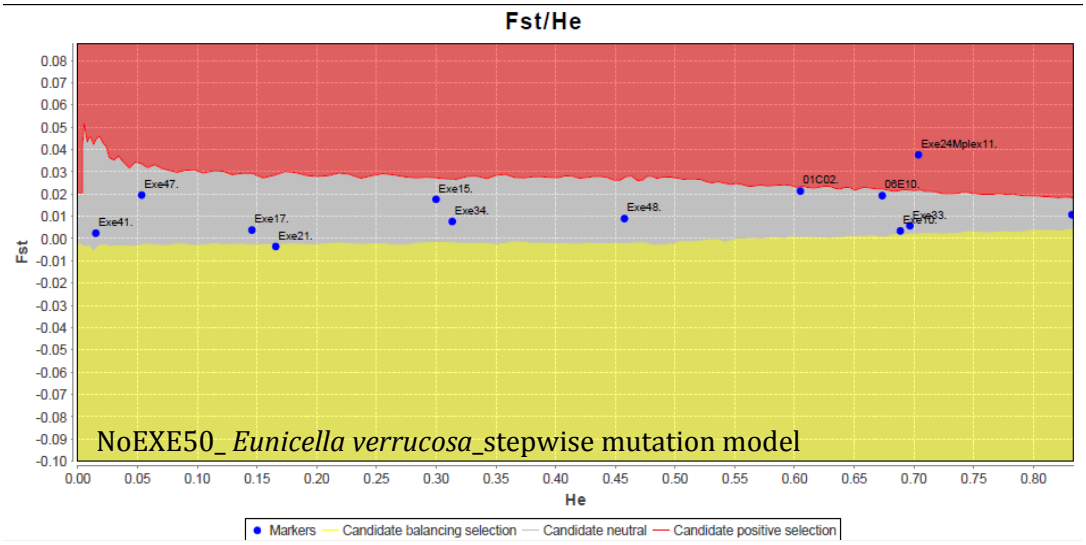
Clone	Locus	UB74 Hobs	N=19 He	Pvalue	Na
85D10	Adig001	0.23529	0.31907	0.06736	4
86B02	Adig002	0.73684	0.78236	0.35058	8
87B11	Adig003	0.17647	0.55793	0.00062	3
87E02	Adig004	0.35714	0.82804	0	9
87H01	Adig005	0.84211	0.90469	0.04067	14
87H03	Adig006	0.36842	0.39972	0.4455	3
88A04	Adig007	0.21053	0.19772	1	3
88C09	Adig008	0.94118	0.93939	0.59731	15
88E08	Adig009	0.73684	0.90327	0.00102	13
88E09	Adig010	0.625	0.96169	0.00007	17
89C09	Adig011	0.84211	0.78094	0.8274	8

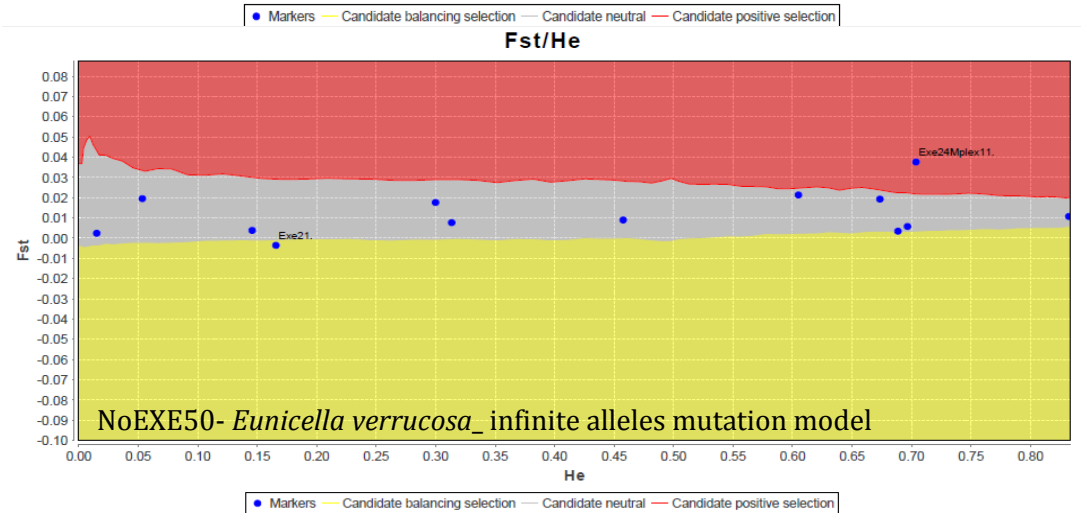
Appendix 4. Tests for outlier loci as calculated using LOSITAN.



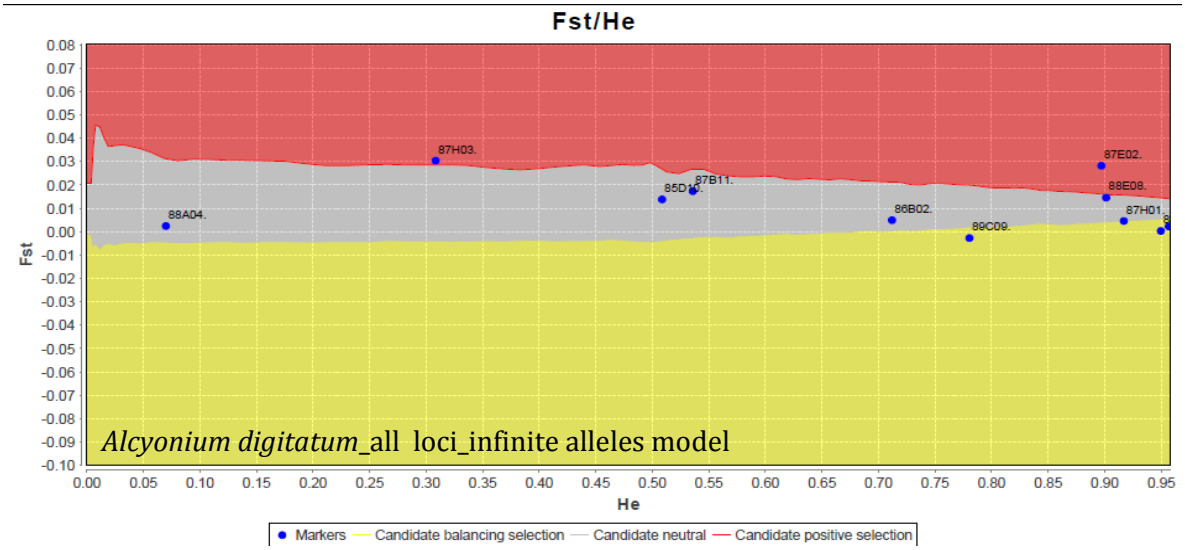


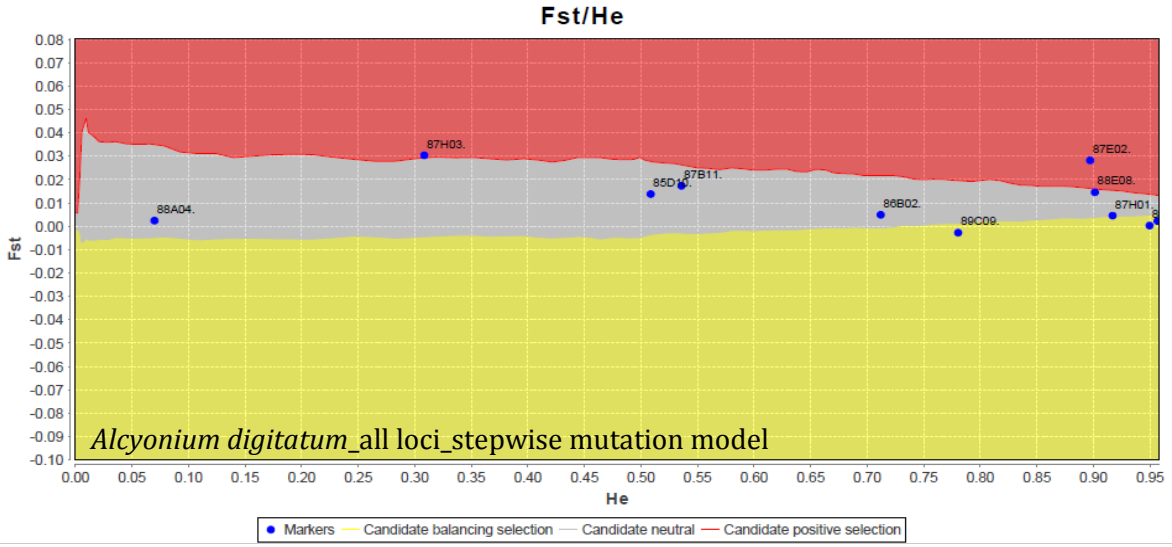
	Ever_955_all loci_SMM			Ever_955_all loci_IAM		
Locus	**Het**	**Fst**	**P-val**	**Het**	**Fst**	**P-val**
Ever001/01C02	0.605	0.021	0.581	0.605	0.021	0.410
Ever002/Exe15	0.300	0.018	0.449	0.300	0.018	0.295
Ever003/Exe21	0.166	-0.004	0.002	0.166	-0.004	0.001
Ever004/Exe33	0.697	0.006	0.013	0.697	0.006	0.000
Ever005/Exe41	0.015	0.002	0.072	0.015	0.002	0.063
Ever006/Exe49	0.832	0.011	0.015	0.832	0.011	0.000
Ever007/Exe10	0.689	0.003	0.004	0.689	0.003	0.000
Ever008/Exe48	0.458	0.009	0.102	0.458	0.009	0.059
Ever009/06E10	0.673	0.019	0.473	0.673	0.019	0.271
Ever010/Exe17	0.146	0.004	0.054	0.146	0.004	0.027
Ever011/Exe34	0.313	0.008	0.095	0.313	0.008	0.038
Ever012/Exe47	0.053	0.019	0.560	0.053	0.019	0.452
Ever013/Exe50	0.767	0.167	1.000	0.767	0.167	1.000
Ever014/Exe24Mplex11	0.704	0.038	0.990	0.704	0.038	0.965





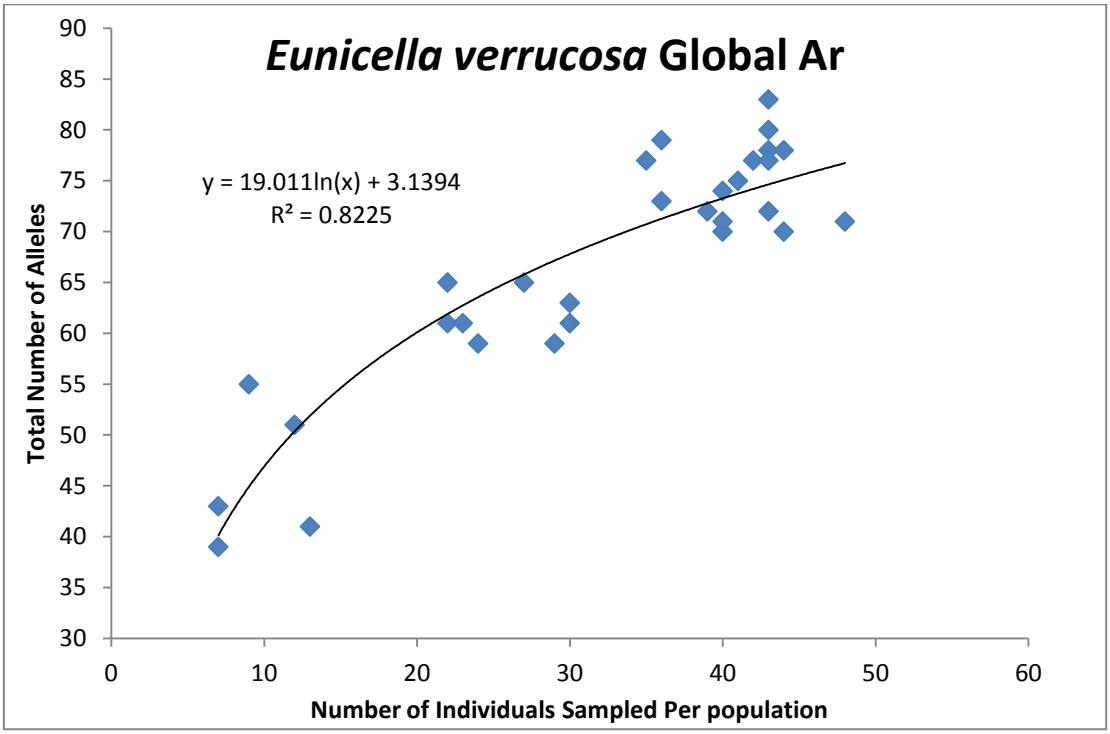
	Ever_955_NoExe50_SMM			Ever_955_NoExe50_IAM		
Locus	Het	Fst	P-val	Het	Fst	P-val
Ever001/01C02	0.605	0.021	0.941	0.605	0.021	0.922
Ever002/Exe15	0.300	0.018	0.807	0.300	0.018	0.757
Ever003/Exe21	0.166	-0.004	0.025	0.166	-0.004	0.007
Ever004/Exe33	0.697	0.006	0.145	0.697	0.006	0.106
Ever005/Exe41	0.015	0.002	0.261	0.015	0.002	0.253
Ever006/Exe49	0.832	0.011	0.481	0.832	0.011	0.358
Ever007/Exe10	0.689	0.003	0.061	0.689	0.003	0.032
Ever008/Exe48	0.458	0.009	0.438	0.458	0.009	0.359
Ever009/06E10	0.673	0.019	0.917	0.673	0.019	0.889
Ever010/Exe17	0.146	0.004	0.211	0.146	0.004	0.147
Ever011/Exe34	0.313	0.008	0.394	0.313	0.008	0.281
Ever012/Exe47	0.053	0.019	0.843	0.053	0.019	0.811
Ever014/Exe24Mplex11	0.704	0.038	1.000	0.704	0.038	1.000

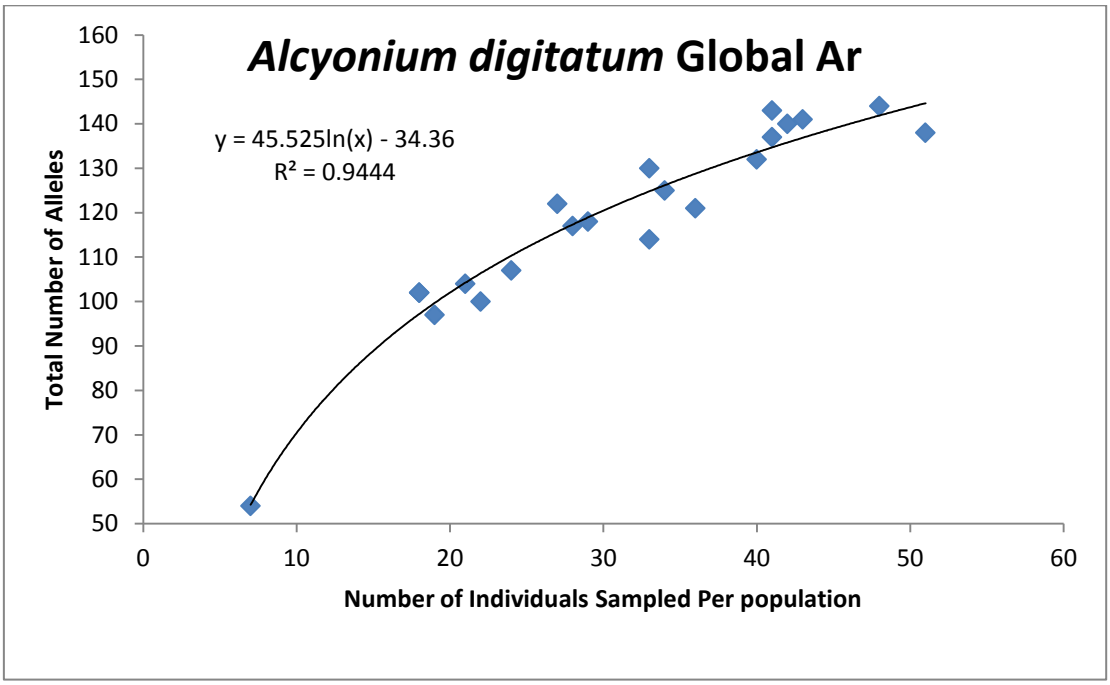




	DMF ALL DATA ALL LOCI IAM			DMF ALL LOCI ALL DATA SMM		
Locus	**Het**	**Fst**	**P-val**	**Het**	**Fst**	**P-val**
Adig001/85D10	0.509	0.014	0.716	0.509	0.014	0.693
Adig002/86B02	0.712	0.005	0.199	0.712	0.005	0.227
Adig003/87B11	0.536	0.017	0.835	0.536	0.017	0.843
Adig004/87E02	0.897	0.028	1.000	0.897	0.028	1.000
Adig005/87H01	0.917	0.005	0.021	0.917	0.005	0.039
Adig006/87H03	0.308	0.030	0.978	0.308	0.030	0.977
Adig007/88A04	0.070	0.002	0.236	0.070	0.002	0.249
Adig008/88C09	0.950	0.000	0.000	0.950	0.000	0.000
Adig009/88E08	0.901	0.015	0.934	0.901	0.015	0.924
Adig010/88E09	0.957	0.002	0.000	0.957	0.002	0.000
Adig011/89C09	0.780	-0.003	0.000	0.780	-0.003	0.000

Appendix 5. Number of alleles observed across each data set compared to number of individuals sampled.





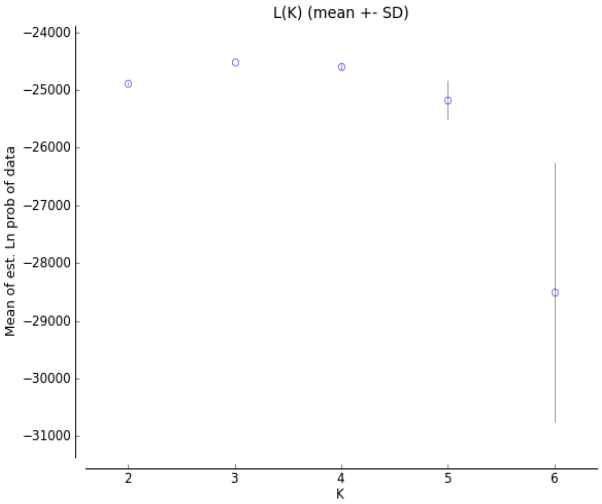
Appendix 6. Allelic richness (Ar) values, all populations and all loci. Top*: Eunicella verrucosa*, bottom: *Alcyonium digitatum*

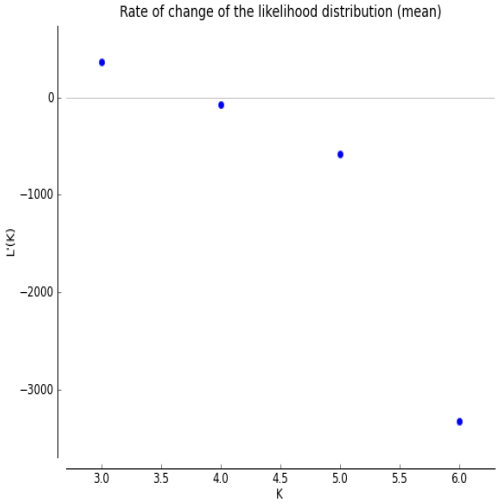
	01C02. Ever001	Exe15. Ever002	Exe21. Ever003	Exe33. Ever004	Exe41. Ever005	Exe49. Ever006	Exe10. Ever007	Exe48. Ever008	06E10. Ever009	Exe17. Ever010	Exe34. Ever011	Exe47. Ever012	Exe50. Ever013	Exe24Mplex11. Ever014	Average
Total	**3.542**	**2.220**	**1.978**	**4.906**	**1.078**	**5.857**	**4.153**	**1.998**	**5.167**	**1.959**	**2.303**	**1.245**	**5.309**	**3.928**	**3.260**
EvMAI	3.13	1.99	1.00	3.79	1.00	4.39	2.70	2.00	2.76	2.34	2.80	1.00	2.26	3.88	**2.50**
EvARM	2.48	1.76	1.52	4.37	1.00	6.29	4.55	2.00	5.98	1.86	1.98	1.62	4.30	4.48	**3.16**
Faro1	2.96	1.85	1.49	5.22	1.00	5.72	3.87	2.00	5.66	1.96	2.39	1.46	3.78	4.40	**3.13**
Faro3	3.27	1.86	2.10	5.61	1.24	6.31	4.85	2.00	5.68	2.09	2.28	1.28	3.36	3.74	**3.26**
Faro2	2.62	1.56	1.93	4.50	1.00	6.20	4.10	2.00	5.40	1.76	2.45	1.17	4.10	4.07	**3.06**
Faro4	3.35	1.96	1.89	5.21	1.15	6.23	4.06	2.00	5.05	2.22	2.16	1.84	3.70	4.12	**3.21**
Faro5	2.59	1.63	2.46	4.67	1.00	6.19	3.84	1.99	5.89	1.98	2.34	1.00	4.42	4.46	**3.17**
Brest3	2.78	2.02	2.20	4.67	1.00	5.49	4.74	2.00	5.14	2.32	2.26	1.00	4.70	3.84	**3.15**
LTGlen	2.86	2.17	1.62	5.42	1.00	4.95	4.62	2.00	3.61	2.81	2.19	1.28	3.86	3.81	**3.01**
MenGlen	3.06	2.16	1.93	4.69	1.00	5.47	4.54	2.00	3.59	2.39	1.95	1.00	4.71	3.38	**2.99**
Ros2	3.17	2.59	2.13	4.98	1.00	5.94	4.69	2.00	5.33	1.98	2.18	1.31	4.90	3.34	**3.25**
Ros1	3.34	2.19	2.09	5.10	1.00	5.67	3.61	2.00	4.92	1.35	2.12	1.17	5.17	3.48	**3.09**
Ire_Blac	3.89	1.37	2.35	5.33	1.00	5.77	3.95	2.00	3.60	1.24	2.44	1.00	1.80	3.23	**2.78**
Ire_Thum	3.90	1.67	1.97	4.98	1.00	5.82	4.61	2.00	4.11	1.43	1.70	1.88	1.00	4.74	**2.92**
DevBF	3.55	2.27	1.93	4.98	1.36	5.38	3.51	2.00	5.52	1.64	2.42	1.00	4.67	2.96	**3.09**
PlyMew	3.49	2.58	2.16	4.50	1.22	5.46	3.22	2.00	5.09	1.83	2.62	1.43	5.44	3.79	**3.20**
HandsDee	4.17	2.84	2.07	5.07	1.14	5.27	4.33	2.00	5.25	1.78	2.28	1.43	5.56	3.64	**3.35**
IoSHath	3.34	2.59	2.64	4.99	1.00	5.69	3.63	2.00	4.42	1.42	2.28	1.62	n.d.	3.67	**2.81**
IoSLR	4.03	2.57	1.86	4.49	1.00	5.16	3.78	2.00	3.84	2.18	2.12	1.00	6.08	3.30	**3.10**
IoSnnw	3.26	1.72	2.21	4.31	1.22	5.48	4.07	2.00	4.75	1.83	1.90	1.00	3.94	3.73	**2.96**
JTEten	n.d.	2.00	2.00	5.00	1.00	5.00	5.00	2.00	5.00	1.00	3.00	1.00	4.00	3.00	**2.79**
Lundy	4.55	2.50	1.32	4.63	1.00	5.43	3.65	2.00	5.63	2.19	2.34	1.00	4.42	3.79	**3.17**
LymeHW	3.63	1.90	2.56	5.87	1.00	5.87	5.73	2.00	5.85	2.56	2.00	1.00	5.95	2.78	**3.48**
ManMo	3.00	2.54	1.90	4.74	1.17	5.44	4.23	2.00	5.13	1.94	2.50	1.00	3.66	3.83	**3.08**
ManRR	3.88	2.86	1.90	4.44	1.00	5.40	3.83	2.00	5.97	1.78	2.45	1.38	4.98	3.93	**3.27**
ManV	4.27	2.37	1.76	4.88	1.00	5.09	4.04	2.00	5.49	1.61	2.58	1.25	3.07	3.60	**3.07**
Sawtooth	3.86	1.76	1.84	4.26	1.42	5.23	3.52	2.00	4.78	2.17	1.58	1.00	3.75	4.00	**2.94**
Skomer	3.58	2.25	1.64	4.17	1.00	5.51	4.21	2.00	5.41	2.54	2.15	1.00	5.41	3.44	**3.16**
WestTen	4.43	2.45	2.18	5.25	1.31	5.55	4.25	2.00	4.94	2.18	2.27	1.00	5.05	3.69	**3.32**
nrPad	4.15	2.00	2.00	5.00	1.00	5.69	3.00	2.00	3.00	1.00	2.00	1.00	6.00	4.00	**2.99**

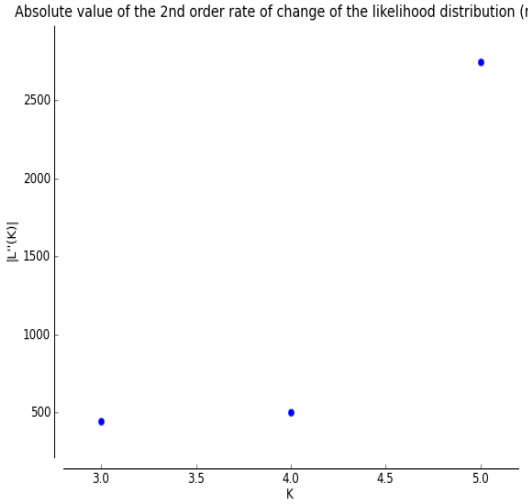
Locus	89C09. Adig011	88A04. Adig007	85D10. Adig001	88C09. Adig008	87E02. Adig004	87H01. Adig005	87H03. Adig006	86B02. Adig002	88E09. Adig010	87B11. Adig003	88E08. Adig009	Average
Total	**6.17**	**1.47**	**4.02**	**10.35**	**7.65**	**8.87**	**2.49**	**5.15**	**10.77**	**3.23**	**4.74**	**6**
Bre2	6.14	2.00	4.73	10.45	7.75	9.06	1.91	4.52	11.27	3.02	4.76	**5.96**
LTGlen	6.56	1.67	3.17	10.67	7.20	8.25	1.71	4.78	9.99	2.72	4.47	**5.56**
Mglen	5.54	1.76	4.78	9.93	7.48	9.09	2.54	4.96	11.17	3.19	4.54	**5.91**
Ros1	6.38	1.48	4.34	10.37	7.28	8.65	1.90	5.17	10.92	3.96	4.88	**5.94**
Ros2	6.33	1.48	4.47	10.36	7.94	9.01	2.07	6.13	10.56	3.58	4.83	**6.07**
IreIT	6.48	1.42	3.98	10.16	7.65	8.78	2.37	4.97	10.59	3.20	4.54	**5.83**
IreTR	6.71	1.00	4.52	10.40	6.72	8.08	3.24	5.09	10.73	4.36	4.37	**5.93**
Dgal	6.00	1.00	3.00	10.00	4.00	8.00	3.00	3.00	10.00	3.00	3.00	**4.91**
DorBA	5.31	1.29	3.84	10.10	7.23	8.97	2.42	4.50	10.14	3.19	4.70	**5.61**
Frog	6.62	1.39	3.79	11.01	7.30	8.69	2.17	5.77	10.85	1.99	4.74	**5.85**
HC	5.99	1.61	3.17	10.36	5.49	9.20	2.31	4.90	10.57	2.49	4.93	**5.55**
Tren	5.89	1.51	3.55	10.56	6.55	8.87	2.13	5.18	10.99	3.84	4.77	**5.80**
Stone	5.95	1.35	3.93	10.57	7.07	8.98	2.26	5.27	10.94	2.67	4.53	**5.77**
ManCD	6.76	1.22	3.63	9.98	6.25	8.79	2.08	5.56	10.74	3.17	4.79	**5.73**
ManV2	6.57	1.50	4.45	10.67	6.53	8.80	2.73	5.03	10.73	2.83	4.70	**5.87**
Lucy	5.91	1.32	3.72	10.23	6.99	8.46	1.58	5.30	10.28	2.28	4.60	**5.52**
PR	6.58	1.26	3.67	10.32	7.71	8.75	3.09	5.08	10.78	2.42	4.53	**5.84**
TR	6.18	1.33	4.52	9.80	6.72	8.89	2.71	4.84	9.63	3.17	4.91	**5.70**
UB74	5.71	2.13	2.88	9.62	5.98	8.36	2.77	5.92	10.81	2.81	4.78	**5.61**
CefMiX	5.07	1.86	2.42	10.52	8.48	9.57	2.70	5.49	10.49	2.97	4.65	**5.84**
CefT342	7.17	1.00	4.28	9.31	8.19	9.51	3.11	4.63	10.13	3.26	4.67	**5.93**

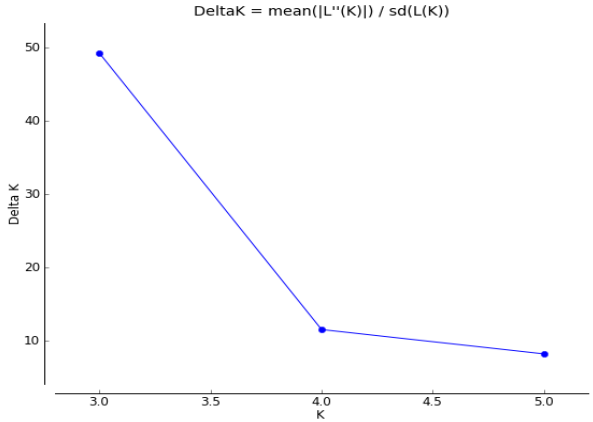
Appendix 7: Evanno Correction results from STRUCTURE HARVESTER (Evanno *et al.* 2005, Earl 2009).

1) *Eunicella verrucosa* all data (N=955, 30 populations) – 10,000 burnin, 10^6 MCMC, LOCPRIOR, correlated alleles and Admixture Model



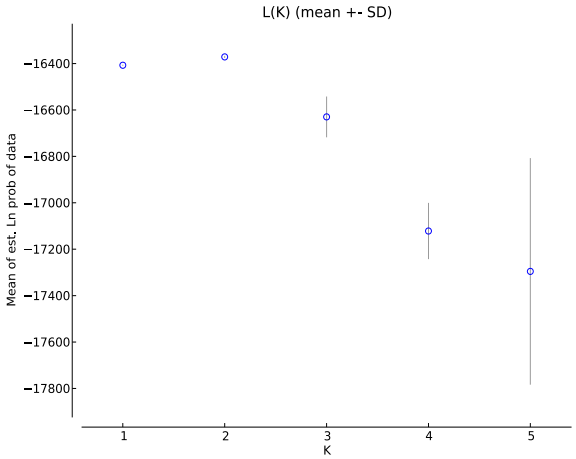


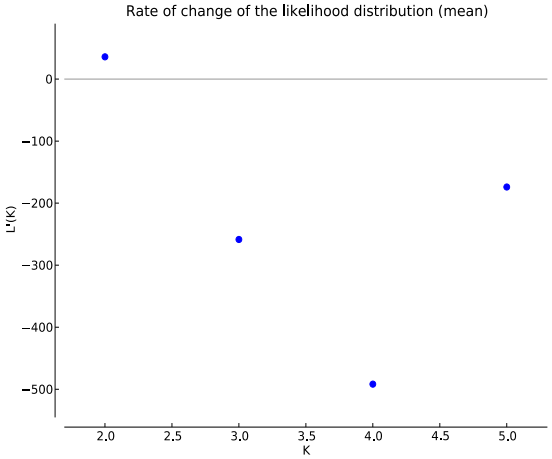


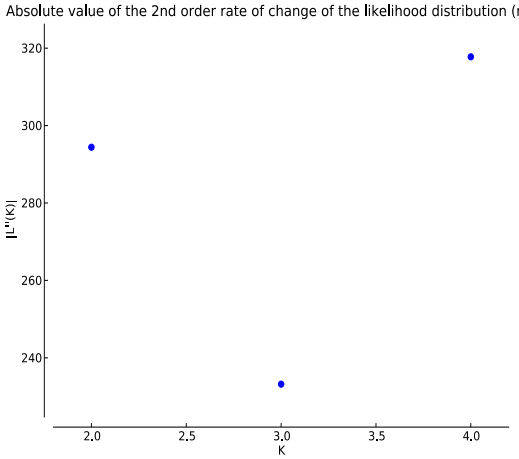


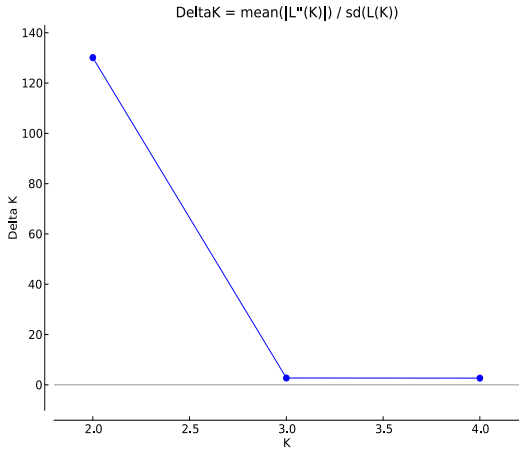
K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
2	2	-24877.650000	20.152543	—	—	—
3	2	-24514.550000	8.980256	363.100000	442.400000	49.263628
4	2	-24593.850000	43.345646	-79.300000	500.750000	11.552487
5	2	-25173.900000	333.188715	-580.050000	2745.250000	8.239325
6	2	-28499.200000	2241.952760	-3325.300000	—	—

2) *Eunicella verrucosa* England & Brittany (N=633, 20 populations) – 10,000 burnin, 10^6 MCMC, LOCPRIOR, correlated alleles and Admixture Model



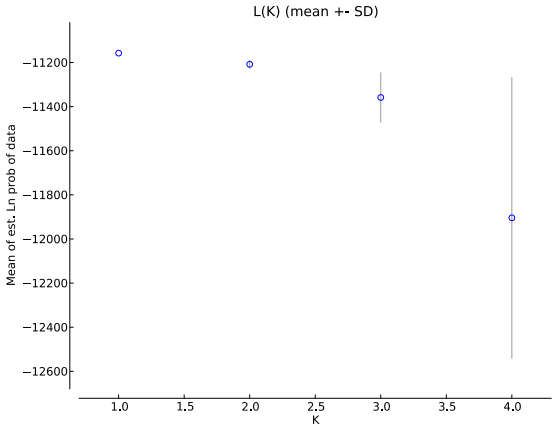


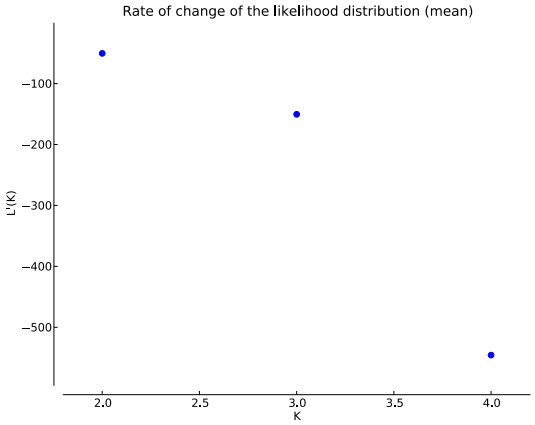


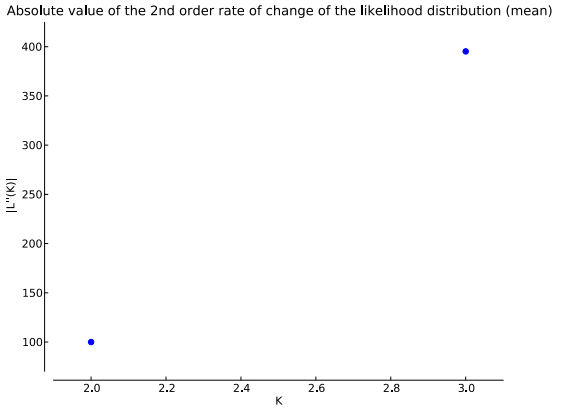


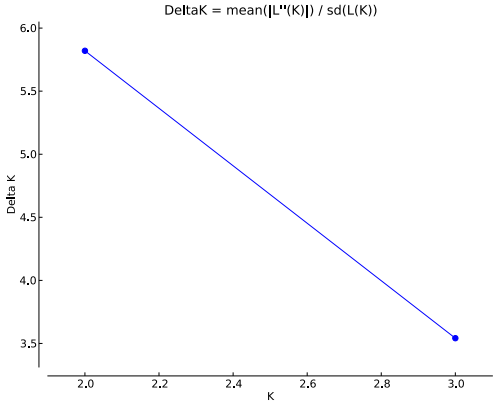
K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	2	-16407.150000	0.070711	—	—	—
2	2	-16371.300000	2.262742	35.850000	294.400000	130.107648
3	2	-16629.850000	85.772053	-258.550000	233.200000	2.718834
4	2	-17121.600000	118.935361	-491.750000	317.750000	2.671619
5	2	-17295.600000	485.923780	-174.000000	—	—

3) *Eunicella verrucosa* England only (N=431, 16 populations) – 10,000 burnin, 10^6 MCMC, LOCPRIOR, correlated alleles and Admixture Model



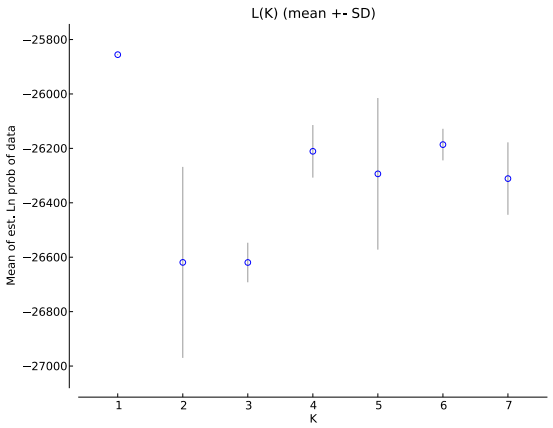


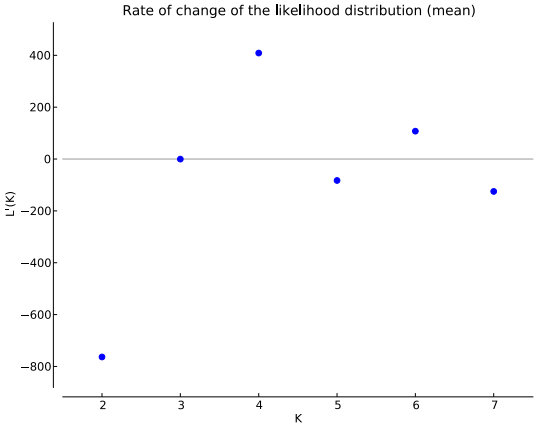


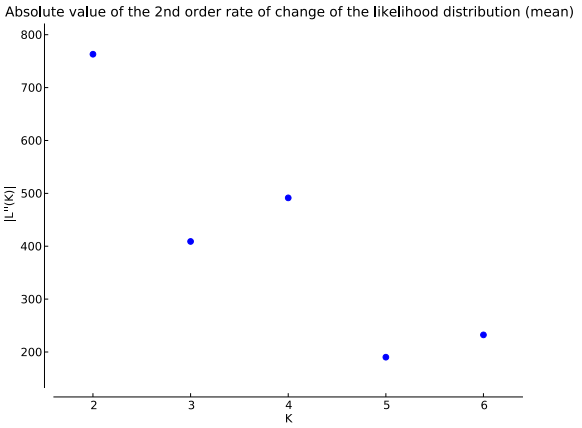


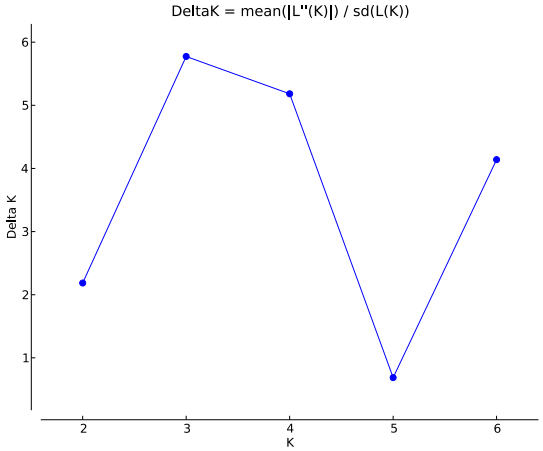
K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	2	-11157.500000	0.000000	—	—	—
2	2	-11207.950000	17.182695	-50.450000	100.000000	5.819809
3	2	-11358.400000	111.581450	-150.450000	395.200000	3.541807
4	2	-11904.050000	636.466814	-545.650000	—	—

4) *Alcyonium digitatum* all populations (N=431, 16 populations) – 10,000 burnin, 10^6 MCMC, LOCPRIOR, correlated alleles and Admixture Model









K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln'(K)	|Ln''(K)|	Delta K
1	2	-25855.550000	0.212132	—	—	—
2	2	-26619.000000	349.169329	-763.450000	763.050000	2.185329
3	2	-26619.400000	70.852099	-0.400000	409.050000	5.773294
4	2	-26210.750000	94.823019	408.650000	491.450000	5.182813
5	2	-26293.550000	276.832305	-82.800000	190.250000	0.687239
6	2	-26186.100000	56.144278	107.450000	232.400000	4.139335
7	2	-26311.050000	131.309729	-124.950000	—	—

Appendix 8. Top= *Eunicella verrucosa* ascending K values as calculated in STRUCTURE (K=2 – K=5). Bottom= *Eunicella verrucosa* K=3 without LOCPRIOR (Note: Population numbers are unlabelled and unordered in bottom image, for comparative purpose to images in thesis, green clusters correspond to Portugal and the blue to Ireland).