A Phylogenetic Study of Vulnerable Batoid Species from the North 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.

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Abstract

Successful resolution of the nomenclature and taxonomy of batoid fish complicated by the high degree of morphological and ecological conservatism in this group. However, both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) phylogenies have been utilised to resolve batoid phylogenies and even to identify cryptic species. As a result, the number of ray species described in recent decades has dramatically increased- although questions still remain regarding the taxonomic status of many batoid species. In chapter one of this thesis, the importance of taxonomy in skate conservation and management is reviewed. In chapter two, control region (CR) and cytochrome oxidase I (COI) sequencing of the blue skate (*Dipturus batis)* and the flapper skate (*Dipturus intermedius)* from across the Northeast Atlantic was performed, in order to clarify their geographical distribution. Although now formally recognised as distinct species, before 2010 these two taxa were classified together as the critically endangered 'common skate', *D. batis*. Although this has important conservation implications, their protection is currently being hindered by a lack of spatiotemporal data. In the present study, the blue skate generally appears to be more common than the flapper skate*,* with a distribution extending from Rockall and Iceland to the Western Approaches and the Celtic Sea. Whilst the flapper skate appears most frequent around northern Scotland, the North Sea and Ireland, novel data also suggests that it may have once had a much wider distribution. For the first time, this species was identified in the Azores, where unique haplotypes were also isolated, potentially highlighting the genetic distinctiveness of the population in this region. In chapter three, nextRAD and mtDNA (concatenated CR and COI) sequencing were utilised to explore the phylogenetics of several vulnerable species of European skate. Whilst the current designation of the Madeiran skate (*Raja maderensis*) and the thornback ray (*Raja clavata*) as distinct species wasn't fully supported, genetically distinct populations were identified in the Azores and surrounding seamounts. The presence of a cryptic *Dipturus* species in the Azores wasn't supported, as suggested by previous work on the longnosed skate (*Dipturus oxyrinchus*)*.* However, Azorean longnosed skate and flapper skate were distinct from their geographically proximate counterparts, and may represent distinct populations. The uniqueness of

the Azores highlights the importance of seamounts as ‘hotspots’ of biodiversity, which has important implications for marine protected areas that include these batoid species as a protected feature. In addition to resolving these phylogenies, this thesis also offered an opportunity to comment on the utility of mtDNA and nextRAD sequencing for batoid phylogenetics, the latter of which has never been applied to skates and rays before.

Chapter 1: General Introduction

1.1 Elasmobranchs: what are they and why are they at risk?

Elasmobranchs are one of the two subclasses of cartilaginous fish in the class Chondrichthyes and include the sharks, skates and rays. They are one of the oldest and most successful lineages of vertebrates; arising some 420 million years ago they survived four mass extinctions, rapidly diverging and monopolizing high trophic levels in most aquatic ecosystems (Compagno, 1990a,b; Kriwet, Witzmann, Klug, & Heidtke, 2008). Worldwide there are at least 1,118 extant species of sharks, skates and rays found from intertidal continental shelf waters to the pelagic ocean and the deep sea, with species being found up to depths of 4,000m (Weigmann, 2016; Dulvy & Reynolds, 2002; Priede *et al.,* 2006). A variety of species have also penetrated freshwater and estuarine habitats and some even exhibit euryhaline lifecycles (Nelson, 2006; Ebert and Winton, 2010; Heupel, & Simpfendorfer, 2011; Moore, 2018). Within these ecosystems, elasmobranchs are thought to play an integral role in regulating community structure and function, particularly in environments where they occupy apex predator roles (Coretes, 1999; Strong, 1991; Heithaus and Dill, 2002, Dill *et al.,* 2003, Heithaus *et al.,* 2008; Heithaus, Wirsing, & Dill, 2012; Heupel *et al.,* 2014).

Despite their ubiquity and ecological importance, around 25% of elasmobranchs are now threatened with extinction (Dulvy *et al.,* 2014). Of all the elasmobranchs, the batoids (skates and rays) are at highest risk of extinction. In a recent systematic analysis of the threats facing 1041 chondrichthyans fish species, five out of seven of the most threatened families were batoids, with 19.9% currently classified as threatened with extinction (Dulvy *et al.,* 2014). This is, in part, due to their life history characteristics; typical of predatory vertebrates, they can be characterised by a large size, slow growth rates, late maturity, and low fecundity. Skates and rays also exhibit some of the highest levels of maternal investment and longest gestation periods of the vertebrates (Cortes,

2000; Dulvy *et al.,* 2014). Such life-history characteristics result in slow rates of population increase, making this group intrinsically at high risk of over-exploitation by fisheries and hindering the recovery of collapsed stocks (Pratt & Casey, 1990). Indeed, many populations have even suffered local extirpations and significant range contractions in recent decades (Brander, 1981; Casey & Meyers, 1998; Dulvy, Metcalfe, Glanville, Pawson, & Reynolds, 2000). The winter skate (*Leucoraja ocellata*), for example, has declined by 90% on the eastern Scotian Shelf, primarily due to being caught as bycatch in demersal fisheries (Kulka *et al.,* 2009). Across other parts of its Canadian range, this species has declined by 98%, and is now considered endangered by the IUCN red list (Kulka *et al.,* 2009).

Batoids are caught for their fins, liver oil, meat, gill rakers and skin, and are an important protein source for many coastal communities (Bonfil, 1994). Industrial elasmobranch fisheries exist in several countries, including the UK and Europe, with an estimated 780 000 t caught in 2007 (Bonfil 2002; Lack & Saint, 2009). A significant number of landings also originate from bycatch or small-scale sustenance fisheries (Bonfil 2002; Lack & Saint, 2009). Perhaps the most significant trend in recent decades has been the use of batoids in the 'shark fin' trade, which uses dried fins in shark fin soup, a delicacy in Asian markets. This industry has expanded rapidly in recent decades and conservative estimates now put the global value of 'shark fin' imports at around $377.9 million per annum, with an average annual volume imported of 16,815 tonnes (FAO 2015). Additionally, dried gill rakes, particularly from manta and devil rays, have recently become a valued commodity in Chinese and South-east Asian markets. Whilst figures on this trade are difficult to estimate, sales of endangered and protected species have recently been detected (Zeng *et al.,* 2016; O'Malley *et al.,* 2017).

The conservation of batoids has been hindered by several factors: (1) fisheries statistics are not accurately maintained or are virtually non-existent for certain species or regions (Bonfil, 1994). Rarely are landings reported to the species-level; the skate fishery in the North-eastern United States, for example, is managed as a complex stock of seven species (Curtis & Sosebee, 2015). Landings have not been reliably reported by species, hindering stock assessments and effective species-level

management (Curtis & Sosebee, 2015). This lack of accurate fisheries data results in an inability to monitor the true scale of decline in exploited populations and developing effective management plans therefore remains a difficult task; (2) Due to their life-history traits, traditional teleost conservation models do not always apply to elasmobranchs, and such limitations are poorly understood (Bonfil, 1994; Camhl *et al.,* 1998); (3) Researching elasmobranchs has associated complexities. Skates, rays and sharks are often highly migratory species, moving across geo-political boundaries (Dulvy *et al.,* 2017). This not only makes managing large-scale fisheries complex, but sampling can become time-consuming and arduous; (4) The high levels of morphological and ecological conservatism among extant orders results in taxonomic confusion and unstable nomenclature (Ebert & Compagno, 2007; Jones *et al.,* 2017).

1.2 Taxonomic confusion

Tackling taxonomic confusion and unstable nomenclature is perhaps the most challenging aspect of elasmobranch conservation (Ebert & Compagno, 2007; Jones *et al.,* 2017).The Chondrichthyes' cartilaginous skeleton separates them from the other major class of extant fishes, the Osteichthyes (comprising around 95% of extant fish fauna), which have skeletons made of bone. Whilst the monophyly of modern elasmobranchs is now generally accepted, it remained heavily debated for some time, with several competing theories at play (reviewed by Maisey, 1984a). Today, however, the elasmobranchs encompass all extant rays, skates and sharks and a select few extinct modern forms such as *Palaeospinax* and *Synechodus* (Schaeffer and Williams 1977; Compagno 1977; Schaeffer 1981; Maisey 1982; 1984a, 1984b; Thies 1983).

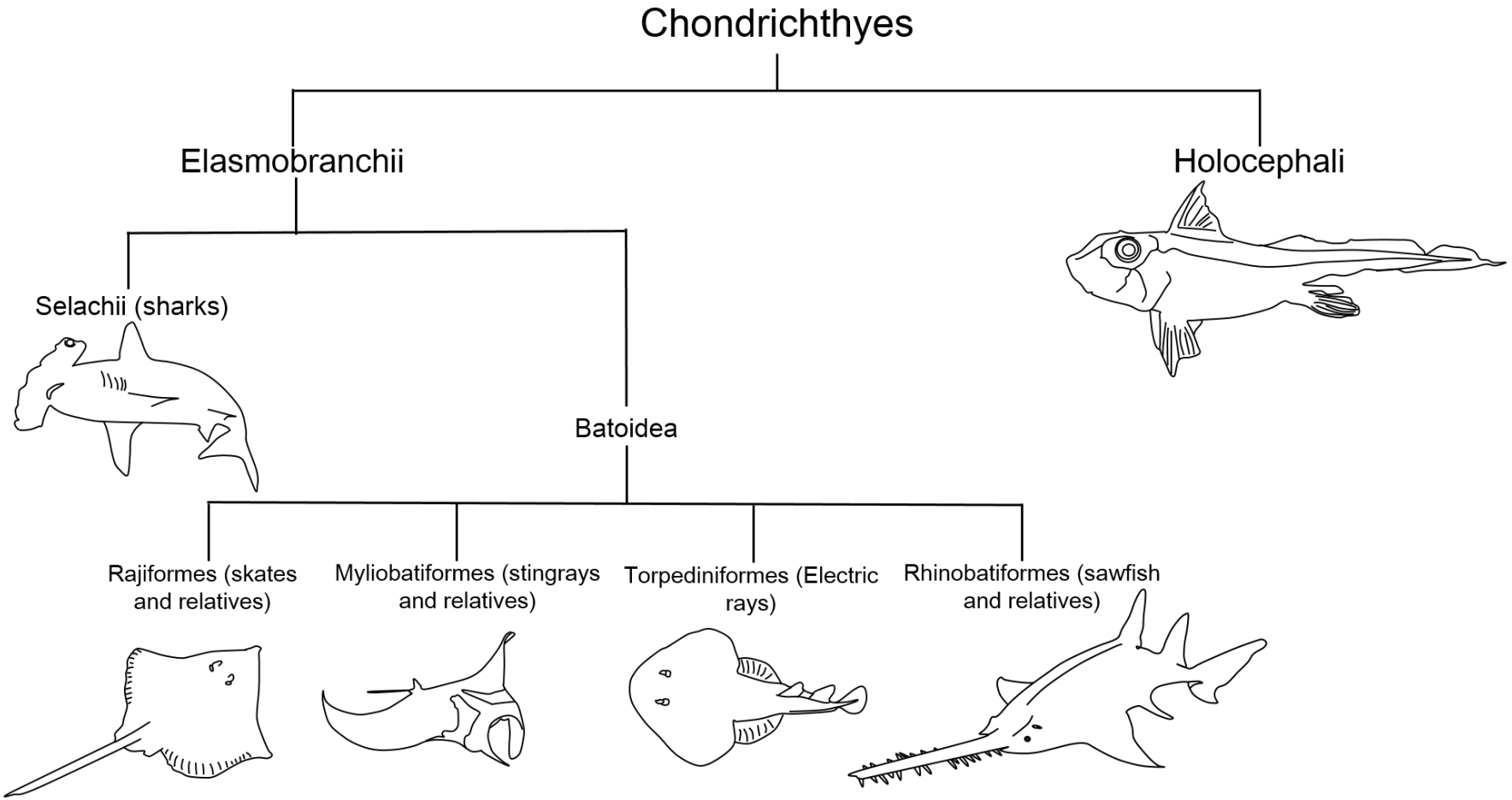

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Figure 1. A simplified diagram of the taxonomic structure of Chondrichthyans and the Batoidea.

Within the elasmobranchs are the superorders Selachii (sharks) and the Batoidea. The Rajiformes (skates), Myliobatiformes (stingrays), Torpendiniformes (electric rays) and Rhinobatiformes (sawfish) comprise the batoids (Nelson *et al.,* 2016; Figure one). The relationships between these orders and superorders have undergone major revisions in recent decades- perhaps one of the most debated theories concerns the higher systematics of batoid fishes (Douady *et al.,* 2003). Whilst early morphological studies supported the separation of the Batoidea and sharks (Bigelow and Schroeder, 1948; Bigelow and Schroeder, 1953; Seret, 1986), based on cladistic analysis of several putative synapomorphies by Shirai (1992a) and endorsement by Carvalho (1996), batoids were classified as derived sharks, grouped with the Pristiophoriforms (saw sharks) and Squatiniformes (angel sharks) in an arrangement was known as the Hypnosqualea hypothesis (Compagno, 1977; de Carvalho, 1996; Shirai 1992b; Shirai 1996). However, recent molecular studies have not supported this interpretation; evidence suggests that the morphological traits used to characterise the Hypnosqualea superorder are either symplesiomorphic (an ancestral trait shared by two or more taxa) or derived from convergent evolution in the batoid, sawshark and angelshark ancestor (Douady *et al.,* 2003). Instead, the reciprocal monophyly of the sharks and batoids has been resurrected, with important implications for the current understanding of elasmobranch life-history and morphological trait evolution (Winchell *et al.,* 2004; Naylor *et al.,* 2012; Amaral *et al.,* 2017; Vélez-Zuazo *et al.,* 2011; Douady *et al.,* 2003; kitamura *et al.,* 1996; Dunn & Morrissey, 1995; Stock, 1992).

1.2.1 Batoid taxonomy and conservation

The uncertainty surrounding elasmobranch higher systematics can primarily be attributed to the morphological characters used to historically assess phylogenetic structure. Anatomical traits can often arise through convergent evolution in elasmobranchs, that often exhibit similar ecological roles even among distantly related taxa (e.g. benthic predators). This, coupled with huge amounts of morphological conservatism (for example, Figure 2), results in largely homoplasious character

distributions (traits shared by taxa but not present in their common ancestor) and ambiguous phylogenetic signal emanating from morphological data (de Carvalho 1996). These issues are particularly true for batoid fish, which represent the most challenging taxonomic problem within the elasmobranchs as they comprise around 633 species, making this group more speciose than all the shark groups combined (Last *et al.,* 2016; Rocco *et al.,* 2007). In addition, many species exhibit ontogenetic variation in their morphology, further complicating field identification (Last *et al.,* 2008a; Manjaji-Matsumoto & Last, 2008; Last *et al.,* 2008b; Last *et al.,* 2008c; Last *et al.,* 2016; Kyne 2016). For example, the giant freshwater whip-ray, *Urogymnus dalyensis,* displays ontogenetic gradients in squamation (scales) and colour pattern and is associated with unstable nomenclature (Manjaji-Matsumoto & Last, 2008).

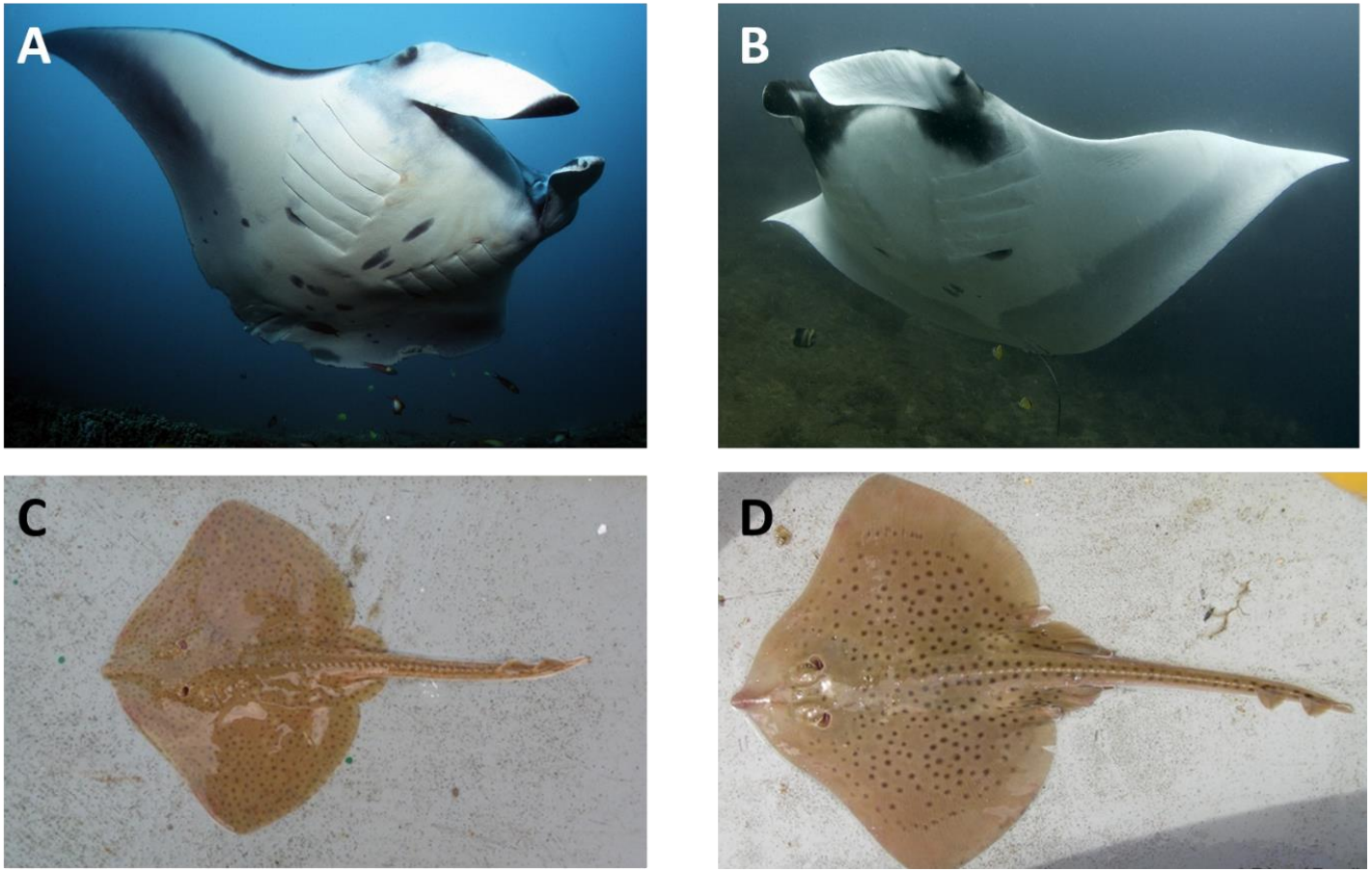


Figure 2. Two examples of morphological conservatism in batoid species. The reef manta, *Manta alfredi* (A; Marshall *et al.,* 2018a) and the oceanic manta, *Manta birostris* (B; Marshall *et al.,* 2018a) exhibit striking levels of morphological conservatism, despite being distinct species. The same is true for the blonde ray, *Raja brachyura* (C; Ellis *et al.,* 2009) and the spotted ray, *Raja montagui* (D; Ellis *et al.,* 2007).

As a result of this taxonomic uncertainty, many relationships within the Batoidea still remain largely unresolved. In a bid to tackle this issue, taxonomists are increasingly utilising molecular characters for phylogenetic inference, which has revealed many novel species within the Batoidea that morphological studies have failed to detect (Whitey, 1939 White, 1930; Beer, 1931; McEachran and Fechhelm, 1982; McEachran and Dunn, 1998; Sandoval-Castillo *et al.,* 2004; Toffoli *et al.,* 2008; Griffiths *et al.,* 2010; Iglésias *et al.,* 2010; Sandoval-Castillo *et al.,* 2011; White & Last, 2012; Weigmann, 2016). As a result, the number of ray species described in recent decades has dramatically increased, even accounting for the converging of several species into single taxa (White & Last, 2012; Figure three).

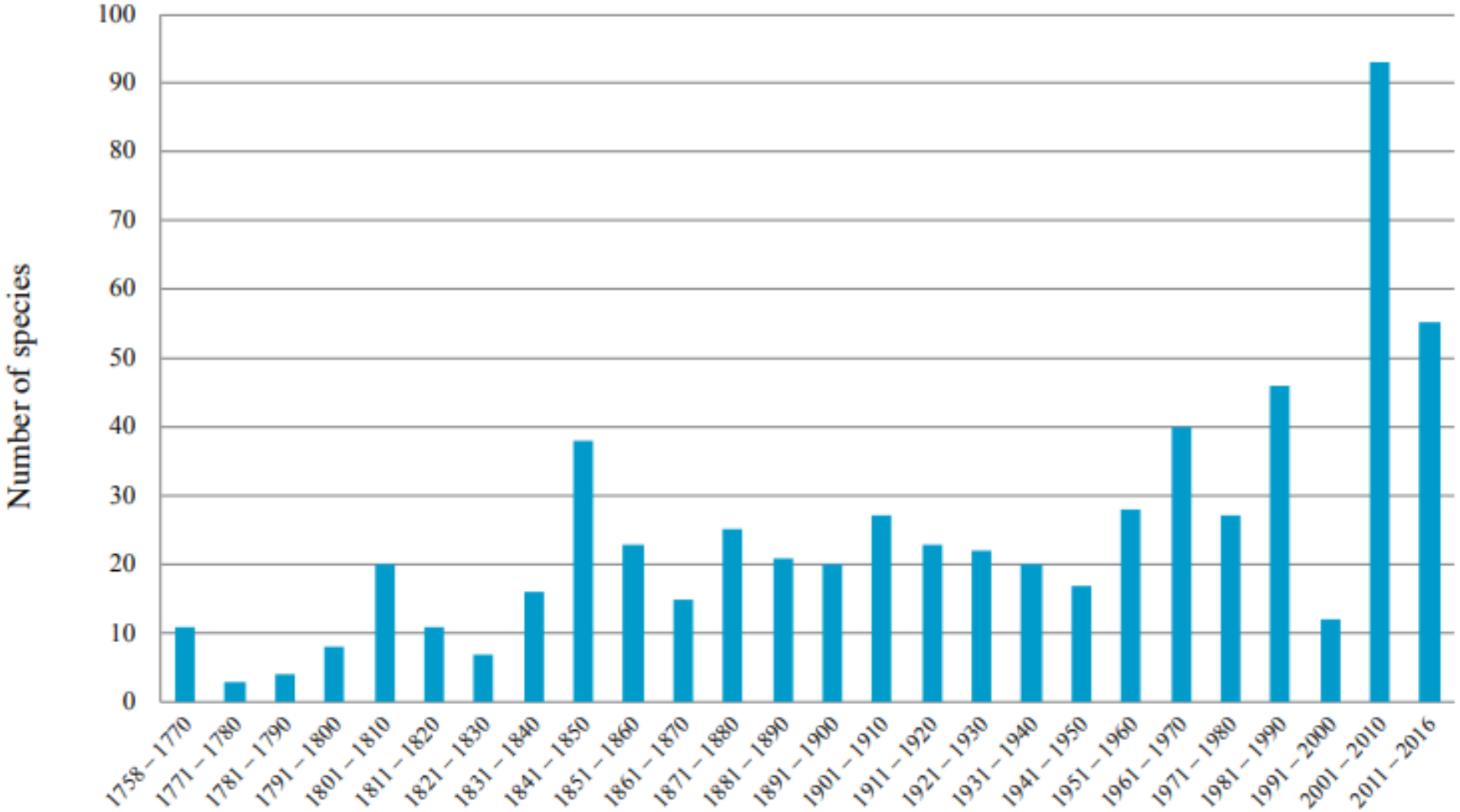


Figure 3. Number of ray species described each decade from Linnaeus in 1758 until 2016 (Last *et al.,* 2016).

A significant phenomenon is the discovery of cryptic species (morphologically indistinguishable species that are genetically distinct), which has important conservation implications, particularly for vulnerable batoids. For example, the spotted eagle ray (*Aetobatus narinari*), a reef-associated and mainly coastal elasmobranch occurring throughout the tropics, is currently designated a single, vulnerable species throughout its range (Compagno and Last 1999). However, geographic differences in its morphology and parasite evolution suggest the presence of cryptic speciation (Compagno and Last 1999; Compagno *et al.,* 2005; Marie and Justine 2005; Kyne *et al.,* 2006). Recent genetic evidence supports this conclusion; one species is thought to have a range extending through the Western and Central Pacific and the other throughout the Central Atlantic and the Eastern Pacific (Richards *et al.,* 2009). As a single species, *A. narinari* was thought to be circumglobal, but these reduced ranges and population sizes for each of the newly described species increase concerns about the already threatened and vulnerable status of these batoid fish, and conservation efforts should be targeted accordingly (Richards *et al.,* 2009).

1.3 Genetic markers in elasmobranch phylogenomics

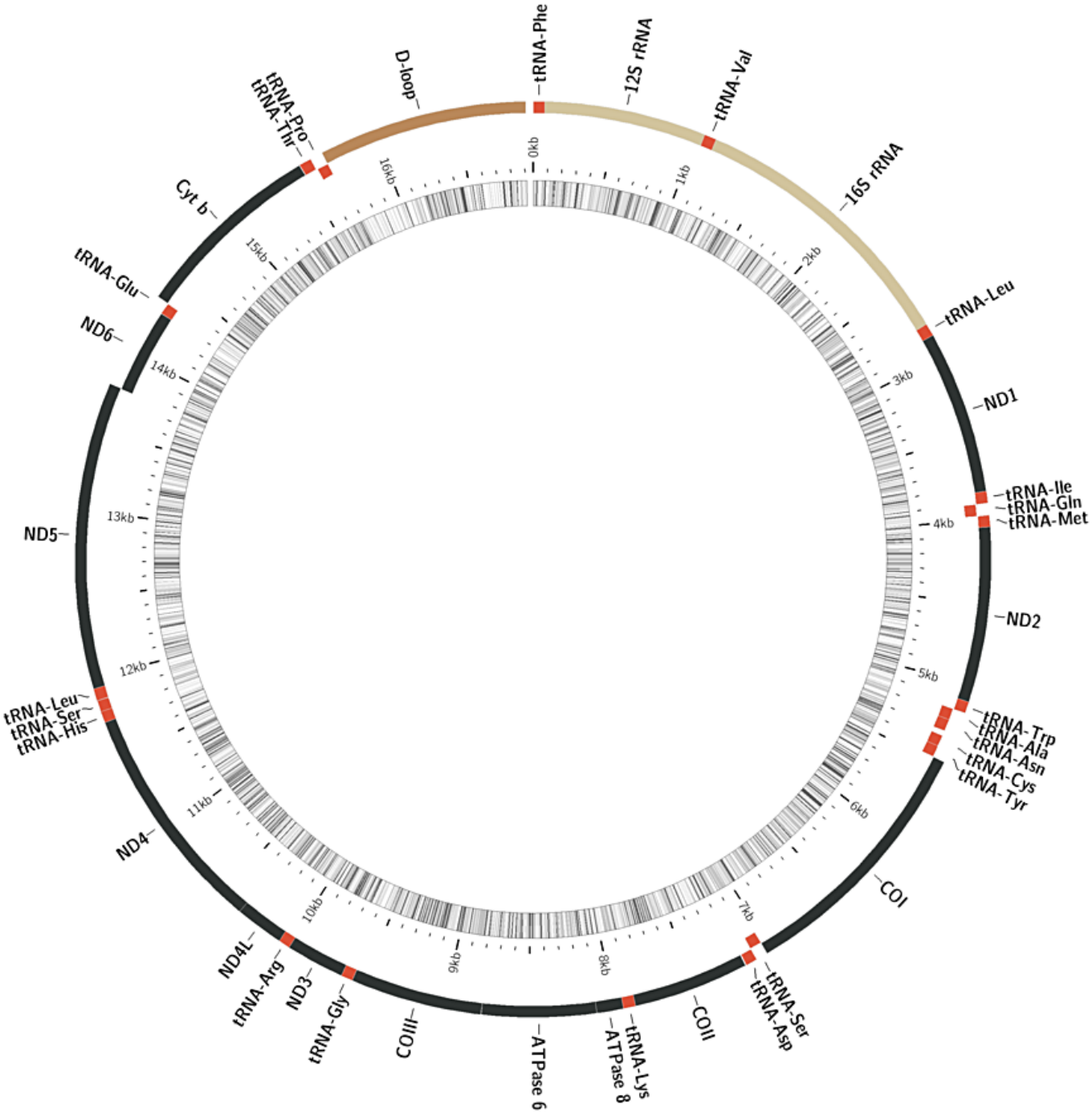
Taxonomists have employed various molecular tools to resolve batoid taxonomy. Broadly, these genetic methods can be categorised into two groups:

- Mitochondrial DNA (mtDNA)
- Nuclear DNA (nDNA)

1.3.1 Mitochondrial DNA

The elasmobranch mitogenome is a circular section of DNA approximately 17kb in length, encoding 37 relatively conserved genes including 13 protein-coding genes, 22 transfer RNA (tRNA), 2 ribosomal RNA (rRNA), a large non-coding control region (also contains the D-loop, the site of mtDNA replication), and an A + T-rich region (example Figure 4; Chai *et al.,* 2016; Chen *et al.,* 2016). Mitochondrial DNA (mtDNA) has been utilised widely to resolve batoid phylogenies; its uniparental, haploid mode of inheritance results in a quarter the effective population size of nuclear genes, resulting in a higher magnitude of genetic differentiation among recently isolated species (Billington, 2003; Birky *et al.,* 1983). Hence, mtDNA provides greater phylogenetic resolution for recently diverged taxa than nDNA, a feature particularly relevant for detecting cryptic speciation in elasmobranchs, which can be characterised by some of the slowest evolving genomes of the vertebrates (Venkatesh, 2014). The earliest mtDNA studies employed whole-molecule analysis of restriction fragment length polymorphisms (RFLPs), lengths of DNA produced by fragmenting whole mitogenomes with restriction enzymes (Heist *et al.,* 1995, 1996a, 1996b). However, as 'universal' PCR primers were developed for mtDNA and the cost of sequencing reduced, direct sequencing of whole mtDNA genes became more common. Early studies date back to Dunn & Morrissey (1995), who showed that a 303 base pair (bp) region of the mitochondrial genome (12s rRNA gene) could be used to distinguish between five recognised elasmobranch taxa (Squalus, Heptranchias, Heterpdontus, Alopias, Urolophus and Hydrolagus; Nelson, 1984). Douady *et al.,* (2003) later used a 2.4 kb segment of mtDNA, predominantly comprising 12s and 16s rRNA genes, to refute the Hypnosqualea hypothesis, with other studies following suit (Winchell *et al.,* 2004; Xiao *et al.,* 2012). One of the largest DNA-barcoding projects on elasmobranchs to date (Cariani *et al.,* 2017) has utilised a single mitochondrial gene for taxonomy, cytochrome c oxidase I (COI), due to its proven effectiveness in distinguishing between species (Herbert *et al.,* 2003; Savolainen *et al.,* 2005; Ball *et al.,* 2016; Velez-Zuazo & Agnarsson, 2011; Moura *et al.,* 2008; Serra-Pereira *et al.,* 2010; Valsecchi *et al.,* 2005; Ward & Holmes, 2007; Wynen *et al.,* 2009; Lim *et al.,* 2015; Pradeep *et al.,* 2018). Indeed, COI-barcoding has

been highly effective in resolving cryptic speciation within the batoids. Borsa *et al.,* (2016), for example, were able to validate the presence of cryptic speciation within the blue-spotted maskray (*Neotrygon kuhlii*) complex through analysis of both mitochondrial COI and cytochrome b genes. Bineesh *et al.,* (2016) utilised COI barcoding to identify two putative new *Dipturus* skate species from the Arabian Sea, along with eight more from other chondrichthyan taxa across the Indian coastline. In the absence of expert taxonomists, COI-barcoding has also been utilised to validate field identifications of elasmobranch taxa (Cerutti-Pereyra *et al.,* 2012).



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157 **Figure 4.** Mitogenome map of the Kwangtung skate, *Dipturus kwangtungensis*. The innermost circle
158 represents GC% per every 5bp of the mitogenome; the darker the line, the higher the GC%. (Jeong
159 *et al.*, 2015)

Despite this proven success, the COI gene's relatively slow rate of evolution has also prompted the use of slightly larger and faster evolving mitochondrial genes for addressing phylogenetic questions within the Batoidea. The mitochondrial control region (CR) is often utilised due to its higher degree of nucleotide polymorphism among elasmobranch species (Valsecchi *et al.,* 2005). Use of the CR has successfully identified species complexes, cryptic speciation, population structure and phylogeographical patterns within the Rajidiae (Griffiths *et al.,* 2010; Serra-Pereira *et al.,* 2010; Li *et al.,* 2014, Ball *et al.,* 2016). In a relatively new addition to the literature, the larger NADH dehydrogenase subunit 2 (NADH2) gene has not only proven to be effective in distinguishing between batoid species (Lim *et al.,* 2015; Henderson *et al.,* 2016), but also in providing reasonable estimates of deeper levels of divergence (Naylor *et al.,* 2012a). Furthermore, the chondrichthyan tree of life project, which is currently the most comprehensive molecular assessment of global elasmobranch taxonomy to date, employs NADH2 for species delineation (Naylor *et al.,* 2012b).

1.3.2 Nuclear DNA

At between 3 to 34 Gbp in size, the nuclear genome of elasmobranchs (with the exception of dipnoans and urodeles) is amongst the largest of the vertebrates and is much larger and more complex than their mitogenomes (Stingo and Rocco, 2001; Heist, 2004). Hence, mtDNA is often used in preference to nuclear DNA (nDNA). However, the use of mtDNA in batoid phylogenetics, and indeed wider taxonomy, has become contentious in recent decades due to incongruence that can occur between individual mitochondrial gene and species phylogenetic trees (Avise, 2004; Avise *et al.,* 1983; Avise and Saunders, 1984). Several studies have argued that phylogenies based solely on mtDNA can be misleading, as mtDNA has been known to obscure species boundaries in taxa (Shaw, 2002; Avise, 1994; Giannasi *et al.,* 2001). One solution to this problem is to include independent markers from the nuclear genome.

At the species level, the use of nDNA is more evident in shark phylogenetics than the relatively under-researched batoids (Abercrombie *et al.,* 2005; Quattro *et al.,* 2013). However, it has proven useful in the resolution of the genus *Manta;* despite their formal separation, evidence suggests that *Manta* is actually nested within the *Mobula* genus (Aschilman, 2014). The earliest evidence supporting this hypothesis came from morphological characters and parasite evolution (Benz and Deets, 1988; Gonzalez-Isais and Dominguez, 2004; Herman *et al.,* 2000), but was later corroborated by analysis of mobulids with the mtDNA genes NADH2 and NADH4, and the nuclear genes RAG1 and SCFD2 (Naylor *et al.,* 2012a; Aschliman *et al.,* 2012). More recently, Poortvliet *et al.,* (2015) used phylogenies of two nuclear genes (RAG1; and Hemoglobin-alpha, HEMO) and full mitogenomes of Mobulidae to support the paraphyly of *Mobula*, with inclusion of *Manta*. These analyses of mtDNA and nDNA together, and not just in isolation, is being used more frequently in phylogenetic studies, due to discordance between nDNA and mtDNA phylogenies (Shaw, 2002). Comparison of nDNA and mtDNA trees provides a useful tool for validating species phylogenies, revealing patterns of hybridization and examining sex-specific dispersal patterns (Shaw, 2002; Roos 2011; Marino 2015).

1.3.3 Nextera-tagmented reductively-amplified DNA (NextRAD)

As next generation sequencing (NGS) technologies become less expensive, sequencing larger segments of the genome for phylogenetic inference has become more feasible. In particular, these advances have allowed scientists to study hundreds of thousands of single nucleotide polymorphisms (SNPs) and their flanking sequences, ideal for high-resolution phylogenetics among closely and distantly related individuals (Levy and Myers, 2016). Crucially, NGS can be applied to the study of non-model organisms, such as batoids, which typically lack many genomic resources (published sequence data, annotated genomes; Levy and Myers, 2016).

One approach is that of ‘Restriction Site Associated DNA sequencing’ (RAD-seq), a reduced-representation technique that isolates a common set of molecular markers and combines two key

principles with NGS: the use of molecular identifiers (MID) to match sequence reads with specific individuals and restriction enzymes to shear DNA into fragments (Davey *et al.,* 2010). Since its publication, various adaptations of traditional RAD protocol have been developed, including ddRAD (Peterson *et al.,* 2012), 2bRAD (Wang *et al.,* 2012; Guo *et al.,* 2014), genotyping-by-sequencing (Elshire *et al.,* 2011), ezRAD (Toonen *et al.,* 2013), and nextRAD (e.g. Russello *et al.,* 2015). Each of these has allowed for refinement of RAD libraries, in alignment with the specific aims of the study in question (Andrews *et al.,* 2016).

Nextera-tagmented reductively-amplified DNA (NextRAD) differs from traditional RAD-seq, and indeed other genotype-by-sequencing methods, in that it doesn't use restriction enzymes to reduce the complexity of the genome. The nextRAD approach instead fragments the DNA with Nextera transposomes, which fragment the DNA and add a short adapter. For a whole genome shotgun library, these fragments are then amplified with primers that also contain a selective primer sequence (8-10 bp) at the 3' end that hybridizes to the short adapter. The primers bind to the adapter sequence, and only those fragments that start with the selective sequence fully bind the primer and are amplified. So just as RAD sequences the genomic DNA next to every restriction enzyme cut site, nextRAD sequences every part of the genome next to a particular 8 bp selective sequence, wherever they occur. SNPs can then be called and filtered bioinformatically just as they are in RAD-seq. The smaller number of steps in the protocol, compared to traditional RAD-seq, reduces loss during library creation, allowing much lower input compared to other methods (Fu *et al.,* 2017; Eric Johnson, personal communication).

NextRAD-seq and RAD-seq have been useful for resolving phylogenies in non-model organisms (e.g. Cruaud *et al.,* 2014, Herrera and Shank, 2015, Leaché *et al.,* 2015), as they allow rapid sequencing of thousands of homologous regions, both with and without an available reference genome (Davey *et al.,* 2010). RAD-seq has been used to construct phylogenies in several marine organisms, including zebrafish (McCluskey & Postlethwait, 2014), swordtail fish (Jones *et al.,* 2013), cichlid fish (Wagner *et al.,* 2013), octocorals (Pante *et al.,* 2015) and the first genome-wide nuclear

marker-based phylogeny of tunas (Díaz-Arce *et al.,* 2016). In a recent study, RAD-seq has also revealed possible cryptic speciation within the eastern Australian sea mullet (Krück *et al.,* 2013). NextRAD has also been a successful tool for phylogenomics; for example, to resolve contemporary measures of population structure and phylogeographic patters in populations of round whitefish (Morgan *et al.,* 2017). Outside of the marine biome, nextRAD loci have been used to construct phylogenies of potato psyllids (Fu *et al.,* 2017), whiteflies (Wosula *et al.,* 2017); neotenic beetles (Bray & Bocak, 2016) and Andean Lupinus (Contreras-Ortiz *et al.,* 2018).

1.4 Aims of this thesis: resolving phylogenetic questions that surround several species of North Atlantic batoids

Using mtDNA and nDNA sequencing, this thesis aims to resolve some of the taxonomic and spatial distribution questions that still surround several species of North Atlantic batoid fish:

1.4.1 Chapter 1

The 'common skate' species complex

The 'common skate' (*Dipturus batis*, L. 1758) was historically found from Iceland and northern Norway, through to Madeira and northern Morocco, including in the Mediterranean Sea and throughout the waters of the British Isles (Dolgov *et al.,* 2005; Ellis *et al.,* 2005). Due to overfishing and being caught as incidental bycatch, this species now appears to be absent from most of its former range, resulting in its listing as critically endangered on the IUCN red list (Dulvy *et al.,* 2006). Recent genetic, morphological and life-history evidence indicates that the 'common skate' actually represents two nominal species: the larger *Dipturus cf. intermedia* and the smaller growing *Dipturus cf. flossada*

(Iglésias *et al.,* 2010; Griffiths *et al.,* 2010). This cryptic speciation has serious implications for the conservation status of the 'common skate', as it is likely that the extinction risk of *D. cf. intermedia* and *D. cf. flossada* is significantly higher than previous estimates that treated *D. batis* as a single homogenous unit (Griffiths *et al.,* 2010). Since 2010, the name *D. intermedius* (flapper skate) has been resurrected for *D. cf. intermedia* and *D. batis* (blue skate) now refers to *D. cf. flossada* (Last *et al.,* 2016). However, the relative distributions of these two species remains unclear, meaning their conservation status cannot be accurately assessed. Chapter one of this thesis aims to resolve the spatial distributions of *D. intermedius* and *D. batis*, utilising CR and COI sequencing to unambiguously distinguish between species.

1.4.2 Chapter 2

The Norwegian skate

The Norwegian skate (*Dipturus nidarosiensis*) is a species of benthic skate found throughout the Eastern North Atlantic: from central and southern Norway, along the slopes off southern Iceland through to western Scotland (including Rockall Trough) and Ireland (Ebert & Stehann, 2013). Its distribution across more southern parts of its range, however, remain uncertain. Although nominal records suggest its occurrence across the shelf edge of the Celtic Sea and the deep slopes of the Bay of Biscay to off North Spain, these records may be misidentifications and are still under investigation (Ebert & Stehann, 2013). Additionally, it is not clear whether records of Norwegian skate in the Mediterranean are distinct from those across the rest of the North Atlantic; morphological evidence suggests that several specimens collected from the Sardinian Channel and off the coast of Portugal could refer to a smaller, undescribed *Dipturus* species (Cannas *et al.,* 2010; Ebert & Stehann, 2013; M. Stehmann, unpubl. data).

The Thornback ray and the Madeiran skate

The thornback ray (*Raja clavata*) is a polytypic species (contains several variant forms) of ray with a wide geographic range, found in Iceland and Norway, the North Sea, the Western Baltic Sea (although sightings in this area are rare), around the British Isles and Ireland, the Mediterranean and through to the coast of West Africa and into the southwestern Indian Ocean (Ebert & Stehann, 2013). Its wide range and polytypic nature mean this species is often misidentified, and it is currently unclear as to whether records of *R. clavata* from Madeira and the Azores actually refer to the endemic Madeiran skate (*Raja maderensis*), a local Azorean form or subspecies (Ball *et al.,* 2016). Despite formal separation of *R. clavata* and *R. maderensis*, mtDNA has been unsuccessful in supporting these species designations (Ball *et al.,* 2016).

The Longnosed skate

The longnosed skate (*Dipturus oxyrinchus*) is a near threatened species of skate found across the Eastern North Atlantic. Historically, the range of this skate stretched from central and southern Norway through to Morocco and the Azores, including the Mediterranean. Due to overfishing, however, this species has suffered significant range restrictions and may have disappeared from the Irish Sea and the Gulf of Lions in the eastern Mediterranean (Ungaro *et al.,* 2007). Similarly to previous research examining populations of skate in the remote seamounts of the Atlantic (Chevlot *et al.,* 2006; Naylor *et al.,* 2012; Ebert & Stehann, 2013; Ball *et al.,* 2016), preliminary mtDNA sequence trees (CR, COI) suggest longnosed skate from the Azores could represent a distinct genetic lineage, or even a cryptic species (Andrew Griffiths, unpubl. data).

Chapter 2 of this thesis will aim to resolve the status of the thornback ray and the Madeiran skate, the Norwegian skate in the Mediterranean, and whether there is a cryptic *Dipturus* species in the Azores, utilising nextRAD and mtDNA (CR, COI) sequencing.

1.3 References

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Chapter 2: Resolving the spatial distributions of *Dipturus intermedius* and *Dipturus batis* - the two novel taxa formerly known as the common skate.

The supplementary materials are available at the end of the chapter.

2.1 Abstract

Batoid fishes (skates and rays) are among the most endangered marine vertebrates, yet conservation efforts have been confounded by unresolved and incomplete taxonomy. Evidence from morphological and molecular characters suggest that the common skate actually represents two species: the flapper skate (*Dipturus intermedius*) and the blue skate (*Dipturus batis*). Despite the species status as critically endangered on the IUCN red list and European Union restrictions on landings, knowledge of the geographic range of these two nominal species is limited. Here, we utilise cytochrome oxidase I (COI) and control region (CR) mitochondrial DNA sequencing to unambiguously distinguish between species, allowing their spatial distributions to be clarified. Samples were obtained from Iceland, Rockall, around the UK in the Atlantic, the North Sea, Azores and the Shetlands. Results suggest that *Dipturus batis* is commonly distributed in the Western Approaches and Celtic Sea, extending out to Rockall and Iceland. The flapper skate generally appears to be much less abundant, but is most frequent around northern Scotland and Ireland, including the northern North Sea, and present in Portugal. Two individuals were also identified from seamounts in remote areas of the Atlantic around the Azores, the furthest south and east the species has been found. This supports reports that the flapper skate historically had a much wider distribution, highlighting the large scale over which

fisheries may have led to local extirpations. Furthermore, these Azorean samples had unique haplotypes, highlighting the importance of seamounts as ‘hotspots’ of biodiversity, with a significant role in the designation marine conservation zones.

2.2 Introduction

Chondrichthyans (sharks, skates and rays) are one of the oldest lineages of vertebrates; arising some 420 million years ago they rapidly diverged to monopolize high trophic levels (Compagno, 1990; Kriwet *et al.,* 2008), playing important roles in structuring ecosystem dynamics (Heithaus *et al.,* 2012). Typical of predatory vertebrates, chondrichthyans can be characterised by a large size, slow growth rates, late maturity, and low fecundity - exhibiting high levels of maternal investment and long gestation periods (Cortes, 2000; Dulvy *et al.,* 2014). Such life history traits, coupled with their ease of catch, make this group intrinsically at high risk of overexploitation by fisheries, with an estimated 25% of species now threatened worldwide (Dulvy *et al.,* 2014; García *et al.,* 2008). In particular, batoids (skates and rays) have suffered rapid abundance declines in recent decades, with many populations suffering local extirpations and significant range reductions (Brander, 1981; Casey and Meyers, 1998; Dulvy *et al.,* 2000). Despite growing concern for their conservation status, effective management of batoids has been confounded by a paucity of species boundaries and geographic range data, often owing to the high level of morphological and ecological conservatism among extant orders (Ebert and Compagno, 2007). Mitochondrial DNA (mtDNA) has, however, proven to be effective in reconstructing batoid phylogenies, and in recent years such molecular markers have also been very powerful in identifying 'cryptic' or morphologically indistinguishable species within this group, suggesting that species diversity is often underestimated (Ball *et al.,* 2016; Cannas *et al.,* 2010; Griffiths *et al.,* 2010; Iglésias *et al.,* 2010).

The common skate, *Dipturus batis* (L. 1758) is one of the most vulnerable of the batoid fishes, now classified as critically endangered on the IUCN red list (Dulvy *et al.,* 2006). Once abundant in the north east Atlantic and a primary constituent of the demersal fish community, this species' former range is thought to have stretched from Iceland and northern Norway, through to Madeira and northern Morocco, including in the Mediterranean Sea and throughout the waters of the British Isles

(Dolgov *et al.,* 2005; Ellis *et al.,* 2005). A more recent assessment by Dulvy and Reynolds *et al.,* (2002) indicated that *D. batis* now appears to be absent from most of its former range and is locally extinct in the southern and central North Sea, west Baltic and the western Mediterranean. This species has also disappeared from the Irish Sea, with just six individuals being caught between 1988 and 1998 in the region, being the first marine fish species to have been formally described as locally extinct due to commercial fishing (Brander, 1981; Dulvy and Reynolds, 2002).

Recent work on the morphological, genetic and life-history characteristics of *D. batis* (Iglésias *et al.,* 2010) suggested that the North-eastern Atlantic *D. batis* actually consists of two nominal species, *Dipturus cf. intermedius* and *Dipturus cf. flossada*. Concurrent work investigating the population genetic structure of common skate around the UK (Griffiths *et al.,* 2010) drew similar conclusions; after genotyping skate with a suite of molecular markers, two reproductively isolated groups were clearly evident. It was further suggested that there may be a degree of latitudinal separation between the groups. These results have serious implications for the conservation status of *D. batis,* as it is likely that the extinction risk of *D. cf. intermedia* and *D. cf. flossada* is significantly higher than previous estimates that treated *D. batis* as a single homogenous unit. Of greatest conservation concern is perhaps *D. cf. intermedia*, the larger growing of the two species, as size has previously been shown to be a powerful proxy for fisheries vulnerability and extinction risk in batoids (Dulvy *et al.,* 2000, Dulvy and Reynolds, 2002). However, in order to verify such conclusions, work still needs to be conducted to clarify the distributions and abundance of these newly revised taxa. Insights into the range sizes of these two species may also provide additional understanding of the scale of their declines and local extirpation. Since 2010, the name *D. intermedius* (flapper skate) has been resurrected for *D. cf. intermedia* and *D. batis* (blue skate) now refers to *D. cf. flossada* (Last *et al.,* 2016). This nomenclature will be adopted herein.

The aim of this study was to resolve the spatial distributions of the flapper and blue skate by sampling a more comprehensive range of these two species' distributions than those examined in Griffiths *et al.,* (2010) and Iglésias *et al.,* (2010). For the first time, sequence data was obtained from individuals collected from Iceland and the Shetlands, and as far south as the Azores. Mitochondrial control region (CR) sequencing and cytochrome oxidase I (COI) barcoding were utilised to unambiguously distinguish between species.

2.3 Materials and Methods

2.3.1 Control Region Sequence Analysis

Fin clips from 44 'common skate' captured during research cruises off southern Iceland were collected, all were putatively identified as blue skate based on their morphology and were analysed with the CR sequence to confirm their identity. A further 37 skate captured from research cruises in the North Sea (Shetlands) were morphologically identified as the flapper skate; a sub-set of 19 were tissue sampled and sequenced for the CR. Additional samples identified simply as *'*common skate' collected from research cruises off the coast of the Azores (n = two; Appendix one, supplementary materials), Western Scotland (n = 22) and the Eastern North Sea (n = one) were also analysed (Appendix two, supplementary materials). Photographs (Appendix one, supplementary materials) of skate in the Azores were subsequently identified as flapper skate, using morphological characteristics outlined by Iglésias *et al.,* (2010). Tissue samples were preserved in absolute ethanol prior to storage at -20°C. Extraction of genomic DNA was undertaken using the Promega (Madison, Wisconsin, USA) Wizard extraction kit. All individuals were sequenced for the same highly variable partial region of the mitochondrial control region, following Griffiths *et al.,* (2010). The PCR products were sequenced by

Source Bioscience, (Nottingham, UK) and the results were checked by eye in BIOEDIT version 7.1.11 (Hall, 1999).

To place results in a broader phylogenetic context additional sequences from other *Dipturus* species were also included in the dataset (Appendix two, supplementary materials). Novel CR sequences were obtained using the methods described above from four longnosed skate captured off Portugal and Norway and two Barndoor skate (*Dipturus laevis*) collected off the South-East Canadian coast (previously COI sequenced in Coulson *et al.,* 2016). An additional 27 CR sequences were obtained from GenBank, including sequences for three additional *Dipturus* species (Appendix three, supplementary materials).

2.3.2 Cytochrome Oxidase I Sequence Analysis

A small subset of six samples were also sequenced for the COI gene following Ward *et al.,* (2005). Given their rarity and the lack of knowledge surrounding whether the blue skate and flapper skate are present in the region (Dulvy *et al.,* 2002), this included the two Azorean 'common skate' samples. For comparison, sequences were also generated from two individuals identified as flapper skate (samples '12.37' and '337') and two as blue skate (samples '12.87' and '3.8'), previously sequenced for the CR and cytochrome b region by Griffiths *et al.,* (2010) (Appendix two, supplementary materials). These six samples represented the total number that were sequenced for both the COI and CR genes. A further 47 COI sequences were included from GenBank, corresponding to an additional 12 *Dipturus* species (Appendix four, supplementary materials).

Due to the high level of morphological conservatism among skate species there is a possibility of misidentification in existing datasets. Hence, when mining CR and COI sequences from GenBank a

conservative approach was taken; only homologous sequences identified to the species level and from peer-reviewed papers in well-established journals were included in phylogenetic analyses.

2.3.3 Phylogenetic and Phylogeographic Analysis

CR and COI sequence datasets for all *Dipturus* spp. were aligned alongside those of outgroups *Mitsukurina owstoni* and *Scyliorhinus canicula* using the CLUSTALW plugin in Geneious 6.0.6 (Biomatters, Aukland, New Zealand). The most appropriate substitution model for phylogenetic analyses was determined using MEGA 7.0.26 (Kumar *et al.,* 2016). TN93 and T92 were identified as the best models for the CR and COI dataset, respectively (Appendix five, supplementary materials). However, these models are not implemented in MrBayes so HKY was selected for the CR and K2 for COI instead (Appendix, five supplementary materials). All phylogenetic reconstruction was conducted in Geneious 6.0.6. Maximum likelihood (ML) trees were constructed using the PhyML (Guindon and Gascuel, 2003) plugin in. The following parameters were used: 1,000 bootstrap replicates, an estimated gamma distribution parameter, an estimated transition/transversion ratio, proportion of invariable sites fixed at 0, 4 substitution rate categories. Bayesian phylogenetic trees were estimated using the MrBayes plugin (Huelsenbeck and Ronquist, 2001); 4 Monte-Carlo chains were run for 1,100,000 generations, with sampling frequency set at every 200 generations. Burn-in length was set at 400,000. Sequence divergence was estimated under the gamma model, enabling the rate variation to be set at 4. Consensus trees were built using the Consensus Tree Builder after removing the initial 10% burn-in; support threshold was set at 50%.

In order to visually assess the distribution of the flapper and blue skate*,* samples corresponding to all CR and COI sequences used for phylogenetic analyses were plotted onto the distribution map (Figure one). This included sequences downloaded from Genbank for which there was latitude and longitude information available (Appendix three, four, supplementary materials). For the CR, all but one

sequence downloaded from Genbank had location information available (Appendix three, supplementary materials). For COI sequences, only one Genbank sequence (flapper skate) from Portugal had location information, although the exact latitude and longitude was not available (Accession number JQ774529; Appendix four, supplementary materials). The distribution map includes samples sequenced in the current study and previously published papers, totalling 201 individuals. To reconstruct genealogical relationships among haplotypes, a minimum spanning haplotype network was creating using PopART V 1.7 (Bandelt *et al.,* 1999). This network was constructed using all CR sequences sequenced in the current study (Appendix two) and those mined from Genbank for which there was location information available (Appendix three). Samples were grouped into the following groups broadly based on sea boarders (World Atlas, 2020): Iceland, Atlantic, Celtic Sea, North Sea, Rockall and Azores. Although Rockall is within the Atlantic, it was grouped as a separate population due to its separation from other Atlantic *D. batis* samples by the Rockall Trough (Figure one). Similarly, although Iceland is within the North Atlantic it is separated from other samples by the Maury Seachannel.

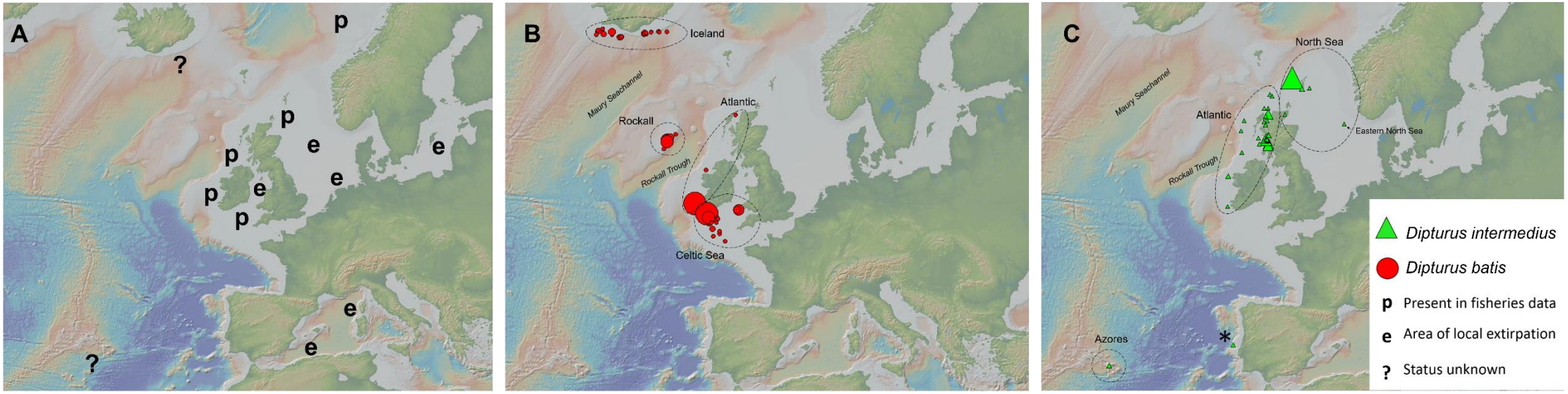


Figure 1. (A) Map detailing the range of the 'common skate' in 2002 (adapted from Dulvy *et al.,* 2002); (B) The geographical locations of all blue skate (*Dipturus batis*) specimens used in phylogenetic analysis, including ones representing CR sequences downloaded from Genbank; (C). The geographical locations of all flapper skate (*Dipturus intermedius*) specimens used in phylogenetic analysis, including ones representing CR sequences downloaded from Genbank. Dotted lines represent samples that were grouped together into geographic units (based on sea boarders) for the CR haplotype network. * indicates that the flapper skate specimen from Portugal did not have exact latitude and longitude information available, and so the proximate location has been indicated. Because this is the only specimen that represents a COI sequence, it was not included in the CR haplotype network. The size of a point is proportional to the number of individuals it represents.

2.4 Results

2.4.1 Phylogenetic and Phylogeographic Analysis

Maximum likelihood and Bayesian probability trees built from COI (Figure two) CR sequences (Figure three) showed that among the *Dipturus* species, most were well resolved with relatively high bootstrap support (54- 96% ML; 51-100% Bayesian). The analyses show the 'northern' and the 'southern' clades of 'common skate' identified by Griffiths *et al.,* (2010) correspond to the species flapper skate (*D. interemedius*) and blue skate (*D. batis*)*,* respectively, and as distinguished by Last *et al.,* (2016). Again, bootstrap support for the separation of these species was high in both the CR and COI trees (Figure two, three). Unexpectedly, within both the Mr Bayes and PhyML COI trees *Dipturus batis* was not reciprocally monophyletic, with two haplotypes grouping with the Barndoor skate from Canada (Figure two). This grouping, however, was not supported in the CR dataset and could well be due to the inherent ability of CR to provide greater resolution for recently diverged taxa (Serra-Pereira *et al.,* 2010; Valsecchi *et al.,* 2005). Furthermore, a greater number of putative blue skate samples from Iceland were sequenced for the CR than COI, which may have contributed to this difference by providing greater resolution. Several other taxa (*Dipturus trachyderma, Dipturus kwantugensis and D. nidarosiensis*) were not reciprocally monophyletic in CR trees (Figure three). The inability to resolve some species in the phylogenetic analyses could reflect the recent divergence of species, confounded by the slow rates of sequence divergence in elasmobranchs.

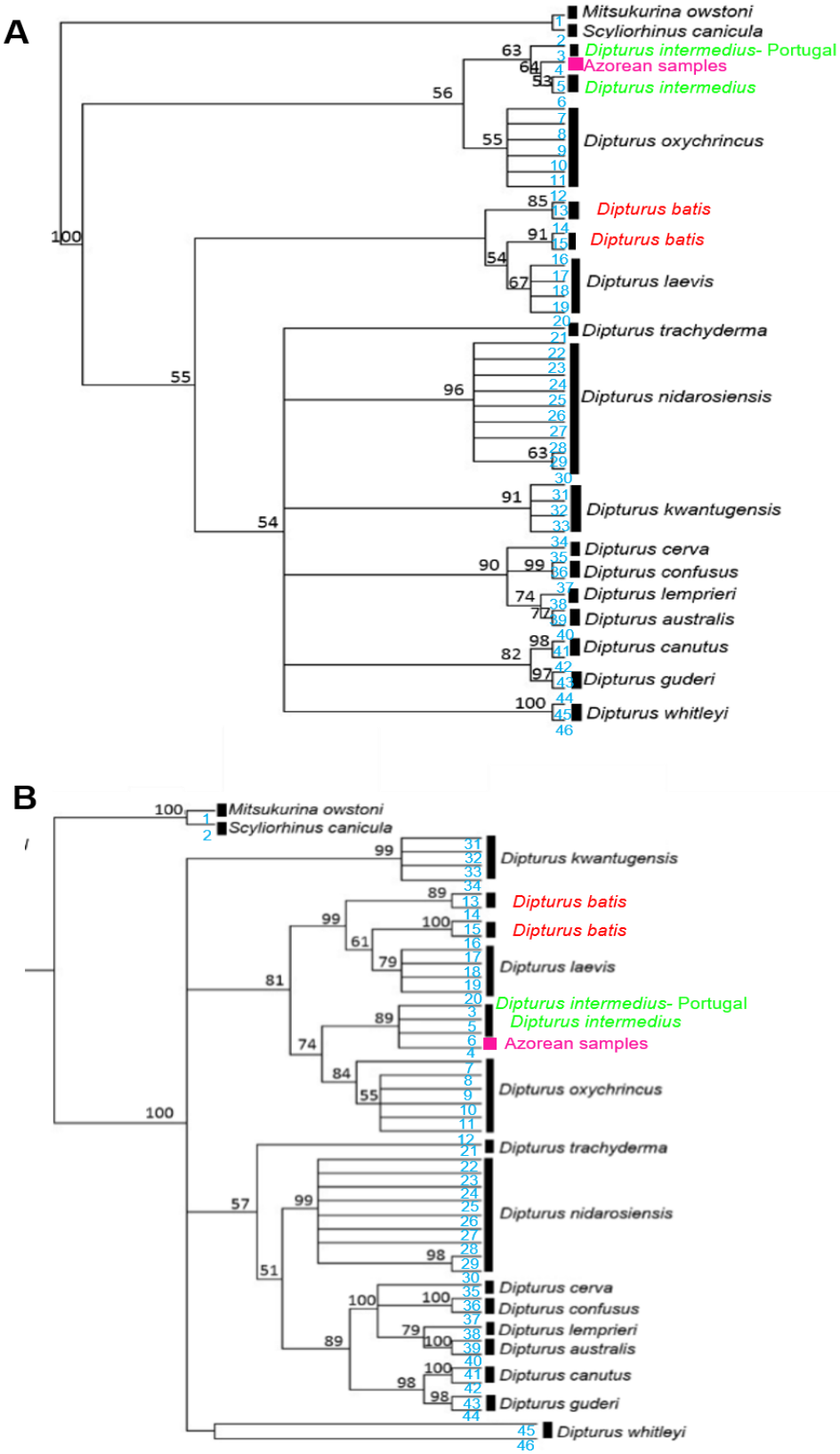


Figure 2. PhyML tree built from COI data (A)**;** Mr Bayes tree built from COI data (B). Numbers above branches represent bootstrap support values. Blue numbers below branches correspond to accession numbers and location information in Appendix two and four, supplementary materials.

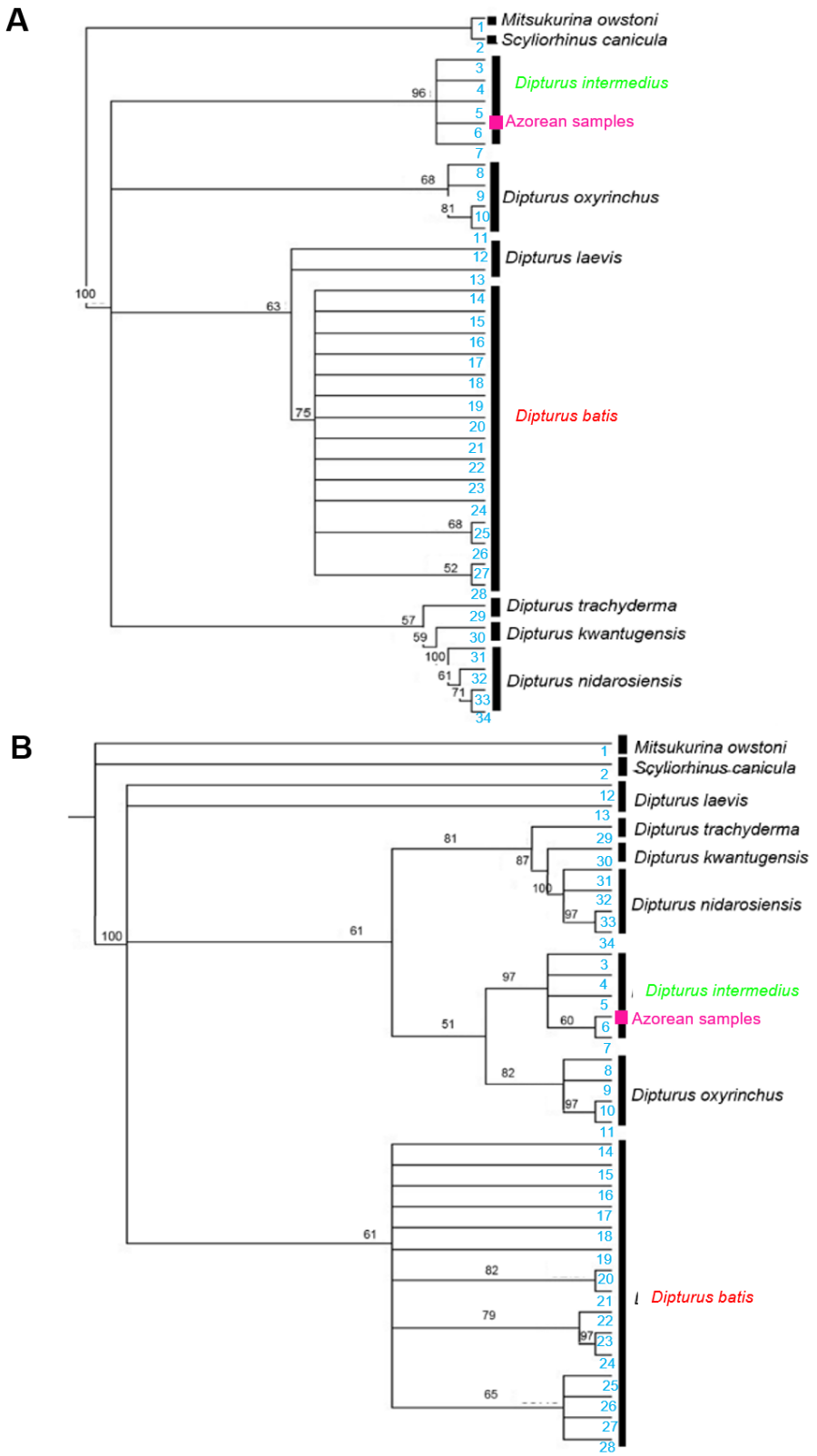
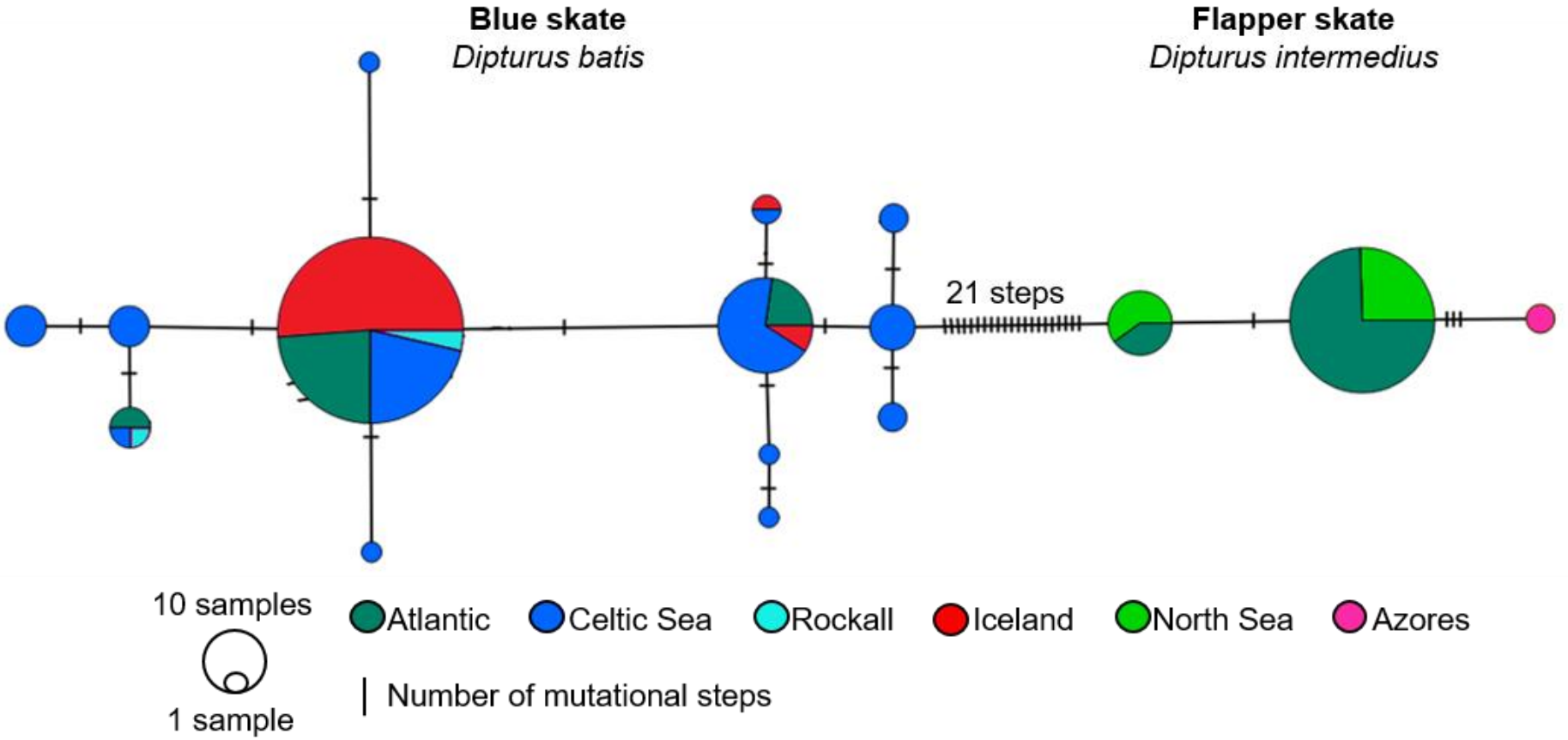


Figure 3. PhyML tree built from CR data (A)**;** Mr Bayes tree built from CR data (B). Numbers above branches represent bootstrap support values. Blue numbers below branches correspond to accession numbers and location information in Appendix two and three, supplementary materials.

The original morphological identification of specimens on research cruises from Iceland (blue skate, *D. batis*), the Shetlands (flapper skate, *D. intermedius*) and the Azorean region (flapper skate, *D. intermedius*) was supported by the phylogenetic analysis. Interestingly, the Azorean flapper skate revealed novel CR and COI haplotypes that have not been identified from previous investigations focused on more northerly regions (Figure two, three). One specimen of *D. intermedius* from Portugal sequenced by Costa *et al.,* (2012) also revealed a unique COI haplotype (Figure two).

In support of results from Griffiths *et al.,* (2010), in the CR haplotype network the blue skate group was proportionally more diverse than the flapper skate, with 13 CR haplotypes shared among 137 individuals (Table one; Figure four). The most common haplotype was found in all populations sampled (Iceland, Rockall, the Celtic Sea and the Atlantic). The flapper skate clade was 21 steps away and was represented by just three haplotypes and 66 individuals, with the most common haplotype present in all populations except the Azores. The rare Eastern North Sea specimen also shared the most common haplotype. The distinctiveness of the flapper skate Azorean population was supported by the CR haplotype network, which was three mutational steps away from the most common haplotype in this clade. For a detailed breakdown of the number of CR haplotypes per location see Appendix six, supplementary materials.



1226

1227 **Figure 4.** Control region (CR) haplotype network of the flapper skate (*Dipturus intermedius*) and the blue skate (*Dipturus batis*) samples collected

1228 from across the North Atlantic and downloaded from Genbank.

Table 1. Haplotype information for all cytochrome oxidase I (COI) and control region (CR) sequences used in phylogenetic analysis for the flapper skate (*Dipturus interemedius*) and the blue skate (*Dipturus batis*).

Flapper skate (*Dipturus intermedius*)		
Parameters	**COI gene**	**CR gene**
Number of samples	8	66
Number of haplotypes	2	3
Polymorphic sites	463	4
Monomorphic sites	157	716
Parsimony informative sites	463	4
Haplotype diversity (Hd)	0.4760	0.5530
Nucleotide diversity (Pi)	0.3569	0.0013
Blue skate (*Dipturus batis*)		
Parameters	**COI gene**	**CR gene**
Number of samples	6	137
Number of haplotypes	5	13
Polymorphic sites	486	9
Monomorphic sites	149	715
Parsimony informative sites	479	5
Haplotype diversity (Hd)	0.9330	0.4260
Nucleotide diversity (Pi)	0.41134	0.0012

2.5 Discussion

The common skate was once one of the most highly abundant demersal fishes of the North Atlantic, with a range that once stretched from Iceland and northern Norway, through to Madeira and northern Morocco, including the Mediterranean Sea and throughout the waters of the British Isles (Dolgov *et al.,* 2005; Ellis *et al.,* 2005). It is now extirpated from much of its former range and listed as critically endangered on the IUCN red list (Dulvy *et al.,* 2006). This high conservation concern has been made even more significant by the recent recognition of the common skate as two distinct species: the blue skate (*D. batis*) and the flapper skate (*D. intermedius*; Griffiths *et al.,* 2010; Iglésias *et al.,* 2010), prompting the investigation of their distributions in the present study. The results are consistent with the conclusions of Griffiths *et al.,* (2010) and Iglésias *et al.,* (2010), suggesting that the common skate actually represents two species. However, the results do not support the suggestion by Griffiths *et al.,* (2010) that there is latitudinal separation between these groups. For the first time, genetic data has supported the occurrence of the blue skate in Iceland and the flapper skate as far south as Portugal and the Azores. The confirmation of the occurrence of flapper skate in seamounts in the Azores is a significant finding, underlining that the distribution of *D. intermedius* was perhaps once much more extensive, and eludes to the large scale of local expiration and decline the species is known to have undergone. The identification of novel haplotypes from these flapper skate is also significant, highlighting the potential role of the Azores as ‘hotspots’ for biodiversity.

The integration of molecular data now permits a synthesis of spatial distribution information concerning these threatened species of skate. Since Dulvy and Reynolds' assessment of the common skate species complex in 2002, it is clear that division of this species has produced two groups with heavily restricted ranges (Figure one). The smaller species, nominally *D. batis*, mainly occurs in the Western Approaches and Celtic Sea and extending out to Rockall (Griffiths *et al.,* 2010), with results of this study demonstrating its occurrence around Iceland. This species abundance in southern UK

1262 waters may, in part, account for the higher frequency of common skate captures at French ports in
1263 2005-2007, that tend to target fisheries associated with these regions (Iglésias *et al.,* 2010).

1264

1265 The larger species, nominally *D. intermedius*, appears to be mainly present off Northern Ireland and
1266 Scotland (Griffiths *et al.,* 2010), including the northern North Sea. This is consistent with previous
1267 research, suggesting common skate were regularly observed off the coast of Northern and North-
1268 western Scotland, Ireland and the Celtic Sea (Dulvy and Reynolds, 2002; Dulvy *et al.,* 2006; Neat *et*
1269 *al.,* 2015). Rare individuals of these species have also previously been reported in the North Sea (e.g.
1270 Ellis *et al.,* 2005; Silva and Ellis*,* 2012; ICES 2012), consistent with the one flapper skate specimen
1271 found in this study. Further north, common skate are known to already occur in the Shetlands (Walker
1272 and Hislop 1998; Dulvy *et al.,* 2006) and the data here suggests that current populations in this area
1273 are mainly comprised of flapper skate*,* consistent with previous research (Griffiths *et al.,* 2010) and
1274 reports from grey literature (Shark Trust, 2009; Shark Trust, 2010). Reports from the grey literature
1275 also suggest this region's role in supporting significant numbers of flapper skate eggs, joining parts of
1276 the western Scottish coast as potential nursery areas for this critically endangered species (Shark
1277 Trust, 2010). Identification and protection of such regions is vital in the conservation of *D. intermedius*.
1278 This is all the more important given the threat of scallop dredging in the region, which remains the
1279 third most important sector of the UK fishing industry (The Scottish Fishermen's Federation, 2018),
1280 that could damage skate eggs sharing the same habitat. Further investigation of the impacts of
1281 dredging on skates is needed to properly assess the conservation implications.

1282

1283 Historically, the range of the common skate has been described as extending much further northwards
1284 than the Shetlands (Figure one). However, previous analysis failed to identify any flapper skate
1285 associated with Rockall (Griffiths *et al.,* 2010) and inclusion of samples from Iceland here has similarly
1286 not identified the species in this region (blue skate are more abundant in these regions). No samples
1287 of common skate could be obtained from Norway in the present study and recent data from the region

suggests misidentification may be at the root of records of its occurrence, at least in more northerly regions (Lynghammer *et al.,* 2014). Analysis of common skate from Norwegian and Swedish museum collections, from both morphology and DNA barcoding, generally suggest the presence of blue skate (Viinamäki, 2010). This implies the current distribution of flapper skate does not extend beyond northern Scotland (and perhaps southern Norway).

Although flapper skate are present in Scotland and Northern Ireland, it appears less abundant in more southerly regions, with one common skate specimen from Portugal being identified as this species. Indeed, blue and flapper skate are thought to be locally extinct in the Black Sea, the Levantine Mediterranean basin (Serena 2005) and scarce throughout the southern British Isles (Dulvy *et al.,* 2006). Further, it is notable that recent inspections of fish markets at ports from 2014-2016 failed to demonstrate the presence of any blue or flapper skate along the Atlantic coast of Morocco (Samantha Hook, personal communication). When contemporary catches of these species from Portugal, France and the English Channel (Machado *et al.,* 2004; Griffiths *et al.,* 2010; Iglésias *et al.,* 2010; Simpson and Sims, 2016) have been reported, they are much rarer than those in Northern UK waters (Simpson and Sims, 2016). Although it is difficult to say if this absence is due to recent decline or historical taxonomic confusion, this northern-bias is consistent with previous research (Dulvy *et al.,* 2006). The two flapper skate individuals collected from seamounts in the Azores and one specimen from Portugal suggest this species once may have had a much wider distribution that extended into more southerly regions. Speculation into the historical distributions of endangered skate is an essential part of informing conservation and IUCN red-list assessments (Dulvy *et al.,* 2006), and these results are consistent with evidence from historical accounts of the occurrence of the common skate (which has been hypothesised to have been confused under the name *Raja macrorynchus*; Moreau, 1881) across Europe (e.g. Clark, 1926; Wells, 1958; Figure one). Indeed, ‘common skate’ were once described from Iceland and northern Norway, through to Madeira and northern Morocco, including the waters of the British Isles (Dolgov *et al.,* 2005; Ellis *et al.,* 2005). These Azorean specimens could represent a relict population, insularised by increased fishing pressure and subsequent large-scale

decline and extirpation of skate in waters associated with the European continental shelf (Dulvy *et al.,* 2002). The relative inaccessibility of the Azores may have protected the skate from over-fishing; the region has some of the earliest designations of marine protected areas (MPAs; Abecasis *et al.,* 2015), meaning it is likely that a population of flapper skate could have persisted here. Given the limited haplotype diversity associated with more northern populations (64 specimens from the Atlantic, Eastern North Sea and the Shetlands shared just two CR haplotypes; Figure four), the identification of novel haplotypes from these Azorean specimens supports this conclusion and is significant, highlighting the general importance of Azorean seamounts as 'hotspots' for biodiversity (Reboleira *et al.,* 2011), including genetic diversity (the most fundamental level of biodiversity (Duffy and Stachowicz, 2006)). The unique COI haplotype from the flapper skate specimen from Portugal (Costa *et al.,* 2012) is also interesting, and reinforces the distinctiveness of Southern *D. intermedius* populations.

The restricted distribution of flapper skate to mainly northern Scotland and Ireland may be a consequence of its morphological and life-history traits, as size can frequently be used as a proxy for vulnerability and local extinction risk in batoids (Dulvy *et al.,* 2000; Dulvy and Reynolds, 2002). Hence, the very large size of flapper skate (up to 2288 mm in length, Iglésias *et al.,* 2010), its low fecundity and long period required to reach reproductive maturity, means it is probably more vulnerable to overfishing than the smaller growing blue skate. Subsequently, this species may have undergone steep declines in number across its range (Iglésias *et al.,* 2010) and even suffered fisheries-induced local extinctions. However, a note of caution should be taken when inferring historical ranges of cryptic species: due to the lack of accurate fisheries data it is impossible to know whether 'common skate' landed across the North Atlantic represent flapper skate or blue skate or both. Reported skate landings are often misidentified or are not reported on a species-specific basis. For example, doubts about the validity of historical identifications of blue skate across France and the Mediterranean region and France have been raised, due to potential misidentifications with the Norwegian skate (*D. nidarosiensis*) and the longnosed skate (*D. oxyrinchus*) (Dulvy *et al.,* 2006; Iglésias *et al.,* 2010).

Nevertheless, results from the current study have strong implications for conservation assessments of the North Atlantic 'common skate' complex. The larger maximum size of flapper skate suggests that it is more vulnerable to extinction than blue skate and conservation efforts should be targeted accordingly. The discovery of two individuals with a novel haplotype from the Azores is significant, and supports evidence from historical accounts that suggest this species may have once had a more southerly distribution. These specimens, combined with the unique haplotype from one Portugal specimen identified by Costa *et al.,* (2012), highlights the distinctiveness of Southern populations of flapper skate.

2.6 Acknowledgements

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2.8 Supplementary materials: Resolving the spatial distributions of *Dipturus intermedius* and *Dipturus batis* - the two novel taxa formerly known as the common skate.

Maisie B. Jeffreys[1], Rachel E. Ball[2], Gui Menezes[3], Jonbjorn Palsson[3], Christophe Pampoulie[4], Jamie R. Stevens[1] & Andrew M. Griffiths[1*]

*Author of correspondence: A.M.Griffiths2@exeter.ac.uk. +44 (0) 1392 725849

Appendix 1: Image of the flapper skate (*Dipturus intermedius*) specimen ‘AZP90’ from the Azores.

Appendix 2: Capture locations for all *Dipturus* species collected and control region (CR) and cytochrome oxidase I (COI) GenBank accession numbers. 'n/a' indicates sequences were not sequenced for this gene. CR and COI trees can be found in Figure three and two of the main text., respectively

Isolate	**Capture date**	**Latitude**	**Longitude**	**Location**	**Clade membership**	**CR accession no**	**Branch number in CR trees**	**COI accession no**	**Branch number in COI trees**
SH183	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392066	3	n/a	n/a
SH184	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392066	3	n/a	n/a
SH186	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392066	3	n/a	n/a
SH194	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392066	3	n/a	n/a
SH181	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392066	3	n/a	n/a

SH199	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	MH581188	5	n/a	n/a
SH195	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH185	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH182	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH193	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH192	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH187	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH191	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH188	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a

SH190	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH189	01/06/2012	60.716	-2.658	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH162	01/06/2012	60.050	-1.417	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
SH163	01/06/2012	60.050	-1.417	*Shetlands*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
AZP90	28/05/2011	37.741	-25.676	*Azores*	*Dipturus intermedius*	MH581186	6	Submit to Genbank	4
D45	02/06/2012	37.741	-25.676	*Azores*	*Dipturus intermedius*	MH581187	6	Submit to Genbank	4
D4471	May 2012	63.330	-22.485	*Iceland*	*Dipturus batis*	GQ392080	14	n/a	n/a
D461	May 2012	63.421	-22.544	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D451	May 2012	63.606	-22.587	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a

D462	May 2012	63.421	-22.544	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4172	May 2012	63.333	-17.396	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4173	May 2012	63.333	-17.396	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4181	May 2012	63.388	-17.306	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4231	May 2012	63.444	-14.520	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4421	May 2012	63.123	-20.329	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4452	May 2012	63.432	-21.551	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4511	May 2012	63.464	-23.241	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D291	May 2013	63.155	-20.575	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a

D463	May 2012	63.421	-22.544	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2971	May 2013	63.155	-20.575	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2101	May 2013	63.135	-20.447	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2102	May 2013	63.135	-20.447	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2301	May 2013	63.464	-15.523	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2331	May 2013	63.456	-15.580	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2371	May 2013	63.315	-17.106	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2402	May 2013	63.153	-20.281	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2403	May 2013	63.153	-20.281	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a

D2441	May 2013	63.276	-21.386	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2472	May 2013	63.422	-21.494	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2542	May 2013	63.480	-23.066	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4178	May 2012	63.333	-17.396	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4182	May 2012	63.388	-17.306	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4391	May 2012	63.425	-16.485	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4451	May 2012	63.432	-21.551	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4481	May 2012	63.283	-23.376	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4521	May 2012	63.482	-23.066	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a

D4522	May 2012	63.482	-23.066	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D4541	May 2012	63.524	-23.183	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5131	May 2011	63.420	-15.472	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5281	May 2011	63.422	-16.494	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5321	May 2011	63.176	-20.204	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5391	May 2011	63.435	-21.411	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5392	May 2011	63.435	-21.411	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5393	May 2011	63.435	-21.411	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5461	May 2011	63.318	-22.457	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a

D5462	May 2011	63.318	-22.457	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5471	May 2011	63.279	-23.384	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D21342	May 2013	63.333	-17.390	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D2345	May 2013	63.392	-17.157	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
D5132	May 2011	63.420	-15.471	*Iceland*	*Dipturus batis*	GQ392068	15	n/a	n/a
Nor	20/02/2009	58.4	3.6	*Eastern North Sea*	*Dipturus intermedius*	GQ392065	4	n/a	n/a
12,37	26/08/2008	60.1	-0.500	*Shetlands*	*Dipturus intermedius*	GQ392065	4	Submit to Genbank	5
337	16/03/2008	56.7	-6.000	*Atlantic*	*Dipturus intermedius*	GQ392065	4	Submit to Genbank	5
12,87	11/2008	50.1	-8.800	*Celtic Sea*	*Dipturus batis*	GQ392068	15	Submit to Genbank	15

3,8	27/01/2008	50.6	-8.300	*Celtic Sea*	*Dipturus batis*	GQ392070	16	Submit to Genbank	16
RJ01	Unknown	38.000	-9.000	*Portugal*	*Dipturus oxyrinchus*	GU595172	8	n/a	n/a
RJ02	Unknown	38.000	-9.000	*Portugal*	*Dipturus oxyrinchus*	GU595172	8	n/a	n/a
RJ03	Unknown	38.000	-9.000	*Portugal*	*Dipturus oxyrinchus*	GU595175	11	n/a	n/a
RJ04	Unknown	38.000	-9.000	*Portugal*	*Dipturus oxyrinchus*	GU595175	11	n/a	n/a
Bar07245	19/02/2006	41.926	-65.809	*Nova Scotia*	*Dipturus laevis*	MH581189	12	n/a	n/a
Bar06125	01/01/2007	42.884	-65.058	*Nova Scotia*	*Dipturus laevis*	MH581190	13	n/a	n/a

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Appendix 3: Information of all sequences mined from GenBank used to construct control region sequence tree. * Indicates 'common skate' sequences for which there were latitude and longitude information available, these were plotted onto the distribution map and used for the haplotype network. Control region (CR) trees can be found in Figure three of the main text.

Species	**GenBank accession number**	**Branch number in CR sequence trees**
Dipturus nidarosiensis	GQ392097	31
Dipturus nidarosiensis	GQ392098	32
Dipturus nidarosiensis	GQ392099	33
Dipturus nidarosiensis	GQ392100	34
Dipturus intermedius	GQ392065 *	4
Dipturus intermedius	GQ392066 *	3
Dipturus batis	GQ392067 *	17
Dipturus batis	GQ392068 *	18
Dipturus batis	GQ392069 *	19
Dipturus batis	GQ392070 *	16
Dipturus batis	GQ392071 *	20
Dipturus batis	GQ392072 *	21
Dipturus batis	GQ392073 *	22
Dipturus batis	GQ392074 *	23

Dipturus batis	GQ392075 *	24
Dipturus batis	GQ392076 *	25
Dipturus batis	GQ392077 *	26
Dipturus batis	GQ392078 *	24
Dipturus batis	GQ392079 *	24
Dipturus batis	GQ392080 *	14
Dipturus batis	GQ392081 *	27
Dipturus batis	GU477349	28
Dipturus oxyrinchus	GU595173	9
Dipturus oxyrinchus	GU595172	8
Dipturus oxyrinchus	GU595174	10
Dipturus oxyrinchus	GU595175	11
Dipturus oxyrinchus	GQ392095	8
Dipturus oxyrinchus	GQ392096	9
Mitsukurina owstoni	NC_ 011825.1:16685-17743	1
Scyliorhinus canicula	Y16067.1:12802-13851	2
Dipturus kwangtugensis	KF318309.2:16032-16753	30
Dipturus trachyderma	NC_027521.1:15660-16907	29

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Appendix 4: Information of all sequences mined from GenBank used to cytochrome oxidase I (COI) sequence tree. * Indicates 'common skate' sequences for which there were latitude and longitude information available, these were plotted onto the distribution map. COI trees can be found in Figure two of the main text.

Species	**GenBank accession number**	**Branch number in COI trees**
Dipturus nidarosiensis	KC262633	25
Dipturus nidarosiensis	KX783029	26
Dipturus nidarosiensis	KX783030	22
Dipturus nidarosiensis	KX783031	27
Dipturus nidarosiensis	KX783032	22
Dipturus nidarosiensis	KX783033	22
Dipturus nidarosiensis	KX783034	22
Dipturus nidarosiensis	KX783035	28
Dipturus nidarosiensis	KX783036	22
Dipturus nidarosiensis	KU761959	29
Dipturus nidarosiensis	KU761958	30
Dipturus nidarosiensis	KF604234	24
Dipturus nidarosiensis	KF604235	24
Dipturus nidarosiensis	KF604236	24
Dipturus nidarosiensis	KF604237	24
Dipturus nidarosiensis	KF604238	23
Dipturus nidarosiensis	KF604239	23
Dipturus nidarosiensis	KF604240	24
Dipturus nidarosiensis	KF604241	23
Dipturus nidarosiensis	KF604242	23

Dipturus nidarosiensis	KF604243	23
Dipturus laevis	JF895055	18
Dipturus laevis	JF895056	19
Dipturus laevis	JF895057	17
Dipturus laevis	JF895058	20
Dipturus laevis	JF895059	17
Dipturus kwangtungensis	EU339344	31
Dipturus kwangtungensis	EU339345	32
Dipturus kwangtungensis	EU339346	32
Dipturus kwangtungensis	EU339347	33
Dipturus kwangtungensis	KF318309.2:5541-6177	34
Dipturus oxyrinchus	KJ709522	11
Dipturus oxyrinchus	HM043215	9
Dipturus oxyrinchus	KY909393	7
Dipturus oxyrinchus	KY909394	8
Dipturus oxyrinchus	KY909395	7
Dipturus oxyrinchus	KY909396	7
Dipturus oxyrinchus	KY909397	7
Dipturus oxyrinchus	KY909398	7
Dipturus oxyrinchus	KY909399	7
Dipturus oxyrinchus	KY909400	7
Dipturus oxyrinchus	KY909401	7
Dipturus oxyrinchus	KY909402	7
Dipturus oxyrinchus	KU761956	12
Dipturus oxyrinchus	JQ774530	10
Dipturus batis	KF604218	14
Dipturus batis	KF604219	14

Dipturus batis	KF604220	13
Dipturus intermedius	KF604221	6
Dipturus intermedius	KF604222	6
Dipturus intermedius	KF604223	6
Dipturus intermedius	KJ204831	6
Dipturus intermedius	JQ774529 *	3
Dipturus australis	DQ108186	40
Dipturus australis	DQ108187	40
Dipturus australis	DQ108188	39
Dipturus cerva	DQ108189	35
Dipturus whitleyi	DQ108179	45
Dipturus whitleyi	DQ108180	46
Dipturus whitleyi	DQ108181	46
Dipturus whitleyi	DQ108190	46
Mitsukurina owstoni	EU398906	1
Scyliorhinus canicula	KM820106	2
Dipturus trachyderma	KR152643.1:5538-6174	21
Dipturus confusus	EU398770	36
Dipturus confusus	EU398771	36
Dipturus confusus	EU398772	37
Dipturus confusus	EU398773	36
Dipturus confusus	EU398774	36
Dipturus canutus	EU398776	41
Dipturus canutus	EU398775	42
Dipturus lemprieri	EU398769	38
Dipturus gudgeri	EU398763	44
Dipturus gudgeri	EU398764	44

Dipturus gudgeri	EU398765	44
Dipturus gudgeri	EU398766	43
Dipturus gudgeri	EU398767	44
Dipturus gudgeri	EU398768	44

Appendix 5: Substitution model analysis for control region (CR) and cytochrome oxidase I (COI) region sequences performed in MEGA 7.0.26.

CR			
Model	**#Param**	**BIC**	**AICc**
HKY+G	70	6481.865	5919.843
T92+G	68	6485.419	5939.443
HKY+G+I	71	6491.895	5921.85
TN93+G	71	6491.899	5921.854
HKY+I	70	6494.68	5932.658
T92+G+I	69	6495.542	5941.543
T92+I	68	6497.111	5951.134
TN93+G+I	72	6501.929	5923.862
TN93+I	71	6504.702	5934.657
GTR+G	74	6514.456	5920.344
HKY	69	6516.206	5962.206
T92	67	6518.362	5980.409
GTR+G+I	75	6524.491	5922.358
TN93	70	6526.24	5964.218
GTR+I	74	6526.288	5932.177
GTR	73	6545.073	5958.983
K2+G	67	6606.359	6068.406
K2+G+I	68	6616.394	6070.418
K2+I	67	6617.147	6079.194
K2	66	6630.748	6100.818
JC+G	66	6652.049	6122.119
JC+G+I	67	6662.084	6124.131
JC+I	66	6663.487	6133.557
JC	65	6670.748	6148.842
COI			
Model	**#Param**	**BIC**	**AICc**
T92+G	92	4734.67	4005.009
K2+G	91	4738.077	4016.338
T92+G+I	93	4740.404	4002.82
TN93+G	95	4740.848	3987.421
HKY+G	94	4742.721	3997.216
K2+G+I	92	4746.348	4016.686
TN93+G+I	96	4750.613	3989.264
HKY+G+I	95	4750.842	3997.415
T92+I	92	4755.899	4026.237
TN93+I	95	4759.593	4006.166
K2+I	91	4761.548	4039.809

HKY+I	94	4766.246	4020.741
GTR+G	98	4770.624	3993.434
GTR+G+I	99	4772.305	3987.193
GTR+I	98	4791.582	4014.391
JC+G	90	4914.487	4200.671
JC+G+I	91	4923.098	4201.359
JC+I	90	4930.72	4216.903
K2	90	4931.768	4217.951
T92	91	4933.152	4211.412
HKY	93	4944.333	4206.749
TN93	94	4949.552	4204.046
GTR	97	4968.974	4199.705
JC	89	5077.071	4371.177

Appendix 6. A breakdown of the number of control region (CR) haplotypes per location for the flapper skate (*Dipturus intermedius*) and the blue skate (*Dipturus batis*). This includes sequences downloaded from Genbank for which there was location information available.

Flapper skate (*Dipturus intermedius*)		
Population	**Number of individuals**	**Number of haplotypes**
Atlantic	43	2
Celtic Sea	No samples obtained from this region	No samples obtained from this region
Rockall	No samples obtained from this region	No samples obtained from this region
Iceland	No samples obtained from this region	No samples obtained from this region
North Sea	21	2
Azores	2	1
Blue skate (*Dipturus batis*)		
Population	**Number of individuals**	**Number of haplotypes**
Atlantic	20	3
Celtic Sea	67	13
Rockall	6	3
Iceland	44	2
North Sea	No samples obtained from this region	No samples obtained from this region
Azores	No samples obtained from this region	No samples obtained from this region

Chapter 3: NextRAD and mitochondrial DNA sequencing reveal hidden diversity within vulnerable species of batoid fish.

The supplementary materials for this chapter can be found at the end of the chapter.

3.1 Abstract

Concern is growing over the conservation status of batoids (skates and rays) worldwide, with around 20% now threatened with extinction. Typically, conservation work is confounded by taxonomic confusion, largely due to the high level of morphological and ecological conservatism within this group. However, molecular markers have proven to be useful in constructing batoid phylogenies, even identifying examples of cryptic speciation. In the present study, we utilise mitochondrial DNA (mtDNA) and Nextera-tagmented reductively-amplified DNA (nextRAD) sequencing to resolve *Raja* and *Dipturus* phylogenies in European species that remain surrounded with taxonomic uncertainty. RaxML trees built from mtDNA and nextRAD data do not support the current separation of the thornback ray (*Raja clavata*) and the Madeiran skate (*Raja maderensis*), consistent with previous studies indicating *R. maderensis* may be a distinct morphotype of the polytypic *R. clavata*. Additionally, results revealed the genetic distinctiveness of skate populations (longnosed skate (*Dipturus oxyrinchus)* and the flapper skate (*D. intermedius*)) in the Azores and surrounding Atlantic seamounts, highlighting the biodiverse nature of the region. Norwegian skate (*Dipturus nidarosiensis*) from the Mediterranean also appeared to be genetically distinct from those in the North Atlantic, but the magnitude of sequence divergence generally argues against the cryptic speciation. Overall, nextRAD data aided in the fine-tuning of

results from traditional mtDNA markers, revealing significant amounts of 'hidden diversity' within these species of vulnerable batoid fish.

3.2 Introduction

Batoids (rays and skates) are one of the most endangered groups of vertebrates; due to their low fecundity, slow growth rate and the late age at which they reach maturity, they are highly vulnerable to overfishing (García *et al.,* 2008; Dulvy *et al.,* 2014). Growing concern is therefore mounting over the status of batoid fish worldwide. In a recent systematic evaluation of elasmobranchs, Dulvy *et al.,* (2014) found that five of the seven most threatened families are rays, with around 20% now threatened with extinction. Batoid fish also represent a significant conservation challenge, as despite being more speciose than all the sharks groups combined, they exhibit huge amounts of ecological and morphological conservatism (Ebert and Compagno, 2007; Last *et al.,* 2016). As a result, effective management is confounded by difficulties in the identification of species and species boundaries, a fundamental concept for conservation and management (Ball *et al.,* 2016).

The application of molecular methods for resolving elasmobranch phylogenies compliments the use of morphological data, particularly as molecular markers have been useful in identifying cryptic species, even within well-studied batoids (Whitey, 1939 White, 1930; McEachran and Fechhelm, 1982; McEachran and Dunn, 1998; Sandoval-Castillo *et al.,* 2004; Toffoli *et al.,* 2008; Griffiths *et al.,* 2010; Iglésias *et al.,* 2010; Sandoval-Castillo *et al.,* 2011; White & Last, 2012; Weigmann, 2016). Perhaps one of the most widely used markers, mitochondrial DNA (mtDNA) offers the advantage of uniparental, haploid inheritance, resulting in a quarter of the effective population size of nuclear genes. Subsequently, mtDNA has a higher magnitude of genetic differentiation among recently isolated species, thus providing greater phylogenetic resolution for recently diverged taxa (Billington, 2003; Birky *et al.,* 1983). This feature is particularly relevant for elasmobranchs, which can

be characterised by some of the slowest evolving genomes of the vertebrates (Venkatesh, 2014). The mitochondrial 'barcoding' gene, cytochrome oxidase subunit I (COI), and the control region (CR), have been utilised significantly for phylogenetics, and can reveal differentiation at fine taxonomic scales (Hebert *et al.,* 2003a; Spies *et al.,* 2006; Griffiths *et al.,* 2011; Serra-Pereira *et al.,* 2011; Coulson *et al.,* 2011; Mabragaña *et al.,* 2011; Lynghammar *et al.,* 2014).

Despite the utility of mtDNA sequencing, several studies have suggested that phylogenies based solely on mtDNA can be misleading, as mtDNA has been known to obscure species boundaries in certain taxa (Avise, 1994; Giannasi *et al.,* 2001; Shaw, 2002). It has been argued that the evolutionary history of the mitochondrial genome, including recombination, hybridization, its small effective population size, introgression, and neutrality, can produce complex patterns of variation (Ballard & Whitlock, 2004). Furthermore, technical problems can arise with a lack of homoplasy in mitochondrial data sets with more homogeneous among-site substitution patterns (Lin & Danforth, 2004; see Rubinoff & Holland, 2005 for a review). One solution to this problem is to include independent nuclear DNA (nDNA) markers. In general, nDNA suffers less from polymerase chain reaction (PCR) artefacts and biased base composition (Lin & Danforth, 2004; Rubinoff & Holland, 2005). Studies employing mtDNA and nDNA have been used to resolve Selachii phylogenies, but have been utilised less for the comparably under-researched batoids. However, the development of reduced-representation sequencing methods has revolutionized the field of phylogenomics and can provide high-resolution genomic data for non-model organisms such as skates and rays (Emerson *et al.,* 2010; Keller *et al.,* 2013; Xu *et al.,* 2014). Nextera-tagmented reductively-amplified DNA (nextRAD), an adaptation of RAD-seq, is a reduced representation technique that allows rapid sequencing of thousands of homologous regions from degraded tissue, both with and without an available reference genome (Davey *et al.,* 2010). It differs from traditional RAD-seq in that it does not use restriction enzymes to reduce the complexity of the genome. Instead, the DNA is fragmented with Nextera transposomes, which also add a short adapter sequence (Appendix one, supplementary materials). The smaller number of steps in the protocol when compared to traditional RAD-seq reduces loss of DNA during library creation, allowing much lower input compared to other methods (Elfekih *et al.,* 2018; Eric

Johnson, personal communication, 2018). Hence, genome-wide information can be obtained from single individuals of non-model organisms utilizing very little tissue (Russello *et al.,* 2015; Filatov *et al.,* 2016; Fu *et al.,* 2017; Elfekih *et al.,* 2018), an important consideration when working with endangered batoids that can be difficult to sample.

Despite the success of molecular markers in phylogenetics, taxonomic questions still surround many species of skates and rays, particularly those inhabiting relatively isolated regions. For example, the polytypic nature and wide geographic range of the thornback ray (*Raja clavata*) means this species is often misidentified, and it is currently unclear as to whether records of *R. clavata* from Madeira and the Azores actually refer to the endemic Madeiran skate (*Raja maderensis*), a local Azorean form or subspecies (Ball *et al.,* 2016). Despite formal separation of the thornback ray and Madeiran skate, mtDNA has been unsuccessful in supporting the distinctiveness of *R. maderensis,* and delineation of these species is currently ambiguous (Ball *et al.,* 2016). Furthermore, unexpectedly highly divergent thornback ray haplotypes have been observed off the coast of Portugal, despite no known barrier to interpopulation gene flow in the region (Ball *et al.,* 2016). Preliminary mtDNA data also suggests a cryptic *Dipturus* species from the Azores could be present, perhaps misidentified as the longnosed skate (*Dipturus oxyrinchus;* Andrew Griffiths, unpubl. data). This is highly consistent with previous research that have observed genetic differences between populations of marine organisms from the Azores and the rest of the North Atlantic (Chevlot *et al.,* 2006; Stefanni *et al.,* 2006; Dominguez *et al.,* 2007; Naylor *et al.,* 2012; Ball *et al.,* 2016). Regional differentiation is also frequently recorded in the Mediterranean, as the Atlantic-Mediterranean transition acts as an important genetic barrier for many marine species (Borsa *et al.,* 1997; Zane *et al.,* 2000; Wilke & Pfenninger 2002; Coyer *et al.,* 2003; Duran *et al.,* 2004; Gysels *et al.,* 2004; Olsen *et al.,* 2004; Baus *et al.,* 2005; Cimmaruta *et al.,* 2005; Nakadate *et al.,* 2005; Provan *et al.,* 2005; Chevolot *et al.,* 2006; Griffiths *et al.,* 2011). This has led to significant spatial genetic structuring across the transition and even examples of cryptic speciation, which further complicate conservation assessments (e.g. Muricy, 1996; Carreras-Carbonell *et al.,* 2005; Remerie *et al.,* 2006). Currently, there is confusion surrounding the taxonomic status of the

Norwegian skate (*Dipturus nidarosiensis*) in the Mediterranean, a near-threatened species of benthic skate found throughout the Eastern North Atlantic (Ebert & Stehann, 2013). It has been discovered in the Mediterranean only recently (Cannas *et al.,* 2010), and it is not clear whether records of *D. nidarosiensis* from the region are distinct from those across the rest of the North Atlantic. Morphological evidence suggests that several specimens collected from the Sardinian Channel and off the coast of Portugal could refer to a smaller, undescribed *Dipturus* species (Cannas *et al.,* 2010; Ebert & Stehann, 2013; M. Stehmann, unpubl. data).

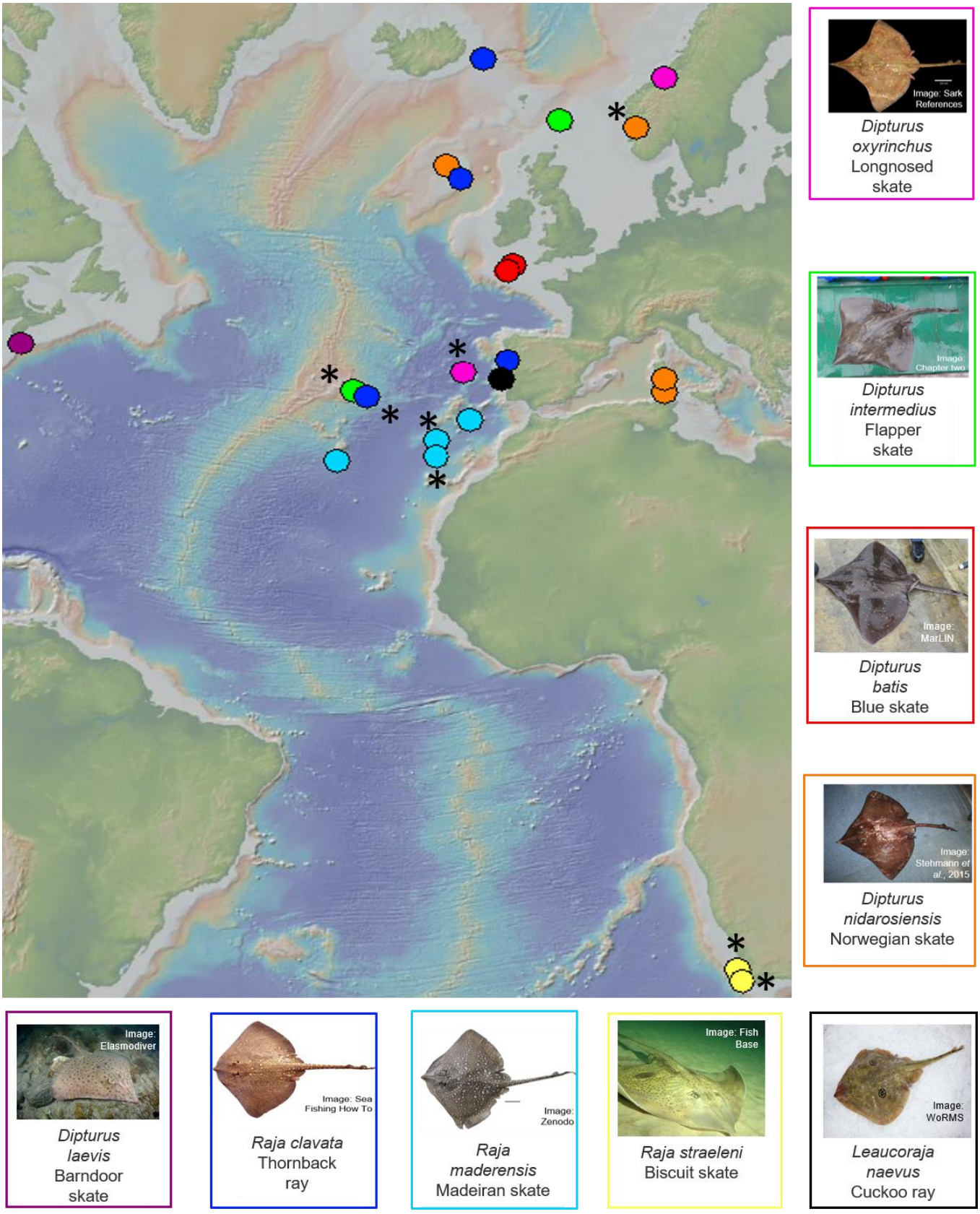
The primary goals of the present study were to (1) assess the utility of NextRAD, in combination with COI and CR sequencing, in providing a high-resolution batoid phylogeny; (2) to resolve the question of whether the thornback ray and Madeiran skate are distinct species or not; (3) to examine if Norwegian skate from the Mediterranean are genetically distinct from those across the rest of the North Atlantic; and (4) to determine if there is an additional cryptic *Dipturus* species present in the Azores.

3.3 Methods

3.3.1 Sample collection and DNA extraction

Samples were obtained from fin clips from *Dipturus* and *Raja* species, taken at various locations across the North Atlantic (Figure one). For the *Dipturus* genus, the following specimens were sampled: two blue skate from the Celtic Sea; one longnosed skate each from the Azores, Portugal and Norway; two flapper skate from the Shetlands and one from the Azores; two Norwegian skate from the Mediterranean, one from Rockall and one from Norway. For the *Raja* genus, two thornback rays from Portugal, one from Rockall, one from Norway and one from the Azores were sampled, along with two

Madeiran skate from Madeira and two from seamounts in the Azores. Two biscuit skate (*Raja straeleni*) samples were also included in analysis as a comparison for *R. clavata/ R. maderensis.* Two cuckoo ray (*Leucoraja naevus*) individuals were also sampled from Portugal which were identified as a suitable outgroup following Naylor *et al.* (2016). For detail latitude and longitude information and GenBank accession numbers for all species, see Appendix two, supplementary materials. All individuals were identified by fisheries biologists upon sampling, using biological keys. Tissue samples were then preserved in absolute ethanol prior to storage at -20°C. A total of 26 individuals were sampled and sequenced. Genomic DNA was extracted from all 26 samples using the Qiagen DNeasy Blood and Tissue Kit (Venlo, Netherlands), including treatment with RNase (2 µl of 100mg/ml), and run on a 1% agarose gel to assess their quality and concentration. The concentration of double stranded DNA (dsDNA) was further quantified with fluorometry using the Invitrogen Qubit Assay.



1793 **Figure 1.** Map detailing the locations of specimens sampled in the current study. * Indicates the exact
1794 latitude and longitude of the sample was unknown, in this instance the point shows an approximate
1795 location. All points represent one specimen.

3.3.2 MtDNA sequencing and phylogenetic analysis

All individuals, except those that already had data on Genbank (Appendix two, supplementary materials), were sequenced for a partial region of the mitochondrial CR, following Griffiths *et al.,* (2010) and the COI gene following Ward *et al.,* (2005). PCR products were sequenced by Source Bioscience, (Nottingham, UK) and the results checked by eye in BIOEDIT version 7.1.11 for ambiguous base calls and sequencing errors (Hall, 1999).

Mitochondrial CR and COI datasets were concatenated and phylogenetic analysis performed in RAxML v.8.2.4 under the GTRCAT and HKY85 model (Stamatakis, 2014). The most appropriate substitution model for phylogenetic analyses was determined using MEGA 7.0.26 (Kumar *et al.,* 2016). All trees were rooted using a *Leucoraja naevus* outgroup (mtDNA accession number CR= AY218369.1, COI= HQ603898.1) and 1000 bootstrap replicates was performed (Stamatakis, Hoover, & Rougemont, 2008). The resulting trees were visualised in FigTree v1.4.3 (Rambaut, 2014), where all branches with bootstrap values below 50% were collapsed to produce the final tree topology. The number of polymorphic and monomorphic sites were calculated in DnaSP v.5.1 (Rozas *et al.,* 2003). Pairwise distances at different taxonomic levels (congeneric and confamilial) were estimated using the Kimura 2-parameter (K2P) distance model (Kimura, 1980), implemented in MEGA 7.0.26 (Kumar *et al.,* 2016)

3.3.3 NextRAD DNA sequencing and phylogenetic analysis

For each sample, an aliquot of DNA was sent to SNPsaurus LLC (Oregon, USA) for nextRAD-seq, due to its ability to produce RAD libraries from lower quantities of input DNA. This was particularly relevant for the degraded samples in the present study, which varied in quality and concentration (Appendix three, supplementary materials). In order to act as a quality control, duplicates of samples Bar06125, D51, C018 and D45 were sent for sequencing, totalling 30 samples. Genomic DNA was converted into nextRAD genotyping-by-sequencing libraries (SNPsaurus, LLC) as in Russello *et al.,*

(2015) and first fragmented with Nextera reagent (Illumina, Inc), which also ligates short adapter sequences to the ends of the fragments (Appendix one, supplementary materials). The Nextera reaction was scaled for fragmenting 25 ng of genomic DNA, although 50 ng of genomic DNA was used for input to compensate for the amount of degraded DNA in the samples and to increase fragment sizes. Fragmented DNA was then amplified for 27 cycles at 74 degrees, with one of the primers matching the adapter and extending 10 nucleotides into the genomic DNA with the selective sequence GTGTAGAGCC. Thus, only fragments starting with a sequence that can be hybridized by the selective sequence of the primer were efficiently amplified. The nextRAD libraries were sequenced on a HiSeq 4000 with one lane of 150 bp reads (University of Oregon).

The quality of the raw reads was checked using FASTQC v (Andrews, 2010), revealing a drop off in quality above 120 bp. The process_radtags programme of Stacks v1.45 (Catchen *et al.,* 2013) was subsequently run with the -no_barcode option to truncate reads to 120 bp using the –t flag. RAD check was disabled using the –disable_rad_check option and any reads with low quality scores or an uncalled base were discarded using the –q and –c flags, respectively. These 'cleaned reads' for each sample were then used to run Ipyrad v.0.6.15 using the denovo assembly method and default parameters (Eaton & Overcast, 2016). The resulting clusters represent putative RAD loci shared across samples. To test the effects of missing data, four datasets with different thresholds for the minimum number of samples per locus (ms) were run. A 'generous' dataset was included where ms was set to two (ms2), because the majority of species (or populations) were represented by at least two samples. Ms30 represented a 'reduced' dataset of loci shared by all individuals. Ms4 (default parameter) and ms12 were used as intermediate thresholds.

Concatenated SNP data was used to infer phylogenetic relationships using the GTRCAT substitution model implemented in RAxML v.8.2.4 (Stamatakis, 2014). All trees were rooted using the *Leucoraja naevus* outgroup and 1000 bootstrap replicates were performed (Stamatakis, Hoover, & Rougemont, 2008). A consensus tree for each dataset was built using the Consensus Tree Builder in

Geneious 6.0.6 (Biomatters, Aukland, New Zealand), support threshold was set to 50% with a 10% burn-in. The resulting trees of all analyses were visualised in FigTree v1.4.3 (Rambaut, 2014), where bootstrap values were plotted onto the most likely tree obtained from RaxML. As a quality control, trees were built both with and without duplicate samples from the ms4 dataset. Given that RAxML violates several assumptions inherent in SNP data (see Leaché & Oaks, 2017 for a review), a phylogenetic tree was also built using SVDquartet, a quartet-based method developed specifically for SNP data (Chhifman & Kubatko, 2014), implemented in PAUP (Swofford, 1993). A multispecies coalescent model was used with 1000 bootstrap replicates, with all trees routed with the *L. naevus* outgroup. The 50% consensus tree produced by SVDquartet was then analysed. Combining RAxML and SVDquartet analysis to RAD-seq SNP data processed via the Ipyrad pipeline has previously been utilised in phylogenetic studies (Anderson *et al.,* 2017). Pairwise distances at different taxonomic levels (confamilial, congeneric and conspecific) were estimated using the Kimura 2-parameter (K2P) distance model (Kimura, 1980), implemented in MEGA 7.0.26 (Kumar *et al.,* 2016).

3.4 Results

3.4.1 Variability in mitochondrial genes

The alignment of the COI and CR genes consisted of 569 and 695 bp respectively. Considering all species in the dataset, overall mean values of pairwise KP2 sequence divergence values were similar for both the COI (0.067%) and CR (0.076%) genes. After confirming homogeneity of phylogenetic signal of the two sequence sets (partition homogeneity test p= 1.00; Swofford, 2000), alignments were concatenated. Other parameters describing variability in the mitochondrial genes can be found in Table one.

Table 1. Characterisation of sequence variation in the cytochrome oxidase I (COI) and control region (CR) mitochondrial genes.

Parameters	COI gene	CR gene
Alignment length (bp)	569	695
Polymorphic sites	110	196
Monomorphic sites	458	480
Parsimony informative sites	77	91
Haplotype diversity (Hd)	0.930	0.987
Nucleotide diversity (Pi)	0.063	0.069

3.4.2 MtDNA phylogenetic analyses

CR (695 bp) and COI (569 bp) genes were concatenated to produce a 1264 bp alignment. The final concatenated data matrix contained 306 polymorphic and 938 monomorphic sites. Within-species K2P mean distance (0.42%) was 10x lower than average congeneric distance in the *Dipturus* genus (4.27%) and 4x lower than in the *Raja* genus (1.81%). Average confamilial distance was 9.40% (Table two, three). The maximum intraspecific distance (1.35%) was observed for the species *D. oxyrinchus,* whilst *D. batis* had the lowest (0%, Table two). Although, this is not surprising given that all *D. batis* individuals in the present study were sampled from the Celtic Sea. A more accurate representation of intraspecific distance for *D. batis* may be obtained by sequencing more individuals from different locations.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 *R. clavata*- Rockall		0.0000	0.0016	0.0121	0.0105	0.0113	0.0089	0.0072	0.0105	0.0153	0.0162	0.0951	0.0942	0.0951	0.0942	0.0950	0.0970	0.0988	0.0988	0.0933	0.0905	0.0905	0.0905	0.0915	0.2094
2 *R. clavata*- Portugal	0.1548		0.0016	0.0121	0.0105	0.0113	0.0089	0.0072	0.0105	0.0153	0.0162	0.0951	0.0942	0.0951	0.0942	0.0950	0.0970	0.0988	0.0988	0.0933	0.0905	0.0905	0.0905	0.0915	0.2094
3 *R. clavata*- Norway	0.1052	0.1437		0.0137	0.0089	0.0097	0.0105	0.0088	0.0089	0.0170	0.0178	0.0951	0.0942	0.0970	0.0961	0.0969	0.0970	0.0988	0.0988	0.0951	0.0923	0.0923	0.0923	0.0933	0.2117
4 *R. clavata*- Portugal	0.1158	0.1440	0.1140		0.0154	0.0162	0.0137	0.0121	0.0154	0.0211	0.0219	0.0990	0.0981	0.0990	0.0981	0.0989	0.1010	0.1027	0.1027	0.0971	0.0951	0.0951	0.0951	0.0942	0.2084
5 *R. maderensis*- Madeira	0.1226	0.1795	0.1121	0.1358		0.0008	0.0016	0.0032	0.0000	0.0186	0.0195	0.0942	0.0933	0.0943	0.0933	0.0934	0.0942	0.0961	0.0961	0.0924	0.0924	0.0924	0.0924	0.0916	0.2107
6 *R. maderensis*- Madeira	0.1174	0.1735	0.1009	0.1418	0.0701		0.0024	0.0040	0.0008	0.0195	0.0203	0.0951	0.0942	0.0952	0.0942	0.0943	0.0951	0.0970	0.0970	0.0933	0.0915	0.0915	0.0915	0.0907	0.2117
7 *R. maderensis*- Irving seamount	0.1104	0.1674	0.0991	0.1314	0.0693	0.0643		0.0016	0.0016	0.0170	0.0178	0.0942	0.0933	0.0924	0.0914	0.0915	0.0942	0.0961	0.0961	0.0906	0.0906	0.0906	0.0906	0.0897	0.2084
8 *R. maderensis*- Siene seamount	0.1377	0.1867	0.1240	0.1526	0.1114	0.0957	0.0850		0.0032	0.0170	0.0178	0.0951	0.0942	0.0933	0.0924	0.0932	0.0952	0.0970	0.0970	0.0914	0.0905	0.0905	0.0905	0.0897	0.2092
9 *R. clavata*- Azores	0.1898	0.2484	0.1814	0.1976	0.1597	0.1454	0.1258	0.1756		0.0186	0.0195	0.0942	0.0933	0.0943	0.0933	0.0934	0.0942	0.0961	0.0961	0.0924	0.0924	0.0924	0.0924	0.0916	0.2107
10 *R. straelini*	0.1799	0.2211	0.1758	0.1859	0.2160	0.2093	0.2040	0.2264	0.2931		0.0008	0.0933	0.0924	0.0906	0.0897	0.0923	0.0942	0.0978	0.0978	0.0923	0.0923	0.0923	0.0923	0.0914	0.2105
11 *R. straelini*	0.1869	0.2204	0.1765	0.1815	0.2135	0.2089	0.1971	0.2258	0.2716	0.0905		0.0924	0.0914	0.0897	0.0887	0.0914	0.0933	0.0988	0.0988	0.0932	0.0932	0.0932	0.0932	0.0924	0.2116
12 *D. oxyrinchus*- Norway	0.9778	0.6576	0.6631	0.5861	0.6689	0.6966	0.6573	0.7060	0.6402	0.6895	0.6543		0.0008	0.0178	0.0170	0.0178	0.0203	0.0327	0.0327	0.0277	0.0514	0.0514	0.0514	0.0523	0.1906
13 *D. oxyrinchus*- Portual	0.7033	0.6632	0.6608	0.5798	0.6838	0.7123	0.6681	0.7143	0.6254	0.7052	0.6704	0.0418		0.0170	0.0162	0.0170	0.0914	0.0318	0.0318	0.0268	0.0523	0.0523	0.0523	0.0532	0.1896
14 *D. intermedius*- Shetlands	0.6582	0.6392	0.6233	0.5416	0.6512	0.6791	0.6318	0.6866	0.5840	0.6617	0.6119	0.1315	0.1280		0.0008	0.0016	0.0080	0.0344	0.0344	0.0260	0.0532	0.0532	0.0532	0.0541	0.1843
15 *D. intermedius*- Shetlands	0.6403	0.6101	0.6143	0.5549	0.6395	0.6600	0.6174	0.6609	0.6154	0.6495	0.6098	0.1157	0.1147	0.0383		0.0024	0.0088	0.0335	0.0335	0.0252	0.0523	0.0523	0.0523	0.0532	0.1832
16 *D. intermedius*- Azores	0.6561	0.6434	0.6488	0.5846	0.6743	0.6872	0.6378	0.6943	0.6339	0.6683	0.6387	0.1215	0.1257	0.0571	0.0441		0.0080	0.0352	0.0352	0.0268	0.0549	0.0549	0.0549	0.0558	0.1851
17 *D. oxyrinchus*- Azores	0.5970	0.5788	0.5728	0.5295	0.5999	0.6221	0.5669	0.6095	0.5633	0.6014	0.5675	0.0880	0.0808	0.1247	0.1141	0.1196		0.0319	0.0319	0.0244	0.0576	0.0576	0.0576	0.0585	0.1917
18 *D. batis*	0.6815	0.6762	0.6618	0.5820	0.6783	0.6992	0.6545	0.7176	0.6471	0.6935	0.6437	0.1552	0.1503	0.1679	0.1565	0.1677	0.1479		0.0000	0.0104	0.0574	0.0574	0.0574	0.0584	0.1911
19 *D. batis*	0.6723	0.6583	0.6436	0.5780	0.6613	0.6811	0.6429	0.6875	0.6521	0.6802	0.6455	0.1501	0.1508	0.1670	0.1533	0.1651	0.1425	0.0049		0.0104	0.0574	0.0574	0.0574	0.0584	0.1911
20 *D. laevis*	0.6715	0.6483	0.6420	0.5606	0.6636	0.6837	0.6229	0.6953	0.6296	0.6671	0.6207	0.1386	0.1374	0.1501	0.1351	0.1468	0.1314	0.0975	0.0886		0.0531	0.0531	0.0531	0.0540	0.1879
21 *D. nidarosiensis*- Mediterranean	0.6649	0.6431	0.6390	0.5596	0.6588	0.6760	0.6294	0.6826	0.6251	0.6669	0.6378	0.1763	0.1718	0.1924	0.1784	0.1887	0.1695	0.1908	0.1819	0.1626		0.0000	0.0016	0.0024	0.1932
22 *D. nidarosiensis*- Mediterranean	0.7075	0.6661	0.6811	0.6008	0.7096	0.7253	0.6775	0.7122	0.6828	0.7047	0.6855	0.1825	0.1806	0.1991	0.1852	0.1989	0.1792	0.2001	0.1949	0.1736	0.0321		0.0016	0.0024	0.1932
23 *D. nidarosiensis*- Rockall	0.6449	0.6379	0.6055	0.5516	0.6382	0.6529	0.6258	0.6534	0.5996	0.6344	0.6139	0.1785	0.1734	0.1967	0.1776	0.1913	0.1739	0.1940	0.1872	0.1683	0.0351	0.0370		0.0008	0.1932
24 *D. nidarosiensis*- Norway	0.6513	0.6401	0.6302	0.5647	0.6300	0.6680	0.6131	0.6629	0.6017	0.6401	0.6211	0.1780	0.1775	0.1989	0.1825	0.1955	0.1713	0.1946	0.1894	0.1658	0.0351	0.0380	0.0422		0.1923
25 *L. naevus*	1.2611	1.2017	1.1751	1.0504	1.1882	1.2286	1.1713	1.2700	1.2081	1.2359	1.2002	0.9849	1.0062	0.9870	0.9505	0.9520	0.8637	0.9862	0.9607	0.9585	0.9441	0.9909	0.9116	0.9187	
26 *L. naevus*	1.2923	1.2151	1.1605	1.1093	1.2060	1.1775	1.1689	1.2918	1.1850	1.2621	1.2168	0.9762	1.0232	0.9623	0.9496	0.9668	0.8768	0.9769	0.9617	0.9568	0.9479	0.9852	0.9159	0.9281	0.0267

0 to 0.02 | 0.0201 to 0.04 | 0.0401 to 0.06 | 0.0601 to 0.08 | 0.0801 to 0.1 | 0.1001 to 0.2 | 0.2001 to 0.4 | 0.4001 to 0.6 | 0.6001 to 0.8 | 0.8001 to 1 | 1.001+

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Table 2. Pairwise KP2 sequence divergence values for each specimen for concatenated control region (CR) and cytochrome oxidase I (COI) data (top right) and nextRAD SNP data (bottom left).

Table 3. Pair-wise concatenated control region (CR) and cytochrome oxidase I (COI) mtDNA distances (expressed in percent; K2P model) of skate species.

Comparison	**Minimum Distance**	**Mean Distance (±SE)**	**Maximum Distance**
Confamilial- between *Raja* and *Dipturus* species of skate	8.87	9.40 ± 0.02	10.27
Congeneric- *Raja*	1.53	1.81 ± 0.04	2.19
Congeneric- *Dipturus*	1.04	4.27 ± 0.19	5.85

MtDNA data failed to support the monophyly of the *Dipturus* genus, and instead produced a topology with three monophyletic groups: the Norwegian skate (*D. nidarosiensis*), all other *Dipturus* species and *Raja* species of skate (Figure 2A). Within these groups, relatively strong bootstrap support for the monophyly of most *Dipturus* and *Raja* species could be seen (bootstrap support= 62-100%), with the exception of the thornback ray (*R. clavata*) and Madeiran skate (*R. maderensis*). Within the latter species, groupings did not appear to be based on current species classifications, but patterns were broadly based on the geographic location of populations, with the exception of thornback rays from Portugal. In the north, thornback rays from Norway, Rockall and one specimen from Portugal (RJC120) formed a monophyletic group (bootstrap support= 100%). In the south, Madeiran skate/ thornback rays from the Azores, Madeira, proximate seamounts and one specimen from Portugal (RJC57) formed the second monophyletic group (bootstrap support= 62%). Within the latter, *R. maderensis* from Madeira and *R. clavata* from the geographically proximate Azores were reciprocally monophyletic. Madeiran skate from the Irving and Siene seamount were genetically distinct from these populations, despite being geographically proximate to Madeira. One specimen from Portugal was genetically distinct from all other thornback rays and Madeiran skate in this clade, although

support for this was only relatively strong (bootstrap support= 62%). One specimen of *D. oxyrinchus* from the Azores appeared to be genetically distinct from other longnosed skate in Norway and Rockall, instead forming a monophyly with flapper skate (*D. intermedius*) with high bootstrap support (bootstrap support= 85%). Genetic divergence between Azorean longnosed skate and their counterparts in the rest of the North Atlantic was 1.99%. Similarly, flapper skate in the Azores were genetically distinct from those in the Shetlands (bootstrap support= 79%, genetic divergence= 0.2%). There was also strong support for the genetic distinctiveness of *D. nidarosiensis* in the Mediterranean in mtDNA sequence trees (bootstrap support= 100%, genetic divergence= 0.2%).

3.4.3 NextRAD quality control and phylogenetic analyses

A total of 1,173,094 low quality reads were filtered by to produce 314,075,645 'cleaned reads' that were used for the Ipyrad pipeline. All filtered datasets contained SNPs varying in number from 1,854,259 to 37, from all 30 individuals. The percentage of missing data was also variable; ms2 had the highest proportion of missing data (82.42%), whilst ms30 represented the lowest (2.60%; Table four).

Table 4. The percentage of missing data and the number of concatenated SNPs in the final fasta alignments for each nextRAD dataset.

Dataset	% Missing data	Number of SNPs in the final alignment
ms2	82.42	1,854,259
ms4	21.22	323,850
ms12	43.66	176,091
ms30	2.60	37

Phylogenetic trees built from the ms4 and ms12 datasets yielded identical tree topologies that differed only in bootstrap support and branch lengths (Appendix four, supplementary materials). Within each tree, two monophyletic clades were produced for the *Raja* and *Dipturus* genera. *D. nidarosiensis* was the only clade that differed slightly in the ms2 dataset; unlike in trees built from ms4 and ms12, ms2 Norwegian skate from the Mediterranean were not reciprocally monophyletic. This is with the exception of the ms30 dataset, which yielded a RaXML tree with unresolved polytomies (a section of a phylogenetic tree in which the relationships cannot be fully resolved into a series of two-way splits) and little phylogenetic support or branch lengths (Appendix four, supplementary materials). All duplicate samples appeared in identical phylogenetic positions in phylogenetic trees, indicating good quality control during nextRAD sequencing (Appendix four, supplementary materials). From this point forward results will be described based on the RaXML tree built from the ms4 dataset (Figure 2B), which has the highest bootstrap support (100%) and the lowest amount of missing data. The SVDquartet tree built from the ms4 dataset was largely congruent with the comparable RaXML tree, with a few minor differences within the *Raja* genus and generally lower bootstrap support (83%-100%; Figure 2B, Figure three). Specifically, the SVDquartet tree did not support the monophyly of Portuguese thornback rays*,* but did place *R. clavata* from Rockall and Norway as reciprocally monophyletic (Figure three). Additionally, Madeiran skate from the Irving seamount and thornback rays from the Azores formed their own monophyly, which was not present in the RAxML tree.

NextRAD confamilial distance was 7x higher than mtDNA (64.45%, Table five), congeneric 12x higher within the *Raja* genus (21.07%) and four times higher within the *Dipturus* genus (16.51%). Intraspecific K2P mean distance (7.14%) was two times lower than average congeneric distance in the *Dipturus* genus (16.51%) and three times lower than in the *Raja* genus (21.07%). Average confamilial distance was 52.95% (Table five). The maximum intraspecific distance (13.58%) was observed for the species *R. clavata/ R. maderensis,* whilst *D. nidarosiensis* had the lowest (3.65%, Table two).

Table 5. Pairwise nDNA (nextRAD-seq data) barcode distances (expressed in percent; K2P model) of skate species.

Comparison	Minimum Distance	Mean Distance (±SE)	Maximum Distance
Confamilial- between *Raja* and *Dipturus* species of skate	52.95	64.45 ± 0.34	72.53
Congeneric- *Raja*	17.58	21.07 ± 0.71	29.13
Congeneric- *Dipturus*	8.86	16.51 ± 0.34	20.01

With the exception of the thornback ray and Madeiran skate, all species formed well-supported monophyletic groups in nDNA sequence trees (bootstrap support= 85 to 100%). With the exception of those from Portugal, geographical proximity, not morphologically identified species, appeared to be the main driver of phylogenetic position within the *R. clavata*/ *R. maderensis* clade. Thornback ray from the Azores formed a distinct group with Madeiran skate from the proximate Irving seamount with high bootstrap support (100%). Madeiran skate from other isolated regions off the coast of Portugal (Madeira and the Siene seamount) were also proximate to Azorean thornback ray in nextRAD sequence trees. Overall, thornback rays and Madeiran skate from seamounts in the eastern Atlantic and Madeira were more closely related to each other than to thornback ray from Norway and Rockall. Interestingly, two *R. clavata* specimens from Portugal formed their own clade with high bootstrap support in RAxML nDNA trees, potentially representing their own distinct genetic lineage (bootstrap support= 100%), but this was not supported in SVDquartet trees. Sequence divergence between these Portuguese specimens and their counterparts in Norway was 10.96% (Table two).

1976

1977

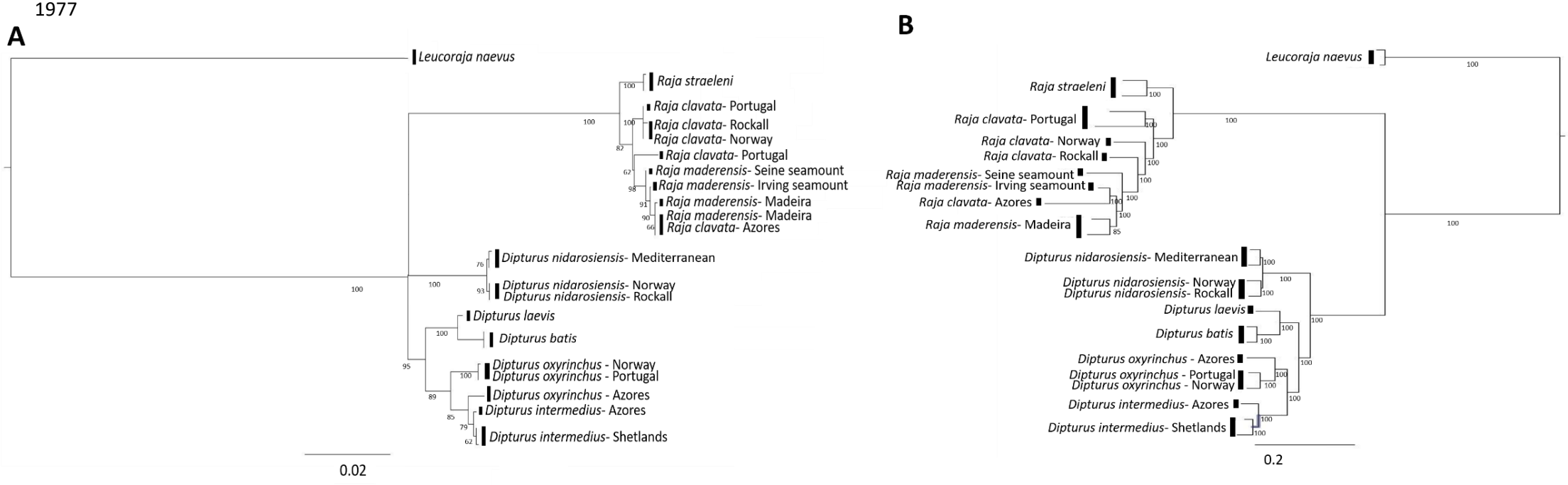


Figure 2. (A) RAxML tree built from concatenated (CR and COI) mtDNA data (ms4); (B) RAxML tree built from nextRAD-seq data (ms4 dataset). Numbers below branches represent bootstrap support values. Scale bars refer to the number of substitutions per site.

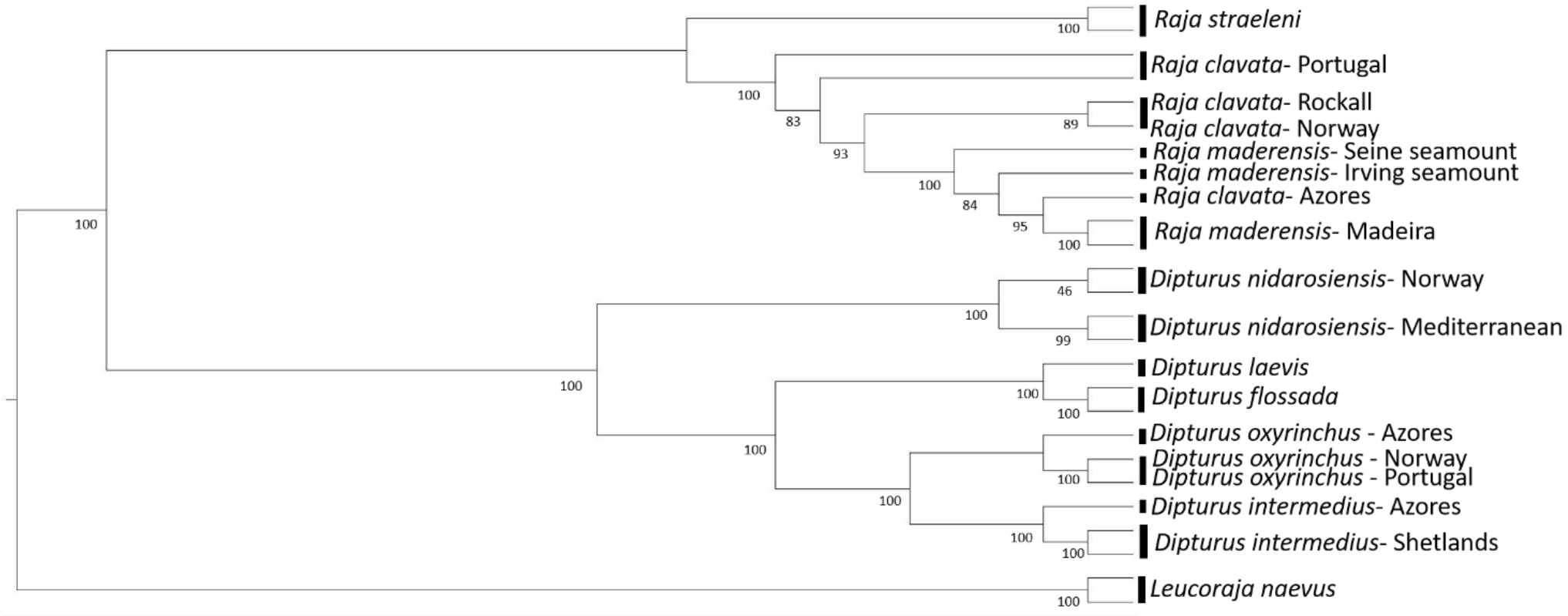


Figure 3. SVDquartet tree built from nextRAD-seq data (ms4 dataset). Support from 1000 bootstrap replicates are shown for branches with support >50%. Branch lengths do not reflect divergence.

Strong support for the genetic distinctiveness of *D. nidarosiensis* from the Mediterranean could be seen in trees built from nextRAD data (bootstrap support= 100%, genetic divergence= 3.65%). Additionally, *D. oxyrinchus* and *D. intermedius* from the Azores appeared to be genetically distinct from their counterparts in the rest of the North Atlantic (bootstrap support= 100%), potentially representing their own genetic lineage (genetic divergence was 8.44% and 5.06% respectively).

3.5 Discussion

3.5.1 Utility of mtDNA-seq and nextRAD-seq for batoid phylogenies

The mitochondrial CR and COI genes have been used extensively in batoid phylogenetics, due to their high degree of nucleotide polymorphism and success in delineating species (Valsecchi *et al.,* 2005; Ward and Holmes, 2007; Moura *et al.,* 2008; Wynen *et al.,* 2009; Serra-Pereira *et al.,* 2010). In the present study, mtDNA phylogenies were generally well-resolved, with relatively high bootstrap support for most monophyletic species (bootstrap support= 62-100%). Although, in several cases, low bootstrap support and poor resolution was observed. The concatenated mtDNA data failed to resolve the monophyly of *Dipturus* species of skate, producing instead 3 monophyletic groups: the Norwegian skate (*D. nidarosiensis*), all other *Dipturus* species and *Raja* species of skate. However, within the *Raja* and *Dipturus* genus, mitochondrial data successfully resolved expected species groupings and revealed fine-scale patterns of genetic structuring.

Of interesting note to taxonomists is the value of the COI and CR genes for phylogenetics in isolation. Whilst the large majority of taxonomists advocate for the use of mtDNA in context with other data sources (morphology, meristic data, nDNA), two extreme viewpoints have also emerged. One position argues for the discontinued use of mtDNA in phylogenetics (Ballard and Whitlock, 2004; Shaw, 2004), the other extreme advocates for the sole use of the 600-bp COI 'barcoding' gene for taxonomic

identification (Hebert *et al.,* 2003b). The latter viewpoint has been a source of significant controversy in recent decades, with many criticising the 'one gene fits all' philosophy for large-scale analysis (Hebert *et al.,* 2003b; Lipscomb *et al.,* 2003; Pennisi, 2003; Scotland *et al.,* 2003; Will & Rubinoff, 2004). In the present study, phylogenetic trees built solely from the COI gene contained several unresolved polytomies, that were well-resolved within the CR, concatenated mtDNA and nextRAD phylogeny (Appendix three, supplementary materials). Namely, COI data failed to resolve the complex genetic structuring within the thornback ray/ Madeiran skate clade, and the genetic distinctiveness of Azorean flapper skate from their counterparts in the Shetlands. Whilst the CR produced a topology that resolved these groupings, generally lower bootstrap support was seen in this tree. Previous authors have highlighted the significance of combining low-supported phylogenies into a single analysis in improving phylogenetic support and resolution (Baker *et al.,* 2002). In the present study, the concatenated COI and CR topology was more congruent with the nextRAD phylogeny and contained higher bootstrap support than the individual gene trees, supporting such an approach. Increased resolution could further be achieved through the sequencing of more mitochondrial markers, or entire mitogenomes (Botero-Castro *et al.,* 2013; Williams *et al.,* 2017). Recent studies, including the most comprehensive molecular assessment of global elasmobranch taxonomy to date, have successfully utilised the NADH2 gene for species delineation in batoids (Naylor *et al.,* 2012; Lim *et al.,* 2015; Henderson *et al.,* 2016). Due to its high level of polymorphism (Naylor *et al.,* 2012; Lim *et al.,* 2015; Henderson *et al.,* 2016), this gene represents a good candidate to fully resolve the mtDNA phylogeny; it is a well-accepted dogma that more data generally improves the accuracy of analysis (Cummings, 1994; Poe & Swofford, 1999; Mitchell *et al.,* 2000).

In the current study, nextRAD-seq was also used to construct phylogenies of members of the *Dipturus* and *Raja* genus of batoids, due to its ability to resolve phylogenies of non-model organisms (e.g. Cruaud *et al.,* 2014; Herrera and Shank, 2015; Leaché *et al.,* 2015). With the exception of the ms30 dataset, all nextRAD-seq trees were well-resolved with high BS, indicating that this method is useful for relatively shallow levels of divergence. In several cases, the inclusion of nextRAD data offered an opportunity to gain further resolution and insight from the mitochondrial phylogeny, due to

discordance between the two datasets. At the lower taxonomic level, longnosed skate from the Azores were placed as a sister species to flapper skate in mtDNA sequence trees; this was not supported by the nextRAD phylogenies, with this specimen grouping with longnose skate from Norway and Portugal. This could reflect the low resolution of mtDNA data, as mtDNA has been known to obscure species boundaries in taxa (Avise, 1994; Giannasi *et al.,* 2001; Shaw, 2002). Furthermore, concatenated mtDNA sequences were much shorter (1,264 bp) than the nextRAD SNP datasets.

In studies employing next-generation sequence data sets for phylogenetic inference, the impact of missing data on the final data matrix is an important point of consideration, particularly as for SNP data, the amount of missing data increases with the number of loci and the sample size (Wagner *et al.,* 2013). In extreme cases, taxa with insufficient data across loci may even be excluded from analysis over concerns that this could lead to poor phylogenetic resolution (Bininda-Edmonds *et al.,* 2002; Wiens 2003). Furthermore, there can be large variation among loci in the amount of missing data, which can arise from mutations at transposome cutting sites, large variations in the number of reads per locus per individual and allocated coverage thresholds in the data processing step (Huang & Knowles, 2016). However, Eaton *et al.,* (2017) suggest that this has only a minor effect on phylogenetic inference. In the present study, four coverage thresholds (ms2, ms4, ms12 and ms30) were used, that vary by the minimum number of samples that must have data at a given locus in order to be retained in the final data set. Ms2, ms4 and ms12 yielded nearly identical RAxML tree topologies, despite having varying amounts of missing data (21.22- 81.42%), indicating RAxML analysis is relatively robust to variation in Ipyrad assembly parameters. Furthermore, although the number of SNPs necessary for phylogenetic resolution can largely be dependent on the variability of loci or study organism (Wagner *et al.,* 2018), the ms2 and ms12 datasets varied in the number of SNPs by over a factor of 10. The unresolved polytomies resulting from the ms30 dataset are perhaps not surprising, given that it represents just 37 SNPs shared by all individuals- the most restrictive dataset in the current study.

3.5.2 Delineating species

An important caveat when interpreting results in the current study concerns the definition of species, which remains one of the most contentious debates is systematics (see Leaché & Oaks, 2017 for a review) . In the current study, species delineation relied on two major principals: reciprocal monophyly (RM) and magnitude of sequence divergence. The concept of RM was first established in the context of lineage sorting- an evolutionary model that explains the incongruence between species and gene trees in mitochondrial data (Avise *et al.,* 1983; Neigel and Avise, 1986; Avise and Ball, 1990; Avise, 2000. Kizirian & Donnelly, 2004). In this model, phylogenies built from mtDNA data may differ from the actual species tree in recently diverged species. Only after sufficient time has elapsed to achieve RM, in a process known as lineage sorting, will the species and gene trees be congruent. RM has been used across systematics, however, several authors have recognised the potential of overlooking significant nested units of diversity in cases where RM is not met (Mortiz, 1994; Paetkau, 1999; Crandall *et al.,* 2000; Kizirian & Donnelly, 2004; Madison & Knowles, 2006). This is particularly true for recently diverged sister species, which may not have time to reach RM (incomplete lineage sorting), resulting in phylogenies that suggest paraphyly for one or both species. Nevertheless, the application of RM has been extended to defining species boundaries, and has even been used to evidence the presence of cryptic batoid species (e.g. Griffiths *et al.,* 2010).

In consideration of RM's wide use in the field of taxonomy and theoretical deficiencies, particularly surrounding sibling or recently emerging species (Paetkau, 1999; Kiziran & Donnelly, 2004; Leaché & Oaks, 2017), the current study also considered pairwise sequence divergence values. Employing a cut-off for sequence divergence from mtDNA data has often been used as a prori for species delineation (e.g. Blaxter, 2004). However, Lohse (2009) argue that such an approach is arbitrary at best, given the large variations in within-species genetic diversity. Indeed, Hebert *et al.,* (2003a) demonstrated that COI divergence among 13,320 congeric species in 11 animal families ranged from 0.0% to 53.7%. Despite this, their results also indicated that sequence divergences at the COI region regularly enable species delineation in all animal phyla except the Cnidaria. Their analysis

demonstrated that 72% of species showed greater than 8% sequence divergence and 98% of species showed more than 2% sequence divergence. This is in concordance with the 2% sequence divergence shown in closely related vertebrates at another mitochondrial gene used widely in phylogenetics, cytochrome b (Avise, 1999). For batoids, 2-3% sequence divergence is regularly seen between congeneric species at the COI and CR genes (Valsecchi *et al.,* 2005; Spies *et al.,* 2006; Diaz de Astarloa *et al.,* 2008; Pasolini *et al.,* 2011). Given this amount of comparable data, sequence divergence cut-offs remain a valuable tool for detecting species partitions for the mitochondrial genome. In the present study, the magnitude of sequence divergence for concatenated COI and CR data was 4.27% and 1.81% within *Dipturus* and *Raja*, respectively, which is in concordance with these values. For the COI and other mitochondrial genes, nucleotide substitution rates in sharks have shown to be seven to eightfold slower than in mammals (Martin *et al.,* 1992), and the relatively shallow intergenic divergence rates for the COI gene in the present study indicate a similar situation for batoids.

In addition to mtDNA, the current study also analysed nextRAD SNP data for species delineation. Whilst SNPs have been employed extensively in population genetic studies, their use in phylogenetics has only become commonplace in recent years (Emerson *et al.,* 2010; Eaton & Ree 2013; Wagner *et al.,* 2013; Herrera & Shank 2015; Leaché & Oaks, 2017). Studies employing SNPs also generally contain more loci than studies utilising capture protocols or Sanger sequencing methods, thus providing more data for phylogenetic inference (Leaché & Oaks, 2017). Indeed, SNPs have been utilised empirically for species delineation in vertebrates at both the shallow and deep levels of divergence (Emerson *et al.,* 2010; Eaton & Ree 2013; Wagner *et al.,* 2013; Herrera & Shank 2015). However, due to their relatively new introduction to the field, concerns remain surrounding their implementation in phylogenetics and phylogeography, including how to deal with missing data, ascertainment bias or violations of modelling assumptions (see Leaché & Oaks, 2017 for a review). In particular, concerns surround the influence of modelling assumptions inherent to methods applied to traditional DNA sequence data on phylogenetic inference from SNPs. The current study applied RAxML and pairwise sequence divergence methods to concatenated SNP data for species

delineation, methods that are common to traditional DNA sequence data. Concatenation remains a commonly used method for phylogenetic inference from SNP data (Edwards *et al.,* 2016, review), however, it suffers from several shortcomings. Because it does not account for incomplete lineage sorting it assumes that all SNPs share the same coalescent history, which can lead to phylogenetic bias when using methods that do not account for this (Liu *et al.,* 2015; Xu & Yang, 2016; Leaché & Oaks, 2017).

New methods have been developed that account for the shortcomings of concatenated SNP data and are implemented in several programs including SNAPP (SNP and amplified fragment length polymorphism phylogenies) (Bryant *et al.,* 2012) PoMo (polymorphism-aware phylogenetic model) (De Maio *et al.,* 2013, 2015; Schrempf *et al.,* 2016) and SVDquartet (Chifman & Kubatko, 2014). The current study utilised SVDquartet for phylogenetic inference, a quartet-based method that accounts for the independent history of each SNP. Whilst this method is coalescent and similar to SNAPP, it has several advantages over its counterparts. Firstly, data is typically present at a locus in each sample within a quartet, which largely accounts for the large amount of missing data that is often inherent to RAD-seq approaches (Wagner *et al.,* 2013; Chifman & Kubatko, 2014; Leaché & Oaks, 2017). Secondly, because it utilises the full data directly without a Bayesian framework it is less bioinformatically and computationally intensive, performing thousands of bootstrap replicates and producing a species tree in minutes (Chifman & Kubatko, 2014). Other sequence-based methods such as SNAPP utilise Bayesian MCMC methods, which increases computational time and requires assessing convergence, which can be challenging. Lastly, SVDquartet has been shown to be useful for resolving deep divergences, contrary to the notion that SNP data becomes less informative the deeper the phylogenetic level (Eaton *et al.,* 2016; Leaché & Oaks, 2017). In the current study, trees produced from SVDquartet and RAxML analysis of the ms4 dataset produced topologies that differed only in phylogenetic support, but were largely congruent in topology. Phylogenetic differences were only found within the *Raja* genus, further increasing confidence in the conclusions drawn from RAxML topologies, particularly for the *Dipturus* genus. This is in support of the application of RaXML to concatenated RAD SNPs, which has previously resolved phylogenies in challenging taxonomic

groups (Massatti *et al.,* 2016; Anderson *et al.,* 2017), and empirical studies have shown consistency between topologies produced from concatenated SNP data and those generated using a range of species tree methods (DaCosta & Sorenson, 2016). However, SVDquartet cannot estimate divergence times, comparison of which with RAxML would be an interesting point of future research to evaluate this methodological approach. The lower level of phylogenetic support in the SVDquartet topology is perhaps not surprising, as RAxML does not account for the genealogical history of SNP data, which has been shown to result in inflated support (Liu *et al.,* 2015; Xu & Yang, 2016; Leaché & Oaks, 2017).

Pairwise sequence divergence methods were also applied to concatenated SNP data in the present study, which, to the author's knowledge have never been applied to nextRAD data in batoids before. This means that, unlike mtDNA, sequence divergence cut-offs for species delineation remain ambiguous for the nextRAD SNP dataset, and hence the results outlined in the following sections should be interpreted with an appropriate degree of caution- relative degrees of divergence are likely to be study and SNP dataset specific. Although limited data is available for comparison, results from the nextRAD data suggest that, at least for the current study, congeneric skate species can be characterised by an average of 16.51% and 21.07% pairwise sequence divergence for *Dipturus* and *Raja* species, respectively. Although, for several sister species this value was significantly lower, namely between the barndoor skate (*Dipturus laevis*) and the blue skate (8.86%), and between the biscuit skate and thornback ray from Norway (17.58%). Given that these are well-established species, this magnitude of sequence divergence is perhaps the most appropriate cut-off for species delineation.

3.5.3 Relationships within the thornback ray (*Raja clavata*) and Madeiran skate (*Raja maderensis*)

Despite the formal separation of the thornback ray and Madeiran skate, mtDNA has been unsuccessful in supporting these species designations (Ball *et al.,* 2016). The current study supports

this conclusion, with mtDNA yielding no distinct separation between *R. clavata* and *R. maderensis.* This is in stark contrast to biscuit skate and *Dipturus* species of skate, that formed well-supported monophyletic groupings in mtDNA trees. Similarly to Ball *et al.,* (2016), generally increased genetic distance was observed among increasingly distant populations, with populations from Madeira and the remote seamounts of the Atlantic more closely related to each other than those from the continental shelf of Europe. In mtDNA phylogenies, thornback rays and Madeiran skate from Madeira, proximate seamounts and one individual from Portugal (RJC57) grouped together with high support, whilst specimens from Rockall, Norway and one individual from Portugal (RJC120) formed a separate clade. Portuguese thornback rays represented their own unique haplotypes, which is in support of previous sequencing that revealed highly divergent CR haplotypes in the region (Ball *et al.,* 2016). Low levels of divergence were observed within the *R. clavata/ R. maderensis* group (0.77%), below the typical 2-3% COI and CR divergence used to delineate species within the genus *Raja* (Valsecchi *et al.,* 2005; Spies *et al.,* 2006; Diaz de Astarloa *et al.,* 2008; Pasolini *et al.,* 2011). Further, this value is significantly lower than the average magnitude of intergenic sequence divergence for *Raja* species in the current study (1.81%), which remains perhaps the most taxonomically appropriate comparison. Therefore, the magnitude of mitochondrial sequence divergence between thornback rays and Madeiran skate is significantly below even the most conservative species cut-offs considered.

The nuclear dataset appears to provide a slightly different pattern of spatial genetic structuring within the thornback ray/ Madeiran skate clade*,* with this group having the highest intraspecific distance (13.58%) of species included in the nextRAD phylogeny, suggesting some degree of genetic diversity. Unlike in mtDNA trees, the nextRAD phylogenies (RAxML and SVDquartet) supported the reciprocal monophyly of Madeiran skate from Madeira, an important consideration when defining distinct evolutionary units. Although closely related to geographically proximate thornback ray/ Madeiran skate, these specimens appear to be genetically distinct, with relatively high divergence from their closest genetic relatives (Azorean thornback rays*;* 15.26%). This study represents the first genetic analysis of the species from the region and suggests that the Madeiran skate is perhaps a distinct

population, recently diverged from the polytypic thornback ray and endemic to Madeira. Based on the current nextRAD phylogeny, this form could also be considered as genetically distinct from Madeiran skate from the waters around the Azores, which grouped with geographically proximate thornback rays in a separate partition. Whilst this supports the possibility of distinguishing Madeiran *R. maderensis* as a distinct species, the magnitude of sequence divergence between this group and geographically proximate specimens is an important point of consideration. Due to the lack of RAD-seq studies on elasmobranchs that employ pairwise sequence divergence methods, an established boundary to define species is not available for comparison. Therefore, following Meier *et al.,* (2008), the lowest distance to the nearest neighbour might be an appropriate cut-off. In the present study, comparison with the level of divergence between thornback rays and their close relative the biscuit skate may be informative. The genetic distance between these species was 21.07%, significantly above the divergence between Madeiran *R. maderensis* and Azorean *R. clavata* (15.26%). This, coupled with the reciprocal monophyly of Madeiran *R. maderensis* and *R. clavata* from the Azores in the mtDNA phylogeny, is not conclusive enough to define Madeiran *R. maderensis* as a distinct species based on the current study. However, completely ruling out the possibility of cryptic speciation based on incongruence between nDNA and mtDNA sequence trees is ill-advised, given that further sampling of additional mtDNA sequences (e.g. the data-rich NADH2 gene; Naylor *et al.,* 2012; Lim *et al.,* 2015; Henderson *et al.,* 2016) may serve to resolve the reciprocal monophyly of Madeiran *R. maderensis,* providing support for its consideration as a distinct species. Furthermore, the contentious nature of utilising species cut-offs remains, as taxonomic groups are unlikely to be stationary, but instead existing on an evolutionary continuum (Trewick 2008).

Although it's identification as a novel species is rather ambiguous in the current study, the results do support the possibility that Madeiran *R. maderensis* represents an earlier stage in the continuum of allopatric speciation, which is in concordance with previous work on the species (Pasolini *et al.,* 2011; Ball *et al.,* 2016). In Mayr's (1954) model of such a process, speciation begins with a polytypic, wide-ranging taxa and ends with a group of geographical species that exhibit isolation with respect to their morphological characteristics. The thornback ray fits this criterion, and, given the lack of a

larval dispersal stage in skates, climatic and oceanographic discontinuities can play an important role in restricting interpopulation gene flow (Pasolini *et al.,* 2011). Furthermore, the Madeiran skate exhibits several morphological differences from the thornback ray*,* which may have led to their classification as separate species (Stehmann and Burkel 1984; Ebert and Stehmann 2013). The thornback ray may have colonised Madeira from the North African coast, and subsequently spread to the Azores and surrounding seamounts where it settled as a population with a distinct morphotype (Ebert and Stehmann, 2013). This may have been an adaptive or plastic response to local environmental conditions, as *R. clavata* has been known to exhibit morphological variation in skin texture and colour across its range (Pritchard 1977; Mnasri *et al.,* 2009). Regardless of taxonomic status, both mtDNA and nextRAD phylogenies reveal the distinctiveness of thornback ray/ Madeiran skate from isolated regions in the Azores, seamounts and Madeira. This is highly consistent with previous studies on both these species that observed genetic differences between populations from the Azores and off the continental shelf of Europe (Chevlot *et al.,* 2006; Naylor *et al.,* 2012; Ball *et al.,* 2016). Certainly, treating these groups as distinct populations would benefit to conserve genetic diversity, the most fundamental level of biodiversity.

3.5.4 Relationships within the longnosed skate (*Dipturus oxyrinchus*) and the flapper skate (*Dipturus intermedius*)

Previous research into the phylogenetic status of longnosed skate from the Azores has suggested a potential cryptic *Dipturus* species in the region (Andrew Griffiths, unpublished data). Current mtDNA evidence supports this conclusion; despite being identified as the longnosed skate, one *D. oxyrinchus* specimen collected from the Azores was reciprocally monophyletic with the flapper skate. The percentage sequence divergence between this clade and longnosed skate from spatially proximate Portugal and Norway was relatively low (1.91%), but is in agreement with the 2-3% magnitude of separation found among other congeneric species of skates (Hebert *et al.,* 2003a; Spies *et al.,* 2006). Under a conventional interpretation of the mtDNA data, the presence of cryptic speciation is supported, however, this was not the case in phylogenies built from nextRAD data. Instead this

Azorean specimen, although genetically distinct, formed a reciprocal monophyly with longnosed skate from the rest of the North Atlantic in both RAxML and SVDquartet trees. For the nextRAD data, the level of divergence between this specimen and longnosed skate from geographically proximate Portugal and Norway (8.44%) was below the average (16.51%) and minimum (8.86%) distance used to delineate *Dipturus* species for the current dataset. Although incongruence between mtDNA and nDNA does not fully support the presence of a cryptic *Dipturus* species in the Azores, populations in this region could represent a distinct genetic lineage. Other marine organisms including white seabream (*Diplodus sargus*; Dominguez *et al.,* 2007) and shanny (*Lipophrys pholis*; Stefanni *et al.,* 2006) have been shown to exhibit these patterns of genetic structuring. Additionally, one Azorean flapper skate specimen (D45) from the current study appeared to be genetically distinct from its conspecifics in the Shetlands. This specimen provides another avenue for future research, as the sequencing of more individuals could reveal it to be a representation of another distinct lineage. The remoteness of the Azores is likely to be a primary cause of its genetic distinctiveness; at 1300 km from mainland Portugal its isolation represents a significant dispersal barrier for skate species that lack a larval dispersal stage (Ball *et al.,* 2016). Furthermore, with some of the earliest designations of marine protected areas being made in the Azores, perhaps the region can act as a refugium for batoids that have been under increasing fishing pressure across the rest of the North Atlantic, meaning vulnerable species could persist here (Abecasis *et al.,* 2015).

3.5.5 Relationships within the Norwegian skate (*Dipturus nidarosiensis*)

In the present study, Norwegian skate from the Mediterranean appeared to be genetically distinct from those from Rockall and Norway, forming their own well-supported reciprocally monophyletic group in both nDNA and mtDNA trees (RAxML and SVDquartet). Mitochondrial sequence divergence between these two clades was significantly lower (0.2%), than between other well-established, sister *Dipturus* species, which does not support the presence of cryptic speciation. This conclusion was well-supported by the nuclear dataset, with *D. nidarosiensis* having the lowest intraspecific level of divergence of all the species included in this study (3.65%). Furthermore, this value is 4.5x lower than

2289 the average (16.51%) and 2.4x lower than the minimum (8.86%) intergenic sequence divergence for
2290 the *Dipturus* genus. This is in congruence with previous studies on the morphometric, meristic and
2291 genetic characteristics of Norwegian skate from the Mediterranean (Carbonara *et al.,* 2019).
2292 Carbonara *et al.,* (2019) reported shared COI haplotypes, including the most common haplotype,
2293 between specimens from the Atlantic and central Mediterranean Basin. However, they did conclude
2294 that additional analyses with more powerful nuclear markers would be needed to investigate possible
2295 intraspecific genetic structuring. Although limited by a small number of individuals, the present study
2296 has provided such an opportunity utilising nextRAD data. The shallow phylogenetic separation
2297 between Atlantic and Mediterranean Norwegian skate indicates the Mediterranean could, although
2298 not cryptic, represent a genetically distinct population. Several phylogeographical studies have
2299 highlighted the Atlantic-Mediterranean transition as an important genetic barrier for many marine
2300 species; regional differentiation has been observed in other batoids (Chevolot *et al.,* 2006; Griffiths *et*
2301 *al.,* 2011), invertebrates (Zane *et al.,* 2000; Wilke & Pfenninger 2002; Duran *et al.,* 2004; Baus *et al.,*
2302 2005), teleosts (Borsa *et al.,* 1997; Gysels *et al.,* 2004; Cimmaruta *et al.,* 2005; Nakadate *et al.,* 2005),
2303 algae and seagrasses (Coyer *et al.,* 2003; Olsen *et al.,* 2004; Provan *et al.,* 2005). Similarly to the
2304 Azores, the Mediterranean may have served as a refugium for Norwegian skate and other batoids
2305 during the last glacial maximum, leading to isolation and restricted gene flow to the rest of Atlantic
2306 (Chevolot *et al.,* 2006).
2307
2308 Although specimens analysed in the present study are likely conspecific with Atlantic *D. nidarosiensis*,
2309 there may still be a cryptic *Dipturus* species in the Mediterranean. The IUCN red list details the
2310 possibility that *D. nidarosiensis* is a composite species with a small and large morphotype similar to
2311 that seen in the 'common skate' complex, the large morphotype represented by *D. nidarosiensis* and
2312 the small an unknown *Dipturus sp.* (Stehmann *et al.,* 2009; Iglesias *et al.,* 2010). This is based on 14
2313 specimens caught in the Sardinian Channel in 2005 (Cannas *et al.,* 2010), two *Raja* (*Dipturus* sp.)
2314 captured within the Rockall Trough (Gordon and Duncan, 1989; Ebert and Stehmann, 2013) and a
2315 possible, yet undescribed *Dipturus* sp. taken off the mainland of Portugal and in the vicinity of the Gulf
2316 of Cadiz (Ebert and Stehmann, 2013). Adult size of these unknown species and the Sardinian

Norwegian skate is significantly smaller, a size at which northern Eastern North Atlantic *D. nidarosiensis* are just approaching sexual maturity. Furthermore, they exhibit a habitus quite different from Atlantic Norwegian skate. The situation is complicated further by a lack of historical fisheries data. The occurrence of Norwegian skate in the Mediterranean was only initially reported in 2010, likely a result of misidentification with other *Dipturus* species of skate (Cannas *et al.,* 2010). Additional genetic analyses is required before formal taxonomic revision, however, such uncertainties highlight the complex nature of this species' nomenclature and taxonomy.

3.5.6 Significance of Hybridization

An important discussion point for the current study is also the possibility of hybridization between species. Hybridization has only been detected relatively recently in elasmobranchs, namely among hammerhead (Marino *et al.,* 2015; Barker *et al.,* 2019) and blacktip sharks (Morgan *et al.,* 2012). This phenomenon has been less explored in batoids, but has nevertheless been detected among river stingrays of the genus *Potamotrygon* (Toffoli *et al.,* 2008) and *Raja* (Frodella *et al.,* 2016). Typically, hybridization can be detected by incongruence between morphology and diagnostic mtDNA sequences, and further information on hybrids can be obtained by parallel analysis of nuclear DNA (see Dudgeon *et al.,* 2012 for a review). Of all the *Raja* species examined in the present study, incongruence between mtDNA and morphological data was only observed in thornback rays from the Azores (Figure 2A), with one specimen from the area grouping with Madeiran skate from Madeira in mtDNA trees. This classification was not supported in nextRAD phylogenies, with Azorean *R. clavata* grouping as genetically distinct from Madeiran *R. maderensis*. Given the ambiguity surrounding whether thornback rays and Madeiran skate are the same species or not (Pasollini *et al.,* 2011; Ball *et al.,* 2016; the present study), hybridization cannot be conclusively identified, particularly as misidentification at the morphological level may occur due to the polytypic nature of *R. clavata* (Ball *et al.,* 2016). Indeed, inter-population, as opposed to inter-species, gene flow may occur between populations of *R. clavata/ R. maderensis.* Furthermore, previous analysis on these species has revealed no evidence on hybridization (Ball *et al.,* 2016).

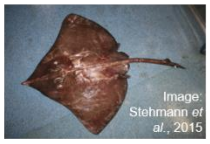
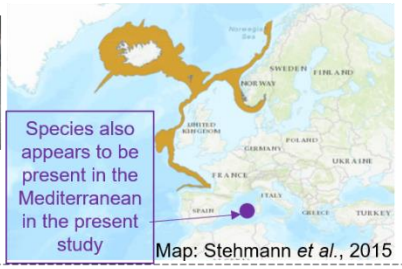
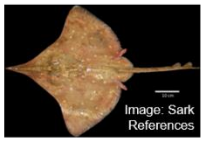
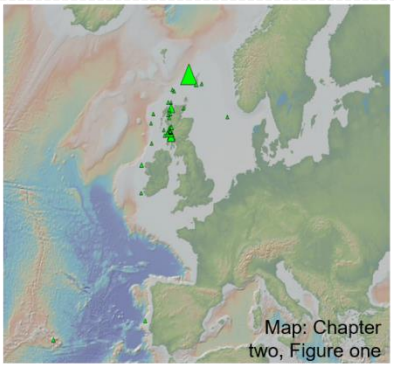
However, further sequencing of more *R. clavata/ R. maderensis* individuals from the Azores, Madeira and surrounding seamounts would help to further validate the presence of distinct genetic lineages of either species in the region, and any patterns of inter-population breeding.

Within the *Dipturus* genus, mtDNA phylogenies supported all morphological identifications, with the exception of longnosed skate from the Azores. This specimen formed a monophyly with flapper skate from the region in mtDNA trees (Figure 2A). Given their position as sister species in phylogenies and overlapping latitudinal and bathymetric ranges (Dulvy *et al.,* 2006; Ellis *et al.,* 2015; the present study), hybridization may indeed be plausible. Indeed, an ancient hybridization event and intogression of mtDNA may account for the incongruence between mtDNA phylogenies and morphological data. However, several factors may this less likely. Firstly, Azorean longnose skate represent a novel mitogenome that isn't seen in flapper skate- this specimen is still genetically distinct from geographically proximate flapper skate. Secondly, the size differences between these two species make it less likely. Flapper skate reach sexual maturity at over double the size of longnosed skate, making copulation difficult (Marine Species Identification Portal; Griffiths *et al.,* 2010; ICES 2012; Kadri *et al.,* 2014). Indeed, hybridization has only been detected within batoids between species of much similar sizes, for example *Raja montagui* (60 cm; Frodella *et al.,* 2016) and *Raja polystigma* (60 cm; Frodella *et al.,* 2016). Furthermore, nextRAD phylogenies actually supported morphological identification, with Azorean longnosed skate grouping with their counterparts from Portugal and Norway (Figure 2B). Nevertheless, the ability to detect hybridization is weakened here due to the limited number of samples analysed. Previous studies that have successfully analysed hybridization in batoids employ a much greater sampling pool of species, and additional admixture analysis, which may aid in drawing more significant conclusions for the species analysed in the present study (Toffoli *et al.,* 2008; Frodella *et al.,* 2016; Vargas-Caro *et al.,* 2017).

3.5.7 Conservation implications

Given the vulnerable nature of all these species of skate, the present study has strong conservation implications (see Table six for a summary). The Madeiran skate is currently listed as a vulnerable species on the IUCN red list, endemic to Madeira and the Azores (Dulvy *et al.,* 2015). However, results based on mtDNA data from the current study and previous mtDNA evidence (Ball *et al.,* 2016) suggest this species is synonymous with the polytypic thornback ray. Contradictory to this, evidence from nextRAD phylogenies (the present study) support the hypothesis that Madeiran *R. maderensis* could represent a novel, cryptic species. This ambiguity highlights the complicated nature of conservation assessments for this species. The thornback ray has a much wider geographic range and is found in Iceland and Norway, the North Sea, the Western Baltic Sea (although sightings here are rare), around the British Isles and Ireland, the Mediterranean and through to the coast of West Africa and into the southwestern Indian Ocean (Ebert & Stehmann, 2013). However, Madeiran skate from Madeira likely represents its own genetic lineage with a heavily restricted range; evidence highlights the genetic distinctiveness of *R. maderensis* populations from Madeira (Ball *et al.,* 2016; the current study). These populations may need to be regarded as a single management unit in conservation assessments, perhaps mandating a different conservation strategy compared to North Atlantic populations, given their isolation. This principal could also be applied to the longnosed skate and flapper skate, as populations from the Azores appear to be genetically distinct from their counterparts in the Northeast Atlantic. Future sampling and subsequent sequencing of more individuals from the region would help to reveal patterns of population structuring, and these Azorean specimens could represent their own genetic lineage. Given the status of longnosed skate as near threatened and flapper skate as critically endangered on the IUCN red list, this finding is significant. Such results elucidate to the importance of seamounts as ‘hotspots’ for genetic biodiversity, which should be considered in the designation of marine protected areas (Samadi *et al.,* 2007; Clark *et al.,* 2010; Morato *et al.,* 2010).

Table 6. Table detailing conclusions drawn for key species focussed on in the present study. Species utilised as outgroups (*Leucoraja naevus*) or as comparisons (*Raja straeleni, Dipturus laevis, Dipturus batis*) for these key species in phylogenetic analysis are not included. However, results support their most up-to-date taxonomy.

Species	Putative geographical range	Conclusions from present study	Questions and future directions
 *Dipturus nidarosiensis* Norwegian skate	 Map: Stehmann *et al.*, 2015	• Norwegian skate appear to be present in the Mediterranean but are not genetically distinct from their counterparts in the North Atlantic.	*Although they may not be a distinct species, are Mediterranean Norwegian skate genetically distinct enough to be treated as a distinct management unit?* • Conduct comparative sequencing of more individuals from the Mediterranean and across their North Atlantic range.
 *Dipturus oxyrinchus* Longnosed skate	 Map: Ellis *et al.*, 2015	• NextRAD-seq data doesn't support the presence of a cryptic *Dipturus* species in the Azores. However, concatenated mtDNA data (CR and COI) did. • Longnosed skate in the Azores could represent a distinct genetic lineage.	*Are longnose skate from the Azores genetically distinct enough to be treated as a distinct management unit?* • Sequencing of more individuals from the Azores may prove difficult, as they are very rare. However, this would prove useful in determining appropriate conservation action, especially given the incongruence between mtDNA and nextRAD data.
 *Dipturus intermedius* Flapper skate	 Map: Chapter two, Figure one	• Flapper skate from the Azores could represent a distinct genetic lineage, given their separation in phylogenetic trees. • This population may have a very restricted range due to the isolation of the Azores and significant distance between their counterparts in the rest of the North Atlantic.	*Should Azorean flapper skate be treated as genetically distinct? What implication does this have for Marine Protected Areas in the Azores?* • Sequencing of more individuals from the Azores may prove difficult, as they are very rare. However, this would prove useful in determining appropriate conservation action.
 *Raja clavata* Thornback ray	 Map: Ellis, 2016	• Data doesn't fully support the separation of *R. clavata* and *R. maderensis* as distinct species. • The genetic distinctiveness of *R. maderensis* from Madeira and *R. clavata* from the Azores suggests they may need to be treated as distinct conservation units to conserve genetic diversity.	*Does R. maderensis from Madeira need to be treated as a distinct population of R. clavata, but with a heavily restricted range? Should the same principal be applied to R. clavata from the Azores?* • Conduct both mitochondrial and nuclear DNA sequencing on these specimens from across their range. In the present study, nextRAD and mtDNA were inconsistent, so this may help to enable conservation managers to draw firm conclusions about which populations should be treated as distinct.
 *Raja maderensis* Madeiran skate	 Map: Dulvy *et al.*, 2015		

The results regarding Norwegian skate are also a point of interest to conservation managers. This species is one of the largest in Europe and is therefore at high risk from overfishing and incidental bycatch, leading to its status as near threatened on the IUCN red list (Stehmann *et al.,* 2009). Despite this, spatiotemporal data remains sparse and little information is available on historic or contemporary populations. Therefore, information such as the results represented here are vital to ensure the effective conservation of the species. The level of divergence between the two *D. nidarosiensis* clades is perhaps too small to indicate the presence of a novel cryptic species in the Mediterranean, however, the genetic distinctiveness of Mediterranean Norwegian skate highlights a 'hidden' population that potentially has a very restricted range, given the importance of the Atlantic-Mediterranean transition as a genetic barrier for batoids (Chevolot *et al.,* 2006; Griffiths *et al.,* 2011). In order to establish whether this Mediterranean population should regarded as a distinct management unit or not, further sampling in the region is needed; ultimately, the conclusions drawn from this study are limited by the small number of individuals analysed.

The conflict between nDNA and mtDNA phylogenies when resolving the taxonomy of *Raja* and *Dipturus* species of skate is an important note for taxonomists, particularly given the wide use of the COI and CR genes for batoid phylogenetics (Valsecchi *et al.,* 2005; Ward and Holmes, 2007; Moura *et al.,* 2008; Wynen *et al.,* 2009; Serra-Pereira *et al.,* 2010). Whilst mtDNA did highlight spatial patterns of genetic structuring, nextRAD data was able to much more clearly resolve the accepted monophyly of *Dipturus* and *Raja*, and in many instances helped to provide greater resolution than those based on traditional mitochondrial markers. In several instances, the interpretation of mtDNA in isolation may have led to inaccurate conclusions, and even the misidentification of a cryptic *Dipturus* species in the Azores. This has important implications for the conservation of batoids, whose morphological and ecological traits make cryptic and polytypic speciation an important feature in their evolutionary history (Hebert *et al.,* 2003a; Spies *et al.,* 2006; Griffiths *et al.,* 2011; Serra-Pereira *et al.,* 2011; Coulson *et al.,* 2011; Mabragaña *et al.,* 2011; Lynghammar *et al.,* 2014). Such discordance could be the result of the inherent low resolution in the current mtDNA dataset; nextRAD SNPs represented a significantly larger sequence, compared to the use of just two mtDNA genes. The evolutionary histories of the

nuclear and mitochondrial genomes may also contribute their incongruence, as characters with different evolutionary rates, patterns of among-site substitution rate variation, homoplasy or base composition often disagree in recovering the underlying topology (Bull *et al.,* 1993; Lin & Danforth, 2004; Rubinoff & Holland, 2005). Additionally, technique-specific issues and PCR artefacts have been shown to cause issues when amplifying highly conserved mtDNA genes, such as the CR (Zhang and Hewitt, 1996; Thalmann *et al.,* 2004; Rubinoff & Holland, 2005). This, however, is not to discount the mtDNA tree entirely. Despite its limitations, the integrated role of mtDNA with nDNA in the current, and indeed previous studies (e.g., Reed & Sperling, 1999; Rubinoff & Sperling, 2002; Caterino *et al.,* 2001), has aided in resolving complex batoid phylogenies, and the confirmation of conclusions drawn from nextRAD data.

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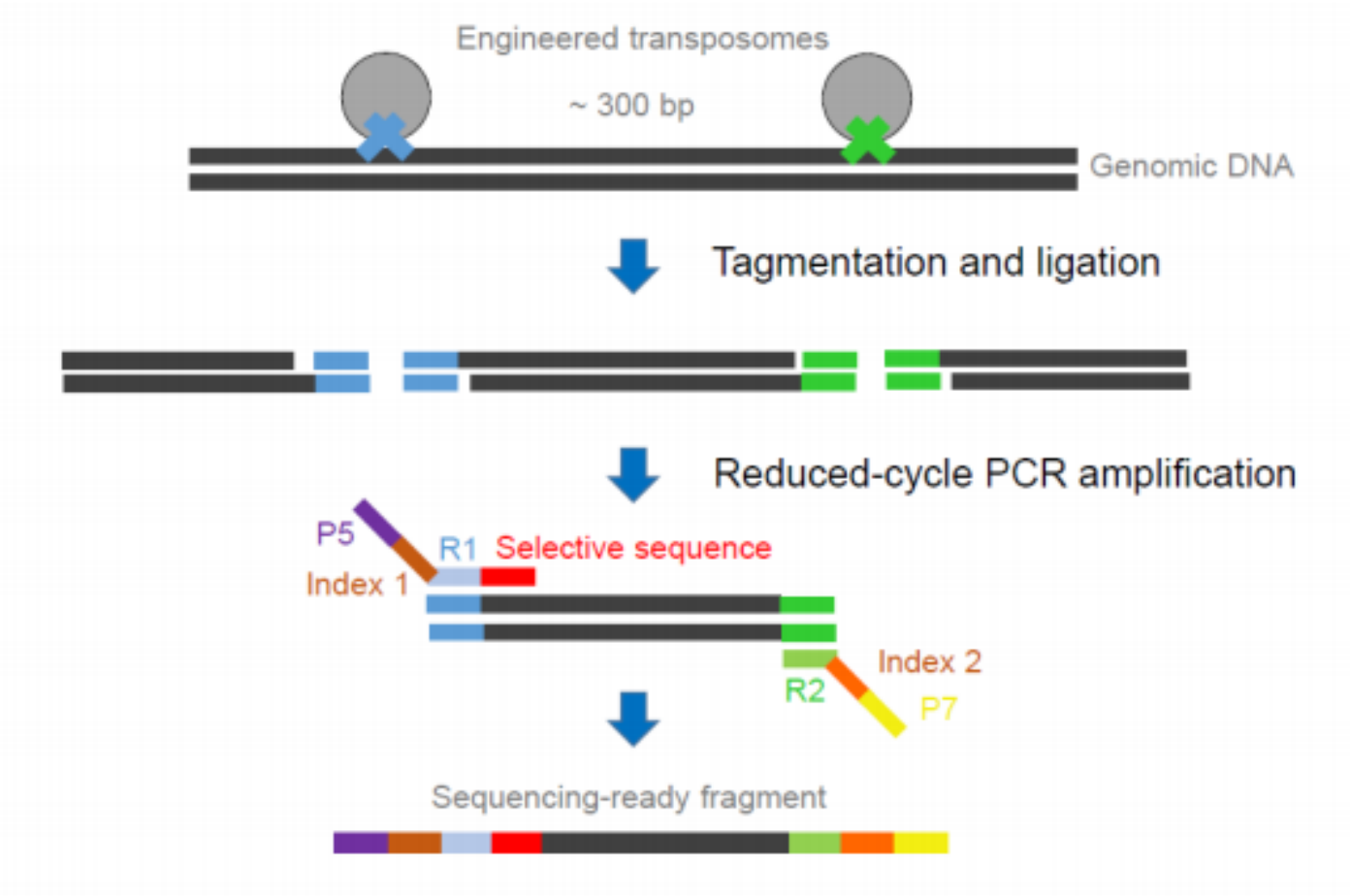
3.7 Supplementary materials: NextRAD and mitochondrial DNA sequencing reveal hidden diversity within vulnerable species of batoid fish.

Appendix 1. Workflow of NextRAD Nextera-tagmented reductively-amplified DNA) sequencing. A small amount of DNA (~10 ng) is mixed with two engineered transposomes of Nextera reagents which tag as well as add short adaptors to genomic DNA. Sequencing primers Read one (R1, light blue bars) and Read two (R2, light green bars), indices (orange and brown bars; small fragments of DNA which allow the customized selective primer (red bar: GTGTAGAGC) to bind), are added by a limited cycle of PCR to generate sequencing-ready fragments, which are compatible for Illumina platforms (this method is taken from Fu *et al.,* 2017).



Appendix 2. Information on the capture locations, control region (CR) and cytochrome oxidase I (COI) accession numbers of samples used in phylogenetic analyses. * Indicates sequences were downloaded from Genbank. 'Submit' indicates sequences do not have accession numbers and need to be submitted to Genbank. To test the effects of missing data, four datasets with different thresholds for the minimum number of samples per locus (ms) were run during analysis- ms2, ms4, ms12 and ms26.

Sample number	Morphological identification	Date collected	Location	Latitude	Longitude	Ms2 Accession number	Ms4 Accession number	Ms12 Accession number	Ms26 Accession number	CR Accession number	COI Accession number
Na2	*Dipturus batis*	Early Dec 2009	Celtic Sea	49.47	-8.72	Submit	Submit	Submit	Submit	GQ392068.1	KF604218.1
8,1	*Dipturus batis*	Early Dec 2009	Celtic Sea	49.47	-8.72	Submit	Submit	Submit	Submit	GQ392068.1	KF604218.1
D51	*Dipturus oxyrinchus*	29/09/2012	Azores	Approx. 40.02	Approx. -13.82	Submit	Submit	Submit	Submit	Submit	Submit
D45	*Dipturus intermedius*	02/06/2012	Azores	37.74	-25.68	Submit	Submit	Submit	Submit	MH581187	Submit
SH190	*Dipturus intermedius*	01/06/2012	Shetlands	60.72	-2.66	Submit	Submit	Submit	Submit	GQ392065 *	KF604221.1
SH194	*Dipturus intermedius*	01/06/2012	Shetlands	60.72	-2.66	Submit	Submit	Submit	Submit	GQ392066.1	KF604221.1
RJC57	*Raja clavata*	Unknown	Portugal	39.35	-9.37	Submit	Submit	Submit	Submit	GQ392108.1*	KY176588.1
RJC120	*Raja clavata*	Unknown	Portugal	41.18	-8.70	Submit	Submit	Submit	Submit	GQ392109.1*	Submit
ARV_011	*Raja clavata*	25/10/2008	Norway	64.45	-11.51	Submit	Submit	Submit	Submit	Submit	KY176588.1
7.12	*Raja clavata*	07/09/2008	Rockall	56.77	-14.14	Submit	Submit	Submit	Submit	GQ392108.1	KY176588.1
CO18	*Raja clavata*	Unknown	Azores	Unknown	Unknown	Submit	Submit	Submit	Submit	Submit	Submit
MAD25	*Raja maderensis*	Unknown	Irving Seamount	30.50	-28.50	Submit	Submit	Submit	Submit	GQ392106.1*	MH547697.1*
MAD19	*Raja maderensis*	Unknown	Siene Seamount	35.00	-13.00	Submit	Submit	Submit	Submit	GQ392107.1*	MH547697.1*
42926	*Raja maderensis*	Unknown	Madeira	Unknown	Unknown	Submit	Submit	Submit	Submit	Submit	HM043185.1
42927	*Raja maderensis*	Unknown	Madeira	Unknown	Unknown	Submit	Submit	Submit	Submit	Submit	HM043185.1
19069_II	*Dipturus oxyrinchus*	19.06.09	Norway	63.37	9.45	Submit	Submit	Submit	Submit	GU595172.1	KY909402.1
RJ04	*Dipturus oxyrinchus*	01/01/2007	Portugal	42.88	-65.06	Submit	Submit	Submit	Submit	GU595175.1	KY909402.1
R06C1	*Dipturus nidarosiensis*	27/09/2006	Mediterranean	38.58	9.46	Submit	Submit	Submit	Submit	Submit	KT307207.1

R08C1	*Dipturus nidarosiensis*	06/03/2008	Mediterranean	38.58	9.46	Submit	Submit	Submit	Submit	Submit	KT307207.1
ARV_02	*Dipturus nidarosiensis*	06/11/2017	Norway	Unknown-locality in Trondheims Fjord	Unknown-locality in Trondheims Fjord	Submit	Submit	Submit	Submit	Submit	Submit
FN2	*Dipturus nidarosiensis*	01/06/2012	Rockall	57.70	-15.72	Submit	Submit	Submit	Submit	Submit	Submit
R24956	*Raja straeleni*	Unknown	Cape Town	Unknown	Unknown	Submit	Submit	Submit	Submit	Submit	Submit
R24958	*Raja straeleni*	Unknown	Cape Town	Unknown	Unknown	Submit	Submit	Submit	Submit	Submit	Submit
Bar06125	*Dipturus laevis*	01/01/2007	Nova Scotia	42.88	-65.06	Submit	Submit	Submit	Submit	MH581190*	JF895055.1
RJN63	*Leucoraja naevus*	30/07/2007	Portugal	39.40	-9.40	Submit	Submit	Submit	Submit	n/a	n/a
RJN52	*Leucoraja naevus*	30/07/2007	Portugal	39.40	-9.40	Submit	Submit	Submit	Submit	n/a	n/a

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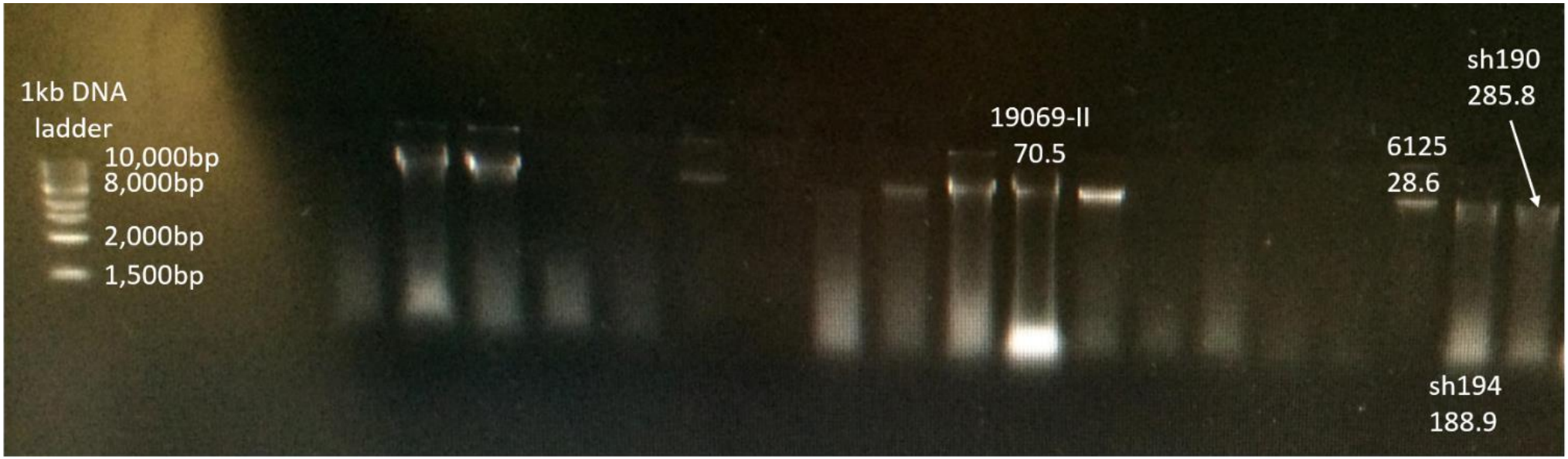
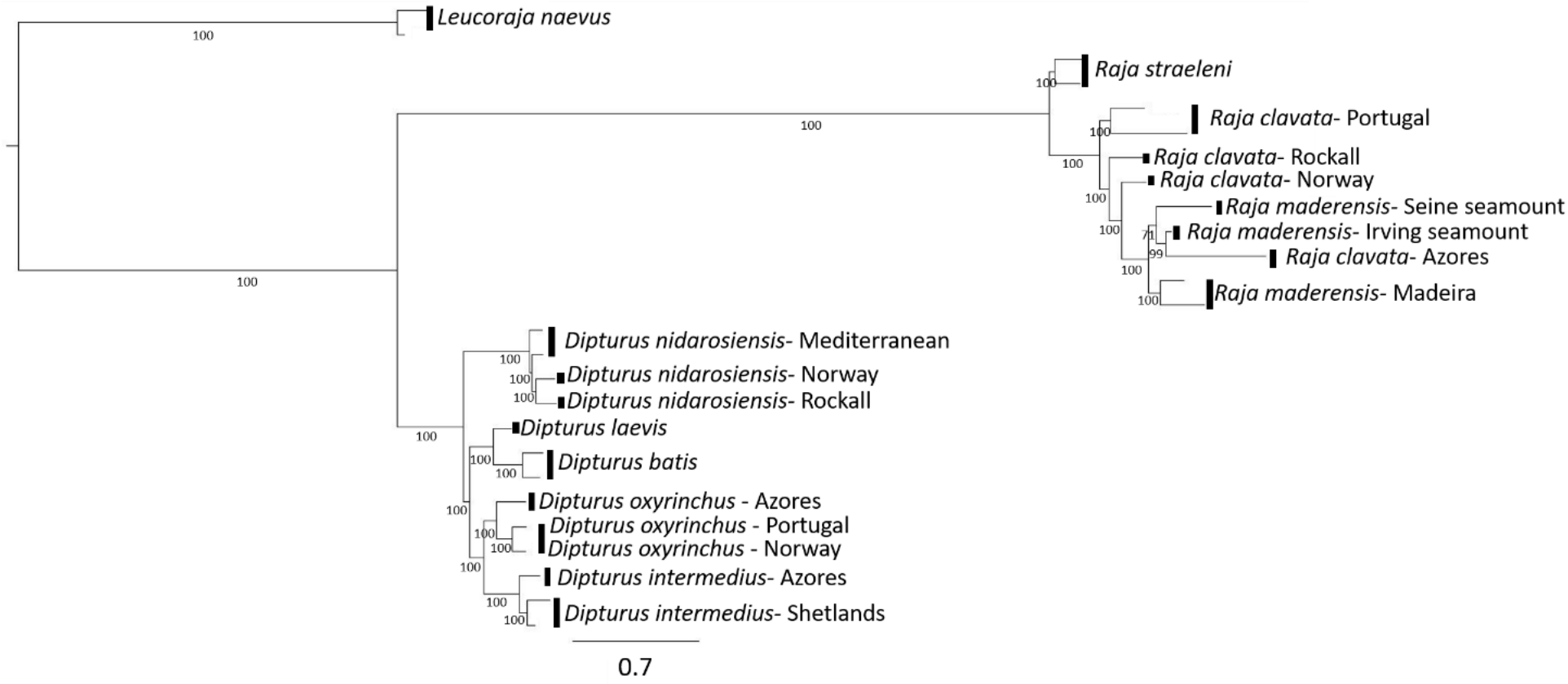
Appendix 3. An example image of a 1% agarose gel electrophoresis performed on total genomic DNA from degraded samples sequenced in the presents study. Samples were run against a 1kb DNA ladder. Numbers represent the concentration of double-stranded DNA in each sample in ng/L, calculated using the Invitrogen Qubit Assay.

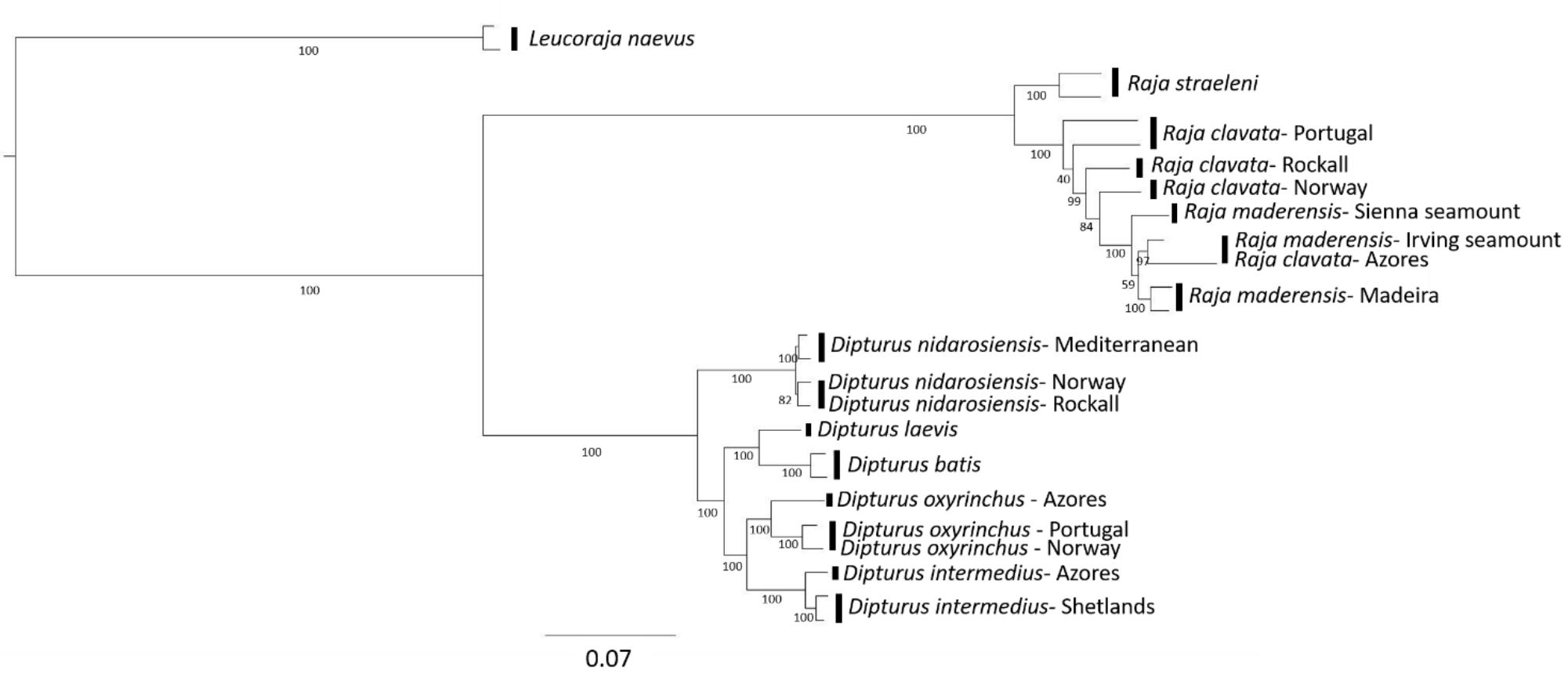


Appendix 4. RAxML trees built from nextRAD and mtDNA sequence data. (A) Phylogenetic tree built from the mc2 dataset; (B) Phylogenetic tree built from the mc12 dataset; (C) Phylogenetic tree built from the mc30 dataset; (D) Phylogenetic tree built from the ms4 dataset including all duplicate samples (n=30); (E) Phylogenetic tree built from control region (CR) data only; (F) Phylogenetic tree built from cytochrome oxidase I (COI) data only. Numbers below branches represent bootstrap support values. Scale bars refer to the number of substitutions per site.

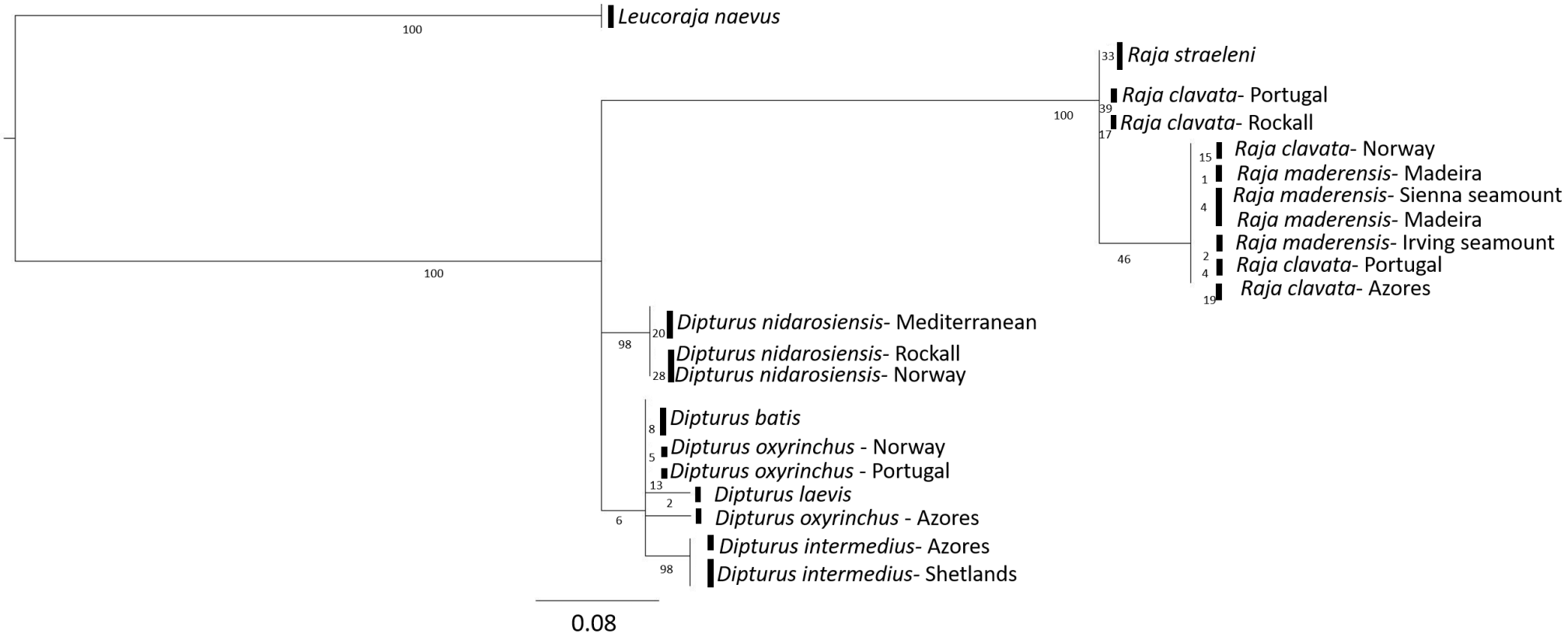
A:



B:


Leucoraja naevus
Raja straeleni
Raja clavata- Portugal
Raja clavata- Rockall
Raja clavata- Norway
Raja maderensis- Sienna seamount
Raja maderensis- Irving seamount
Raja clavata- Azores
Raja maderensis- Madeira
Dipturus nidarosiensis- Mediterranean
Dipturus nidarosiensis- Norway
Dipturus nidarosiensis- Rockall
Dipturus laevis
Dipturus batis
Dipturus oxyrinchus - Azores
Dipturus oxyrinchus - Portugal
Dipturus oxyrinchus - Norway
Dipturus intermedius- Azores
Dipturus intermedius- Shetlands
0.07

C:


Leucoraja naevus
Raja straeleni
Raja clavata- Portugal
Raja clavata- Rockall
Raja clavata- Norway
Raja maderensis- Madeira
Raja maderensis- Sienna seamount
Raja maderensis- Madeira
Raja maderensis- Irving seamount
Raja clavata- Portugal
Raja clavata- Azores
Dipturus nidarosiensis- Mediterranean
Dipturus nidarosiensis- Rockall
Dipturus nidarosiensis- Norway
Dipturus batis
Dipturus oxyrinchus - Norway
Dipturus oxyrinchus - Portugal
Dipturus laevis
Dipturus oxyrinchus - Azores
Dipturus intermedius- Azores
Dipturus intermedius- Shetlands
100
100
33
39
100
17
15
1
4
46
2
4
19
20
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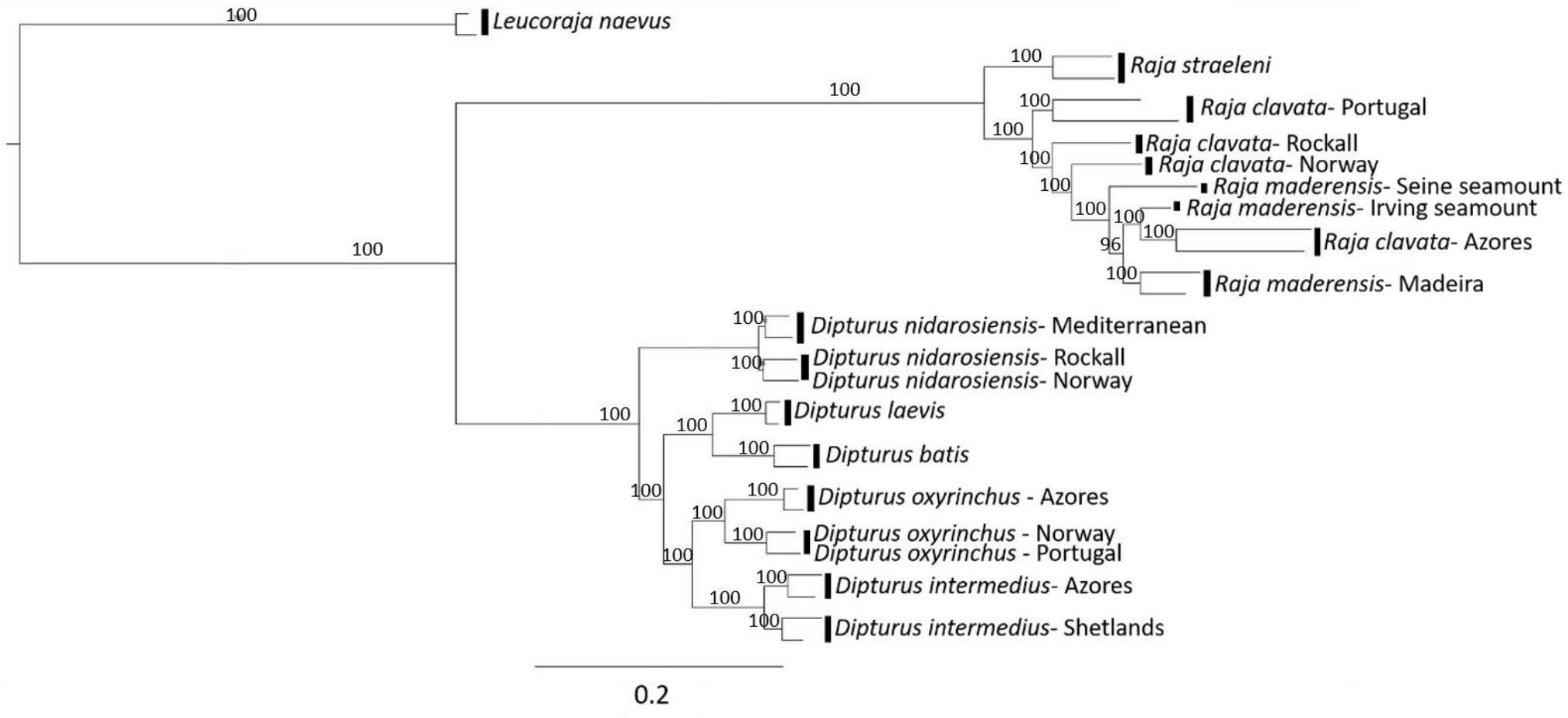
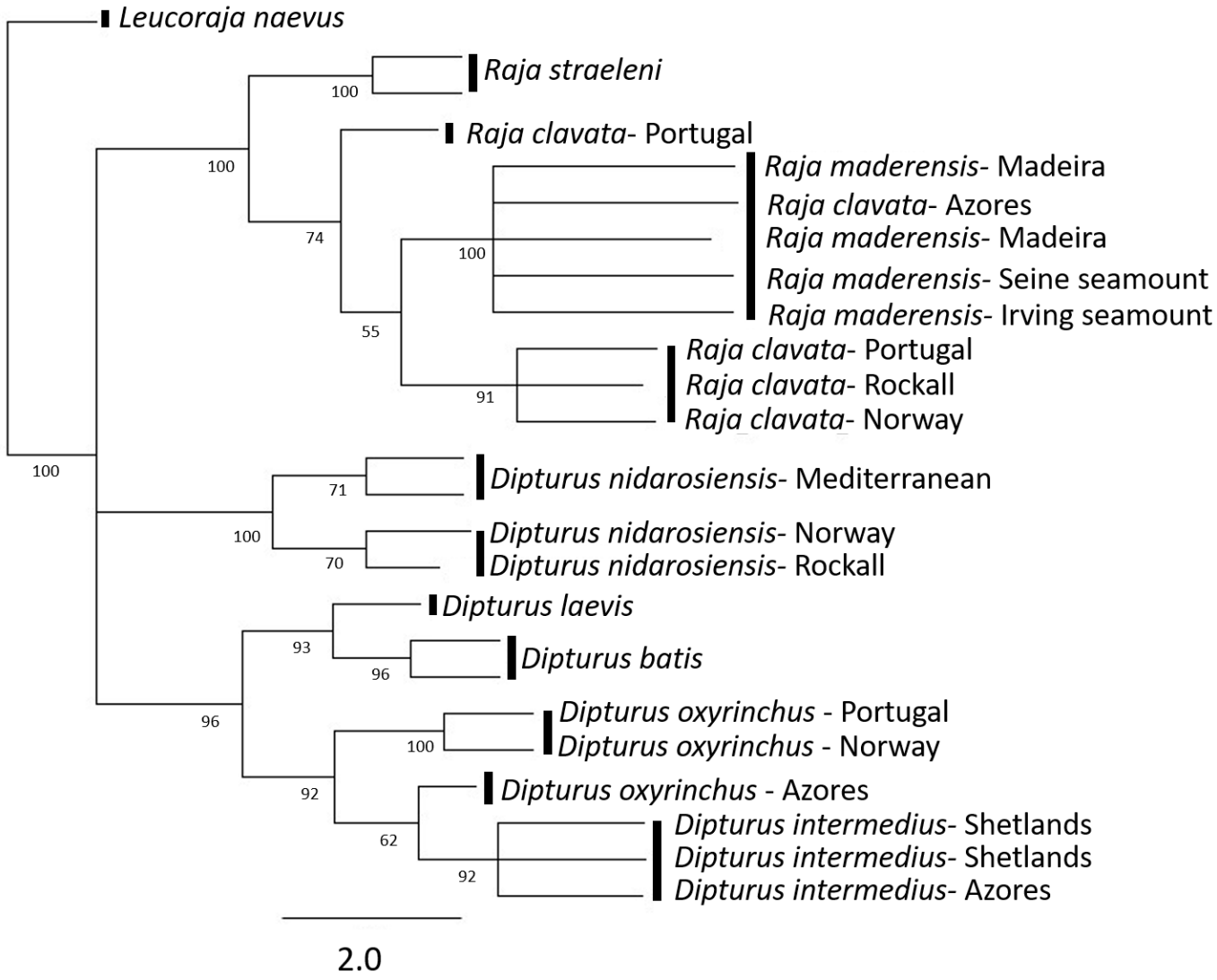
28
8
5
13
2
6
98
0.08

D:

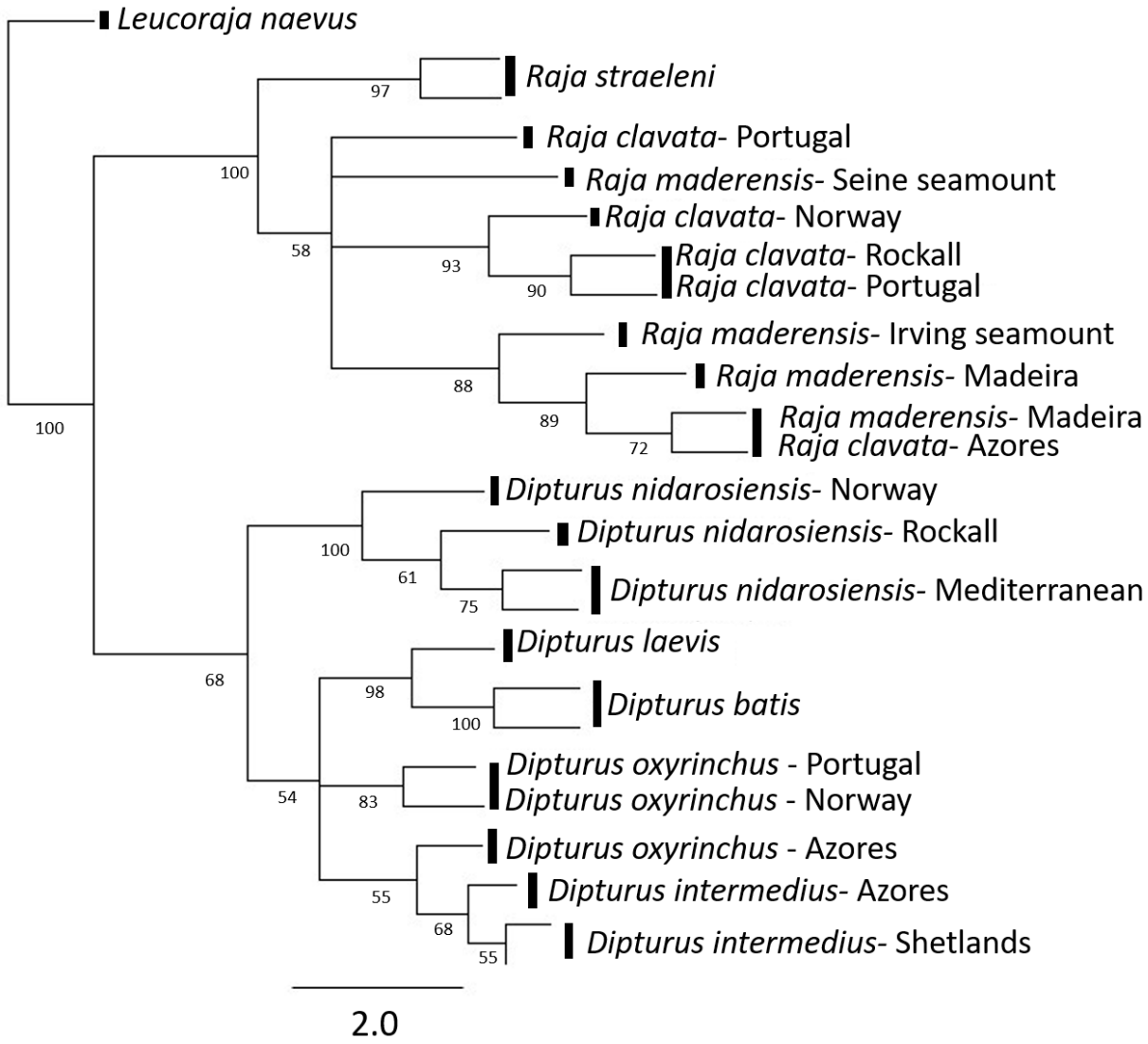

Leucoraja naevus
Raja straeleni
Raja clavata- Portugal
Raja clavata- Rockall
Raja clavata- Norway
Raja maderensis- Seine seamount
Raja maderensis- Irving seamount
Raja clavata- Azores
Raja maderensis- Madeira
Dipturus nidarosiensis- Mediterranean
Dipturus nidarosiensis- Rockall
Dipturus nidarosiensis- Norway
Dipturus laevis
Dipturus batis
Dipturus oxyrinchus - Azores
Dipturus oxyrinchus - Norway
Dipturus oxyrinchus - Portugal
Dipturus intermedius- Azores
Dipturus intermedius- Shetlands
0.2

3094 **E:**


Leucoraja naevus
Raja straeleni
Raja clavata- Portugal
Raja maderensis- Madeira
Raja clavata- Azores
Raja maderensis- Madeira
Raja maderensis- Seine seamount
Raja maderensis- Irving seamount
Raja clavata- Portugal
Raja clavata- Rockall
Raja clavata- Norway
Dipturus nidarosiensis- Mediterranean
Dipturus nidarosiensis- Norway
Dipturus nidarosiensis- Rockall
Dipturus laevis
Dipturus batis
Dipturus oxyrinchus - Portugal
Dipturus oxyrinchus - Norway
Dipturus oxyrinchus - Azores
Dipturus intermedius- Shetlands
Dipturus intermedius- Shetlands
Dipturus intermedius- Azores
100
100
74
100
55
91
100
71
100
70
93
96
96
100
92
62
92
2.0

3095

3096 **F:**


Leucoraja naevus
Raja straeleni
Raja clavata- Portugal
Raja maderensis- Seine seamount
Raja clavata- Norway
Raja clavata- Rockall
Raja clavata- Portugal
Raja maderensis- Irving seamount
Raja maderensis- Madeira
Raja maderensis- Madeira
Raja clavata- Azores
Dipturus nidarosiensis- Norway
Dipturus nidarosiensis- Rockall
Dipturus nidarosiensis- Mediterranean
Dipturus laevis
Dipturus batis
Dipturus oxyrinchus - Portugal
Dipturus oxyrinchus - Norway
Dipturus oxyrinchus - Azores
Dipturus intermedius- Azores
Dipturus intermedius- Shetlands
97
100
58
93
90
88
89
72
100
100
61
75
68
98
100
54
83
55
68
55
2.0

3097

Chapter 4: General discussion

This thesis has addressed two main research components surrounding the taxonomic status of batoid species across the North Atlantic: (i) chapter two utilised mtDNA sequencing to assess the distribution of the flapper skate (*Dipturus intermedius*) and the blue skate (*Dipturus batis*)- the two taxa formally known as the 'common skate'; (ii) chapter three generated novel nextRAD and mtDNA data from several species of skate in order to resolve their taxonomy. Specifically, this chapter addressed whether the thornback ray (*Raja clavata*) and the Madeiran skate (*Raja maderensis*) are the same species or not, the genetic distinctiveness of Norwegian skate (*Dipturus nidarosiensis*) in the Mediterranean and whether there is a cryptic *Dipturus* species in the Azores. NextRAD sequencing has never been applied to batoids before, so this section offered an opportunity to comment on the utility of this method for resolving elasmobranch phylogenies. In addition to these taxonomic questions, this thesis also touched on the conservation implications of our findings for these threatened species of skate.

4.1 Conclusions and future directions

4.1.1 The blue skate (*Dipturus batis*) and the flapper skate (*Dipturus intermedius*)

Currently, the IUCN led list does not include the flapper and blue skate as separate species, despite evidence existing since 2010 (Griffiths *et al,* 2010; Iglésias *et al.,* 2010). Therefore, *D. intermedius* and *D. batis* are still being treated as one homogenous unit in IUCN conservation assessments (Dulvy *et al.,* 2006). Given the evidence that the 'common skate' actually represents two distinct, critically endangered species (the current study; Griffiths *et al,* 2010; Iglésias *et al.,* 2010), a new formal assessment into the conservation status and distribution of *D. batis* and *D. intermedius* is urgently needed. In the current thesis, the distribution and taxonomic status of these species was explored in

both chapters two and three. Results from CR and COI sequencing in chapter two suggest that the blue skate is commonly distributed in the Western Approaches and Celtic Sea, extending out to Rockall and Iceland. The flapper skate generally appears to be much less abundant, but is most frequent around northern Scotland and Ireland, including the northern North Sea. In particular, the current thesis represents the first genetic evidence of flapper skate in the Azores (chapter two; chapter three), an area that has long-established Marine Protected Areas (Abecasis *et al.,* 2015). This is the furthest East and South this species has been found to date; although exciting, it does suggest that *D. intermedius* once may have had a much wider range that extended into more southerly regions. Whilst historical fishing of 'common skate' may have led to its local extirpation across much of the continental shelf of Europe, the Azores may have acted as a refuge for flapper skate. Additionally, these Azorean samples had unique haplotypes, highlighting the importance of the area as a 'hotspot' for the most fundamental level of biodiversity- genetic diversity. Future investigation of the population genetics of skates in this area could help to determine if these Azorean flapper skate need to be treated as a separate conservation unit due to their genetic distinctiveness. At 1300 km from mainland Portugal, the remoteness of the Azores could act to isolate this population from other specimens across the North Atlantic (chapter two, Figure one). For example, this is analogous to the critically endangered angelshark. Having undergone huge declines across its range in Europe, the remote Canary Isles represents a stronghold and growing focus for conservation in this species (Barker *et al.,* 2015). In the case of flapper skate in the Azores, only a single specimen is recorded in the tissue bank collected over approximately 5 years of research cruises in the region and evidence from fisheries surveys does not suggest the species is abundant in this area (Menezes *et al.,* 2006). However, further surveys, especially in deeper seas, may well be required to comprehensively answer this question.

In the rest of the North Atlantic, increased protection for blue and flapper skate is likely needed. Although there exists a ban on landing either species across the UK, MPAs are largely limited to Scotland (Scottish Natural Heritage). The Lock Sunart to the Sound of Jura Nature Conservation Marine Protected area, for example, is used by flapper skate as important egg-laying grounds and

juvenile habitat (Scottish Natural Heritage), a demographic identified for protection in order to enable population recovery (Dulvy *et al.,* 2006). Furthermore, although the current study has highlighted the presence of blue skate in the North Sea, there exists no designated sites for the protection of these skates in the area (Marine Conservation Society), which may need to be considered. Distribution information such as the results represented in this thesis could help to identify more population 'centres' for blue skate and flapper skate in the rest of the North Atlantic as candidates for protection, helping to conserve these critically endangered species. Indeed, the utility of spatially restricted MPAs in the protection of highly mobile species such as batoids has been the focus of debate in the literature (e.g. Kaiser, 2005; Wearmouth & Sims, 2009; Knip *et al.,* 2012; Schofield *et al.,* 2013) and may perhaps be most effective when targeted at critical life-stages e.g. foraging habitats, migration corridors (Griffin *et al.,* 2013). The limited tagging data for this group has generally been collected before the 'common skate' was spit into *D. intermedius* and *D. flossada* (Sutcliffe, 1994; Little, 1995, 1998; Wearmouth & Sims, 2009), so uncertainties remain regarding its applicability. However, tagging programmes such as those already being conducted in Scotland (Scottish Shark Tagging Programme, 2018) may help provide more clarity on the effectiveness of MPAs in conserving this group.

4.1.2 The thornback ray (*Raja clavata*) and Madeiran skate (*Raja maderensis*)

Based on combined interpretation of mtDNA and nextRAD phylogenies in chapter three, the distinctiveness of Madeiran skate from the thornback ray still remains ambiguous. This is in concordance with previous mtDNA studies that have not supported their classification as distinct species, but instead a broad genetic pattern based on correlation between genetic and spatial proximity (Chevlot *et al.,* 2006; Naylor *et al.,* 2012; Ball *et al.,* 2016). Overall, Madeiran skate from Madeira and thornback ray/ Madeiran skate from the Azores and the surrounding seamounts appear to be genetically distinct from conspecifics in the rest of the North Atlantic (Chevlot *et al.,* 2006; Naylor *et al.,* 2012; Ball *et al.,* 2016; chapter three). These populations may mandate a different conservation strategy due to their genetic distinctiveness, which should be considered in future conservation

assessments. Currently, European Union catch limitations do not extend to the Azores or Madeira (Dulvy *et al.,* 2015), but additional protection for this area would certainly help to conserve genetic diversity. Additionally, nuclear and mitochondrial DNA evidence indicates that Portuguese *R. clavata* may be genetically distinct from other North Atlantic populations (Chevlot *et al.,* 2006; Ball *et al.,* 2016; chapter three), however, this is based on a limited number of samples (n= two). Future mitochondrial and nuclear sequencing of more thornback ray from Portugal may help to determine if this region should be treated as a distinct genetic unit in conservation assessments.

4.1.3 Longnosed skate (*Dipturus oxyrinchus*)

Chapter three explored the possibility of the presence of a cryptic *Dipturus* species in the Azores, based on previous mtDNA evidence (Andrew Griffiths, unpubl. Data). The ideas of further cryptic Dipturus species has also been highlighted in the wider literature (ref). Whilst the presence of a cryptic *Dipturus* species in the Azores wasn't fully supported, longnosed skate from the region could represent their own genetic lineage, distinct from their counterparts in geographically proximate Portugal and Norway. Such consistent distinctiveness of Azorean skate populations throughout this thesis is a strong point of interest to conservation biologists and has the potential to inform the designations of MPAs that include batoids as a protected feature. Sequencing of more longnosed skate from the Azores, for example, may help to provide essential baseline data relating to levels of genetic diversity and population differentiation.

It is important to note that the small samples sizes of *Dipturus* specimens analysed from the Azores does limit useful biological interpretation (and partially reflects the scarcity of material from these threatened groups). Therefore, looking to evidence from species with similar ecologies or taxonomic affinities can be a valuable additional approach in making conservation recommendations. Results from the analysis of thornback rays that are included in this thesis and elsewhere (Chevolot *et al.,* 2006; Ball *et al.,* 2016) support the highly distinct nature of rays in this area. This combined with the

limited evidence of the genetic distinctiveness of longnose and flapper skates does broadly support the importance of the Azores to conservation of genetic diversity of European skated more generally.

4.1.4 Norwegian skate (*Dipturus nidarosiensis*)

Given the previous hypothesis that Mediterranean Norwegian skate may represent a cryptic species, chapter three analysed individuals from the North Atlantic and the Mediterranean (Cannas *et al.,* 2010; Ebert & Stehann, 2013). Although evidence does not generally support the presence of cryptic speciation (the current study; Carbonara *et al.,* 2019), the genetic distinctiveness of *D. nidarosiensis* from the Mediterranean in the current study highlights a potentially distinct population that may have a very restricted range. Results suggest the Atlantic-Mediterranean transition may act as a genetic barrier to gene flow in Norwegian skate, as has been shown with other batoid species (Chevolot *et al.,* 2006; Griffiths *et al.,* 2011). Similarly to the Azores, the Mediterranean may have served as a refugium for Norwegian skate and other batoids during the last glacial maximum, leading to isolation and restricted gene flow to the rest of Atlantic (Chevolot *et al.,* 2006). However, the small number of individuals used in the current study result in a weak ability to detect population structuring, and therefore further sequencing of Norwegian skate from this region would be an interesting point of future research. Nevertheless, given the sparse nature of available information on this threatened species of skate, results such as those represented here are vital to ensure the effective conservation of the species and should be considered in conservation assessments.

4.1.5 Methodologies

This thesis has not only provided resolution of vulnerable batoid taxa, but also an opportunity to evaluate the methodological process from which taxonomists draw conclusions. Recently, the role of mtDNA for phylogenetic inference has become contentious, with one viewpoint advocating it's future elimination from the field (Ballard & Whitlock, 2004; Shaw, 2004) juxtaposed against the DNA 'barcode' movement, which solely uses the COI gene to assign unknown individuals to species

(Hebert *et al.,* 2003, see Rubinoff & Holland, 2005 for a review). In chapter two, COI barcoding successfully confirmed the allocation of individuals by morphological experts in the field to either blue or flapper skate, thus allowing their distributions to be clarified. Although the presence of cryptic speciation in batoids has been suggested as a limitation to the successful application of DNA barcoding (Cerutti-Pereyra *et al.,* 2012), these results advocate for its use to confirm the presence of cryptic species. This is not a novel concept and has been successfully applied in previous studies on batoids (e.g. Ward *et al.,* 2008; Richards *et al.,* 2009; Ball *et al.,* 2016). This study utilised COI barcoding to confirm morphological identification, and indeed the species identity of unknown specimens (e.g. flapper skate from the Azores). By combining different data sources (*in situ* morphological identification, mtDNA), the present study allowed the biological context of the COI phylogeny to be clarified. Furthermore, sequencing of the CR produced a similar topology, thus increasing confidence that these results represent the true evolutionary history of the blue and flapper skate. This is the most effective way to understand the evolutionary history of taxa; without biological context, or combined data sources, there can be limited confidence in a topology derived from a single gene that includes unknown specimens (Rubinoff & Holland, 2005).

Chapter three also utilised the principal of analysing taxonomic partitions across datasets, combining *in situ* morphological identification with nDNA (concatenated nextRAD SNPs) and mtDNA (concatenated CR and COI data). Whilst the utility of mtDNA for resolving batoid phylogenetics is well-documented, concatenated SNP data has been traditionally used for population genetics (Valsecchi *et al.,* 2005; Ward and Holmes, 2007; Moura *et al.,* 2008; Serra-Pereira *et al.,* 2011). Due to their cost efficiency, high abundance and genome-wide distribution, SNPs are playing an increasingly important role in phylogenetic and phylogeographical studies (Emerson *et al.,* 2010; Eaton & Ree 2013; Wagner *et al.,* 2013; Herrera & Shank 2015; Leaché & Oaks, 2017). However, several concerns remain regarding their utility in this context. An important issue is how to analyse concatenated SNP data, as it violates assumptions inherent in models that are often applied to traditional DNA sequence data. In extreme cases, this violation can lead to overestimated support or inaccurate topologies (Liu *et al.,* 2015; Xu & Yang, 2016; Leaché & Oaks, 2017). Therefore, Chapter

three applied two methods for constructing batoid phylogenies from nextRAD-seq data for comparison: RAxML and SVDquartet analysis. The former of these has been applied extensively to analyse traditional DNA sequence datasets, whilst the latter is specifically built to handle SNPs, accounting for their unique genealogical history and predisposition to large amounts missing data (Chhifman & Kubatko, 2014). Both phylogenies were largely congruent, with only minor differences within the *Raja* genus and lower phylogenetic support in the SVDquartet tree. Further, the mtDNA and nextRAD SNP datasets successfully resolved expected species groupings and patterns of fine-scale genetic structuring within the challenging batoid taxa analysed. However, incongruency between the classification of Azorean longnosed skate in mtDNA and nextRAD phylogenies could be seen. Namely, mtDNA data supported the presence of a cryptic *Dipturus* species in the Azores, placing longnosed skate from the area as closely related to flapper skate from the Azores and Shetlands. However, in all trees built from nextRAD data, Azorean longnosed skate were grouped with their counterparts from Norway and Portugal. This highlights the dangers of interpreting mtDNA in isolation, and supports previous studies that have utilised concatenated SNPs for species delineation in vertebrates, applying RAxML and other SNP-specific methods such as SNAPP and PoMo (Emerson *et al.,* 2010; Eaton & Ree 2013; Wagner *et al.,* 2013; Herrera & Shank 2015; DaCosta & Sorenson, 2016; Massatti *et al.,* 2016). Furthermore, nextRAD SNPs offered an opportunity to gain finer resolution from traditional mitochondrial markers in Chapter three, accounting for mtDNA's weaknesses whilst utilising its strengths. An interesting point of future research would be to explore the utility of nextRAD-seq data in identifying areas of the genome under selection in these vulnerable batoid species. RAD-seq has previously been used to this end in other organisms, for example, studies in sea anemones (Reitzel *et al.,* 2013) and stickleback (Hohenlohe *et al.,* 2010) have identified novel genomic regions under selection, which correlate with local adaptation in natural populations. Given the genetic divergence of populations of batoids from the Azores and surrounding seamounts in the current study, and the unique morphology of Madeiran skate, this would be an interesting hypothesis to explore for these species. Currently, there is no reference genome available for batoids, however, several sources could make this possible in the future, including the North East Bioinformatics Collaborative's project to sequence the genome of the Little Skate

(http://skatebase.org/) and the Sanger Institute's 25 Genomes project (https://www.sanger.ac.uk/science/collaboration/25-genomes-25-years).

4.2 Conclusion

Overall, this thesis' exploration into the taxonomic status of vulnerable species of skate has provided us with somewhat of a paradox. Whilst we have gained more genetic information essential to the conservation of these batoids, this study has simultaneously revealed the complex nature of their taxonomy and management. The polytypic nature of the thornback ray and subsequent taxonomic confusion highlights the complicated task of accurately conserving and managing batoid species, particularly those that exhibit intra-species morphological variation. The genetic distinctiveness of skate populations from the remote seamounts in the North Atlantic and Norwegian skate in the Mediterranean suggests that biodiversity, a fundamental component of conservation assessments, is often underestimated in the batoids. However, mtDNA and nextRAD sequencing have proven to be effective in resolving batoid phylogenies in the current study and provide useful tools for future research. By combining the use of mtDNA and nDNA, one can examine patterns of intogression, selection, population genetics and demographic structuring, all of which can provide information that is currently lacking in batoid fish.

4.3 References

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