Divergence and reproductive isolation in the bushcricket *Mecopoda elongata*

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

Signature: .....

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Abstract

The evolution of isolating mechanisms within a species population impedes gene flow. This allows isolated populations to diverge along different trajectories, which may ultimately lead to the formation of new species. Our attempts to understand the evolution of isolating barriers have benefited enormously from studies of divergent populations that are still recognized as members of the same species. The co-occurrence of five acoustically distinct populations of the bushcricket *Mecopoda elongata* in south India provided us with the opportunity to study one such divergence of sympatric populations of a single species. In sympatric populations that share identical ecology, sexual selection has the potential to play a prominent role in the maintenance of reproductive isolation. Based on a previous traditional morphometric study, *Mecopoda elongata* in India were thought to be a morphologically indistinguishable cryptic species complex. The lack of morphological divergence suggests a less significant role of ecology in the divergence of the group. One possibility is that songtypes may be maintained by the preference of *Mecopoda elongata* females for mating with a specific songtype. In this thesis I show that female phonotaxis to their 'own' call has the potential to contribute to behavioural isolation among the songtypes and in particular between two songtypes with overlapping temporal call parameters. This finding is supported by an independent no-choice mating experiment utilizing the same two songtypes. To investigate the cues other than song that *Mecopoda elongata* females' may use to exercise preference for their own type, I examined the composition of cuticular lipids in the cuticle and the detailed structure of secondary sexual characters. I was able to differentiate all *Mecopoda elongata* songtypes with high probability based on CHC profiles and geometric morphometrics of the sub genital plate and cerci. My study reveals that divergence in sexual traits other than acoustic signals, although dramatically less obvious in nature, is present among *Mecopoda elongata* populations. This provides potential mechanisms for premating isolation among *Mecopoda elongata* songtypes in the wild suggesting that reproductive isolation is maintained by female preferences for male sexual signals. Additionally, I discovered a parasitoid Tachinid fly responsible for infecting three different songtypes of *Mecopoda elongata*, namely Double Chirper, Two Part and Helicopter. This Tachinid fly appears to have specialized hearing organ to track down calling *Mecopoda elongata* males throwing light on potential selection pressure and possible mechanism for *Mecopoda elongata* song divergence.

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Speciation research

It has been postulated that substantial progress in speciation research has occurred in three phases and that it has suffered stagnation in between these phases (Coyne and Orr, 2004). The last phase of advancement (since the 1980s) has been characterised by a spurt of research activity related to understanding the evolution of barriers to gene flow and their role in causing divergence in populations ultimately resulting in speciation. The overall basis for speciation has been attributed to a small number of processes [see figure 1.1] including the primary evolutionary processes of selection (including sexual selection) and random evolution including genetic drift.

To understand the processes influencing speciation, scientists have broadly followed three approaches (Tregenza, 2002) all having their share of advantages and disadvantages. These three approaches are laboratory based experimental studies, comparative studies on different but already established related species and comparative studies of divergent populations of the same species or populations in hybrid zones. While the first two approaches have obvious drawbacks of either removing the effects of the natural environment or studying groups that have already speciated, the study of divergent populations of a single species has proved to be particularly valuable in attempting to understand actual speciation processes in nature. While we can be very confident that hybrid zones are a product of secondary contact of already existing divergent populations, sympatric populations that lack clear tension zones and are not fully reproductively

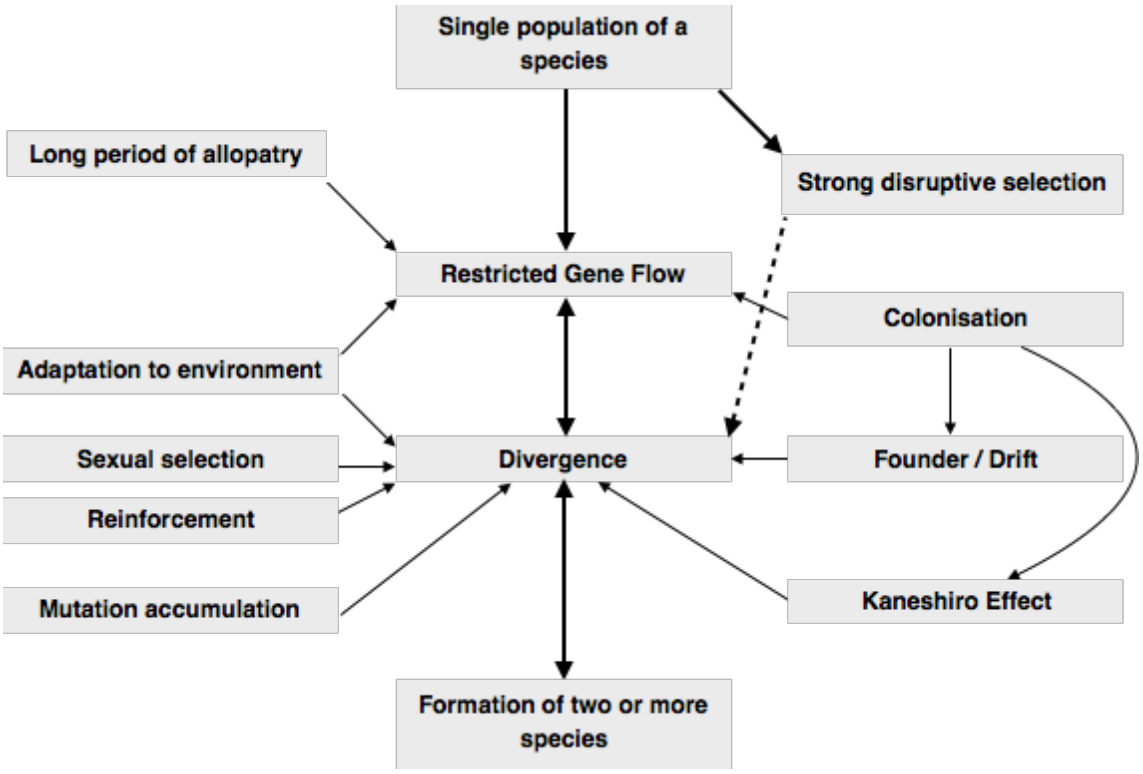


Figure 1.1: A scheme showing different factors contributing to speciation at different stages of the process (adapted from Tregenza (2002))

isolated may throw light on the mechanisms leading to speciation. These studies, however, fail to judge the relative importance of various processes in nature leading to speciation. To achieve this, we have to test multiple hypotheses in a single divergent study system, which can be very challenging particularly when generation times are lengthy. Another way to overcome this problem would be to find a naturally occurring study system in which it is possible to rule out major influences of many different processes such that one is left with few factors to test with and hold accountable for the observable divergence. One such unique system is that of the strikingly different songtypes of bushcrickets in India belonging to the genus *Mecopoda* (Orthoptera: Tettigoniidae: Mecopodinae) [see figure 1.2] whose unique natural history makes it a conducive system to ask many questions related to the evolution and maintenance of reproductive isolation.

1.2 Speciation

How a seemingly continuous process of adaptation within a single population leads to the formation of discrete species has been one of the central questions

Figure 1.2: *Mecopoda* in its natural surrounding

in evolutionary biology. It took a long time to recognise that without a barrier to gene flow new species cannot form (Dobzhansky, 1935) and an even longer time for studies into the evolution of these isolating mechanisms to begin (Coyne and Orr, 2004). The concept of a "barrier to gene flow" is a central idea in evolutionary biology because of its essential role in the process of speciation.

Speciation is the process by which new species are formed from an existing one. The ability to assign distinct groups of individuals into species predates any academic consideration of the notion of a species in a formal sense. It has been difficult to provide a conclusive and foolproof definition of what a species is; almost invariably, there have been a number of limitations to each definition formulated based on philosophical concepts (Coyne and Orr, 2004). A similar problem is also faced during the practical identification of species or even higher classification taxa: different analytical methods have led to incongruent grouping of the individuals under study (Balakrishnan, 2005), most strikingly in the case of the Order Orthoptera itself (Song et al., 2015). The most popular definition of 'species' has been one that stems from the Biological Species Concept (BSC) propounded by Ernst Mayr in 1942 (De Queiroz, 2005). According to the BSC, "species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr, 1942). It is based on the possibility that if a group of individuals develops a barrier to gene flow to other similar groups of individuals such that they share a separate gene pool than the

parent gene pool, then over a period this group may diverge to form a unique group. This unique group may be designated as a new species if the uniqueness is maintained even when there is a possibility of mating (gene flow) through the potential for direct interactions between individuals of the two incipient species.

If we follow figure 1.1, a single population of one species may split into two species if there is restriction to gene flow between two isolated groups. In case the restricted gene flow leads to divergence, the divergent populations are seldom found to differ in only a single trait and we can expect to find signatures of isolating mechanisms in many traits. Notable exceptions to this observable divergence are the cryptic populations of a species that appear to be morphologically indistinguishable but upon a more intensive search of divergence in other traits, these cryptic divergent populations can be identified. If these divergent populations accumulate enough differences such that the probability of leaving progeny after successful mating between two random individuals of different divergent populations varies from negligible to nil, then we can assume that the process of speciation has ended due to complete reproductive isolation between the divergent populations. The strength of reproductive isolation can be determined by studying the differences among the divergent populations based on the divergent traits. It is interesting that speciation may still occur even with limited or widespread gene flow (Papadopulos et al., 2011) and does not necessarily need complete reproductive isolation.

Reproductive isolation is classified as pre-zygotic and post-zygotic depending on whether the barriers to reproduction between two divergent populations occur before or after fertilisation of eggs by sperm. Pre-zygotic isolation is further divided into ecological and non-ecological isolation. Ecological isolation, include isolation due to difference in habitat, pollinating agents in the case of plants or due to differences in life history. Non-ecological isolation includes mating system, behavioural, mechanical and post mating pre-zygotic isolations (Coyne and Orr, 2004). Generally the evolution of pre-zygotic isolation is considered critical for initiation and maintenance of difference among divergent populations of a species (Kirkpatrick and Ravigné, 2002). One cannot, however, ignore the immense role of post-zygotic isolation in the case of reinforcement (maintenance of isolation between divergent populations that are not species yet through selection against the creation of hybrids) and reproductive character displacement (maintenance of isolation between species through selection against mating with the wrong species).

The formation of new species can occur whilst the diverging groups are in allopatry (where there is no overlap of distribution due to a geographical barrier to gene

flow) or when they remain in contact, either through parapatry or sympatry (where there is overlap of their spatial distribution). While speciation by allopatry remains well addressed and easily explained due to independent evolutionary trajectories of the divergent populations separated by geography (Turelli et al., 2001), this thesis mostly addresses the potential for sympatric speciation since *Mecopoda* songtypes are largely sympatrically distributed in southern India [see table 1.1 and figure 1.3]. Sympatric speciation is defined as the process where the barriers to gene flow evolve intrinsically within the average dispersal distance of the members of an interbreeding population of a species (Coyne and Orr, 2004). This definition underlines the fact that the uniqueness of sympatric speciation lies in not being caused by either geography or distance, factors that are the main determinants of allopatric and parapatric speciation.

1.3 Role of sexual selection in sympatric speciation

Traditionally speciation research has been classified based on the distribution of divergent groups under study into allopatric speciation, sympatric speciation and parapatric speciation. Nevertheless, speciation research may also be classified according to the major processes such as natural selection, sexual selection and genetic drift that leads to the evolution of reproductive isolation between populations (Turelli et al., 2001). In nature, it is often seen that individuals (mostly, males) in a population possess certain traits associated with competition with other males and attracting mates that do not appear to have an adaptive advantage in their environment. These traits, called secondary sexual traits however, confer an advantage to the individuals possessing them in gaining fertilisations. The process by which traits that are advantageous in relation to gaining matings and fertilisations rather than in relation to survival evolve is called sexual selection (Darwin, 1874). Sexual selection can drive divergence between allopatric populations if male adaptations to attract mates and female adaptations to choose among males evolve in different directions in allopatric populations which subsequently come into contact. In individual allopatric population, female preference may initially arise for an arbitrary male trait. This arbitrary trait may be a character affecting ecology of the population under study and hence under direct selection or it might be neutral and not under direct selection. In case the trait is under neutral selection, it should get associated with beneficial traits that are under direct natural selection at some point of their evolution for strong isolating mechanisms

to evolve between the two allopatric populations (Dieckmann and Doebeli, 1999; Turelli et al., 2001). On the other hand, sexual selection appears to play a more pivotal role in the population divergences in sympatry.

The main problem addressed in sympatric speciation is to explain how reproductive isolation during sympatric speciation can be stably maintained over a long time when there is potential for hybridisation especially at the initial stage. Empirical evidence and mathematical models of sympatric speciation show that it takes place by disruption of either natural selection or sexual selection (Coyne and Orr, 2004). In disruptive natural selection, initial divergence is due to divergence in the ecology of the populations. However, once this ecological divergence is achieved there must be assortative mating between the sexes within each ecological niche to strengthen the speciation, otherwise the divergence would not sustain itself in the face of possible gene flow (Barton, 2010; Dieckmann and Doebeli, 1999). Assortative mating is not always equivalent to mate choice where an individual of one sex preferentially mates based on preferred traits with an individual of the opposite sex. In a simplistic scenario, assortative mating may be a byproduct of natural selection itself (Kirkpatrick and Nuismer, 2004). For example, if a female has a natural tendency to mate with males having similar traits to its own where population differentiation has happened due to variation of such traits, there may be no genetic basis for preference for a particular trait involved, at least in the initial stage of speciation (Bolnick and Paull, 2009). However, assortative mating may be in the form of mate choice when it arises from non-random mating between individuals with a preferred genetic trait or a small group of traits spread over one locus or multiple loci (Maan and Seehausen, 2011).

In speciation where divergence arises due to disruption in sexual selection, there is also the problem of coexistence of sister groups in the face of potential hybridisation. There has been debate about the plausibility of speciation occurring through disruptive sexual selection. It is generally agreed that speciation due to preference for a secondary sexual trait initiates when different groups of females have preference for different secondary sexual characters resulting in the evolution of reproductive isolation among them. This presumes that these secondary sexual characters should have a very high variability within the population. This condition appears inexplicable at first in a stable population since these traits are associated with reproduction. However, numerous models now have shown that there may be disruption in population continuum by frequency dependent competition even when the population is at equilibrium under the assumption that

the population under study is a panmictic one (Kirkpatrick and Nuismer, 2004). Besides, when divergent populations get established, maintenance of population differences seems unlikely unless there appears some sort of ecological differentiation among the divergent populations since gene flow will sooner or later dissolve the population differences (Maan and Seehausen, 2011; Ritchie, 2007; van Doorn et al., 2009). There have been recent efforts to model all processes leading to sympatric speciation based on empirical evidences: a few of these evidences are discussed as subsections below.

As seen in models of speciation through disruptive natural selection, assortative mating is important for the completion of speciation (Dieckmann and Doebeli, 1999). We may consider no-genetic-basis condition of assortative mating as the null model (Coyne and Orr, 2004); however, in nature only heritable characters play an important role in the evolution of species over generations. In models where assortative mating is based on one locus, preference for a trait may be based on allelic forms in the population. In case the preference of females is based on a single allele (and there is no preference for the other allelic forms) that represents a divergent population then speciation may take place very easily. In the two allele situation, each of the two divergent populations is represented by either of the two allelic forms. The chance presence of the alternative allele in one population does not coerce preference from the opposite sex of the same population (Barton, 2010; Felsenstein, 1981). This results in rapid sympatric speciation if the preferred allele additionally gets linked to traits under natural selection (Felsenstein, 1981). Multilocus models are more realistic models since genetic basis for traits under assortative mating are more commonly found to be spread over more than one locus. These models also assume that all these loci are tightly linked such that recombinations do not dissociate preferred traits from traits under natural selection (Johnson et al., 1996).

1.3.1 Example: host race formation in insects

The theoretical models of speciation by disruptive natural selection involve selection of the habitat-preference-genotype, adaptation to preferred habitat and subsequently assortative mating (Johnson et al., 1996). Individuals that are genetically predisposed to prefer a new habitat may get adapted to it over generations through natural selection since their fitness is increased in the new habitat. This increased fitness in the new habitat may also cause decreased fitness in the previous habitat.

This condition is likely to give rise to incipient species due to intraspecific conflict over resource use (Barton, 2010; Bolnick, 2011). These probable species groups may then transform into new species if individuals of one habitat group develop preference for mating with individuals of the same habitat. For example, in *Rhagholetis pomonella* races that show assortative mating due to habitat preference, hawthorn race and apple race undergoes 20-30 % reduction in gene flow due to the mating habits of the race and seasonality of the host species (Feder, 1998; Feder and Filchak, 1999). However, ambiguity about phylogenetic relations and past distribution makes it difficult to test if the present distribution and population differentiation is due to allopatry or sympatry. Two races of pea aphid in northeastern America, *Acyrthosiphon pisum* is another cited example of host race formation where phytophagous exploiters specialise in utilising a particular host due to intrinsic genetic advantage and subsequent assortative mating creates isolation in microhabitats within a geographical boundary. These two races are well adapted to clover and alfalfa hosts with each race having significant fitness on their respective host even over race hybrids (Via, 2001). However, sympatric speciation in these incipient species is also not foolproof due to lack of proper documentation of their biogeographic history that appears to be a common problem regarding support of speciation in sympatry.

1.3.2 Example: adaptive radiation in cichlid fish

Lake Victoria, Lake Malawi, Lake Tanganyika and other lakes of east Africa appear to possess numerous species of cichlid fish forming species complexes. For instance, Lake Malawi consists of 659 known endemic species of cichlid fish that seem to be monophyletic and many of which have distinct nuptial male colouration (Albertson et al., 1999). It has been found that sexual selection through sensory drive may have led to reproductive isolation in the cichlid fish complex in Lake Victoria (Seehausen et al., 2008). In Lake Malawi, cichlid fish were found to differ in feeding habits between genera but species within genera were found to differ little in food habits, habitat use and mating site preference. This indicates that recent radiation is most likely to have been initiated by mate choice based on male colouration. Interestingly, this preference for colour morphs broke down leading to hybridisation when visibility of the colour was hampered (Allender et al.,

2003). Thus the rapid development of reproductive isolation of closely related cichlid populations within a small geographical area without prominent hybridisation make them a stunning example of sympatric speciation through sexual selection.

1.3.3 Example: speciation in threespine stickleback

The threespine stickleback *Gasterosteus aculeatus* presents a condition where theoretically sympatric speciation seems plausible given their natural history but empirical studies along with simulation studies show otherwise (Bolnick, 2011). Under strong intraspecific competition, sticklebacks show adaptation to varying resources with individuals showing specialisation to different prey size (Bolnick, 2004). This kind of specialisation may lead to divergence of the population due to disruptive selection based on feeding habits that can be enhanced by environmental conditions such as habitat size and higher interspecific competition. In these conditions assortative mating due to co-ocurrence in same habitat, mating season, mating between similar phenotypes or directed preference from both sexes may lead to incipient speciation. However, although disruptive selection and assortative mating seem to co-occur in sticklebacks, weak disruptive selection followed by weak assortative mating appears to not allow much differentiation in sympatric populations, rather these population shows adaptation to fluctuating environment instead (Bolnick, 2011).

1.4 Trade off between natural selection and sexual selection in sympatric speciation

The scheme of classification of speciation research according to evolutionary process is unconventional since it is very rare to find a single evolutionary process entirely responsible for impeding gene flow in a speciating system (Turelli et al., 2001). In allopatric speciation, populations separated by a geographic barrier take independent evolutionary trajectories and accumulate differences. The interaction between natural selection and sexual selection in allopatric speciation is limited to the accumulation of such differences and reaffirmation of the divergences through reinforcement. Female preference may evolve for random male traits by sexual selection in allopatric populations undergoing speciation. In the event that the

preferred trait is already under natural selection, divergence of the population will follow the evolutionary trajectory of the preferred trait without any hindrance where as if the preferred trait is neutral under selection an added step of linkage between preferred trait and ecologically beneficial trait is necessary for the preferred trait to diverge enough to cause speciation (Dieckmann and Doebeli, 1999). Thus the preferred male traits may simultaneously be under both natural selection and sexual selection at some point of their evolutionary path: the evolution of this male trait will be then a product of trade-off between natural and sexual selection where the end product is contingent on the conditions in which it exists. For example, sexual conflict in the population may result in predominance of sexual selection in driving divergence between the two allopatric populations while on the other hand, exploitative interactions may lead to natural selection influencing the divergence of the concerned trait. Reinforcement, on the other hand, may enhance the evolution of secondary sexual characters leading to reproductive character displacement, should the allopatric populations come in contact with each other.

In no process of speciation do we see an evident interaction of sexual selection with natural selection as seen in sympatric speciation. Most models of sympatric speciation have shown that natural selection and sexual selection are both necessary for sympatric speciation irrespective of initiation by disruptive natural selection or disruptive sexual selection (Coyne and Orr, 2004). In models that assume initiation of population divergence of a stable population in sympatry through ecological differentiation, a strong assortative mating is necessary for divergent populations to establish as separate species (Barton, 2010). Speciation in this way is a possible scenario when the mating probability is equal for all individuals in a population such that even rarer divergent traits finds mate and have progenies (Kirkpatrick and Nuismer, 2004). This is an unrealistic assumption since it is expected that the individuals with rarer traits will find it more difficult to find mates than those individuals which have mean trait values. Therefore if the number of females with preference for mean trait value is more in number than those with divergent traits, then assortative mating based on variation of the male traits may lead to establishment of the stable population again. This scenario is possible when the cause for divergence is competition for resources, which is frequency dependent. However, if the cause for divergence is habitat association, the result of speciation might be completely different since habitat association evokes strong selection on the genetic basis of habitat preference as well as adaptation in the new habitat (Johnson et al., 1996). Again if the assortative mating is replaced by simultaneous evolution of female preference for a divergent trait, then sympatric speciation seems likely.

This means under different conditions sympatric speciation may be enhanced or hindered by both natural selection and sexual selection. Apart from this, the secondary sexual characters are costly traits that will be inevitably weighed down by natural selection. Sympatric speciation by disruptive sexual selection takes place when these secondary sexual characters diverge and female preference evolves for each of the divergent forms simultaneously. The divergence and preference of the secondary sexual character is greatly enhanced when they are also under natural selection. Probability of all these conditions occurring at the same time is low making speciation by disruptive sexual selection unrealistic (Arnegard and Kondrashov, 2004). This explains why magic traits (traits that are preferred by females and are also linked to survival of the organism in its surroundings) are thought to be rare in nature and why sympatric speciation remains controversial.

1.5 Conflict between sexes in speciation

In the last two decades, various studies have established that sexual conflict has tremendous potential to cause evolution of reproductive isolation and hence act as a mechanism for speciation (Gavrilets, 2014b). In sexually reproducing species, fitness gained through mating may vary between the sexes since the investment in such mating varies between males and females of the species. For example, the females are usually limited by the energy they spend in producing eggs while males are limited by the number of mates they can inseminate. Sexual conflict has been found to occur based on mating rate, parental care, size of the offspring, sperm use and epigenetic developmental control (Gavrilets, 2014a). In *Drosophila*, males release peptides in the female genital tract along with seminal fluid to manipulate the female endocrine and reproductive system (Chapman and Partridge, 1996). The females commit more eggs for fertilisation upon peptide release and this helps males to fertilise as many eggs as possible. However, there is also an increase in death rate of females due to secreted peptides (Chapman and Partridge, 1996). In case the females in allopatric populations evolve adaptations to avoid harmful matings, such sexual conflict may lead to genetic divergence and reproductive isolation among the allopatric populations as a byproduct of the rapid coevolution of reproductive traits to minimise intersexual conflict, ultimately leading to allopatric speciation (Gavrilets, 2000). In an alternative scenario with respect to speciation, females of the parent population may diverge genetically

based on mating rate while males continue to have lower fitness with both populations thus ending conflict. Subsequently, males may also diverge corresponding to the female genetic clusters leading to sympatric speciation of the divergent population (Gavrilets and Waxman, 2002). These are only two outcomes with possibility of speciation occurring out of the six possible outcomes where sexual conflict leads to genetic differentiation among populations (Gavrilets and Hayashi, 2005). Although these two outcomes of sexual conflict may lead to evolution of reproductive isolation in theory, speciation research has so far failed to find any definitive evidence of reproductive isolation (except in Martin and Hosken (2003)) arising out of sexual conflict (Gavrilets, 2014a).

1.6 Reinforcement and reproductive character displacement

The role of reinforcement in completion of speciation process has been a highly debated topic in speciation research (Noor, 1999). There is dearth of measures of its pervasiveness in nature and it has been difficult to assess exact role of reinforcement in driving speciation. In spite of this difficulty, where four different criteria (Noor, 1999) have to be met for reinforcement to be implicated for the observed increased isolation, studies have started showing evidence that reinforcement may result in strengthening of prezygotic isolation (Higgie and Blows, 2007; Hopkins and Rausher, 2011; Matute, 2010; Ortiz-Barrientos et al., 2004). It has also been argued that reinforcement is only relevant when the species pair in question is sympatric (Servedio, 2004). As such in absence of a definitive biogeographic history, most study models of sympatric speciation have an alternative explanation. The alternative explanation centers around the possibility that two or more related species currently in sympatry have speciated allopatrically in the past and in absence of a barrier, these allopatric species have come to coexist together within a geographical boundary. It is expected that proximity would increase the chance of hybridisation between the two species especially if the reproductive isolation is not complete and in extreme cases may lead to dissolution of species boundaries. More often it is found that the isolation is maintained and in fact strengthened further due to lower fitness of the hybrids and inability to adapt

well in the respective niches of the parent population (Servedio, 2004). This phenomenon of selection acting against mating between individuals of isolated populations is called reproductive character displacement or reinforcement depending on whether the isolated populations concerned are already defined species or are divergent populations with uncertain species status (Coyne and Orr, 2004). In both cases, speciation is considered to be complete resulting in differing species able to maintain their discrete characters. Reinforcement by definition then should allow evolution of stronger reproductive isolations in sympatric species than those evolving allopatrically (Matute and Ortiz-Barrientos, 2014). It is important to note that the evidence for reproductive character displacement is quite widespread in nature (Pfennig and Pfennig, 2009). This has led to the belief that reinforcement may also be more common than thought since reproductive character displacement is one of the outcomes of reinforcement. However, reinforcement is not always followed by reproductive character displacement especially in recent sympatric speciation (Servedio, 2004) and it will be interesting to examine if we can find evidence of reproductive character displacement in *Mecopoda*. We can assume that divergence in *Mecopoda* may not have been allopatric since there is no evidence of independently accumulated ecological differences. In case of divergent populations that were allopatric initially and later become sympatric, we would expect signatures of reproductive character displacement in response to hybridisation. An apparent lack of preferences for habitat, absence of morphological divergence that usually accompanies differentiation of general ecology and no evidence of hybridisation in the wild supports the assumption that *Mecopoda* divergence may be recent and not be allopatric in origin.

1.7 Genetics of signal and female preference

In the context of speciation by sexual selection, it is often assumed that divergence in traits and corresponding female preference for such traits within a population has an underlying genetic basis and is heritable. It is of paramount importance that the genetic basis is established since speciation primarily depends on blocking the flow of genes. Much of the work on finding genetic basis has been done on *Drosophila* (Laturney and Moehring, 2012) with few additions from orthopteran insects such as the cricket *Laupala* (Coyne and Orr, 2004). It is seldom found that genetic basis of sexual signals and preference for that trait is limited to a

single gene. One unique example of preferred trait (pheromonal blend) resulting from change in one locus and one or two preference genes for that trait is that of the pheromonal races of the European corn borer *Ostrinia nubilalis* and two closely related noctuid species of *Spodoptera* (Monti et al., 1997; Roelofs et al., 1987). In most other cases studied in *Drosophila*, however, preferred traits involve changes in more than one locus (Laturney and Moehring, 2012; Mallet, 2006). Chromosomal mapping has been carried out to figure out genetic basis for female receptivity in *Drosophila* species pairs and it has been found that most of these loci (most probably preference traits for auditory and olfactory cues) lie where probability of recombination is least, such as regions of species inversion polymorphisms, telomeres and centromeres. Reduced recombination of these loci may give rise to population specific heritable gene associations which may accumulate further mutations to form the basis for sympatric speciation (Laturney and Moehring, 2012). In introgression analysis involving 5 quantitative trait loci (QTL) of *L. paranigra* contributing to species difference with *L. kohalensis* based on song pulse rate, it was found that all 5 QTL had small but significant effects on differences in pulse rate (Ellison et al., 2011). Four out of these 5 QTL of *L. paranigra* were also found to be genetically linked with preference of females for pulse rates (Wiley et al., 2012). Such direct evidence is quite rare in speciation research and it shows that “run away” selection based on coevolution of preferred trait and female preference is possible and results in rapid speciation as seen in *Laupala*.

1.8 *Mecopoda* as a model system for studying speciation

The Tettigoniids, also called Bushcrickets or Katydids are important model systems in animal behaviour, ecology, evolutionary biology and taxonomy (Bailey and Rentz, 1990). Their usually broadband calls, specialised ears, varied reproductive biology and the fact that they are a large and diverse group adds to their interest (Bailey and Rentz, 1990). Being a bushcricket, *Mecopoda elongata* possesses many of the features of the Tettigoniids and a few unique features of its own.

Many Tettigoniid species are broadband callers with carrier frequencies ranging from 0.6 kHz to more than 100 kHz. These calls are species specific and along

with differences in mostly male genital morphology act as essential species recognition traits (Heller, 2005). The reproductive biology usually involves phonotaxis by females over a considerable distance. Upon contact or once in close proximity, the male stops calling and both sexes engage in antennation and other physical contact presumably to ensure conspecificity. After a period of this behaviour typically the pair mate or separate. There is usually no specific call associated with mating itself in bushcrickets. During mating, bushcricket males frequently provide the female with a nuptial gifts in the form of a spermatophylax (Heller, 2005).

Song et al. (2015) determined the higher taxonomic relationships of 36 out of 40 major orthopteran families that include all 12 ensiferan families and 24 out of 28 caeliferan families. This study included only the Family Tettigoniidae as a representative of the Superfamily Tettigonioidea and had to be satisfied with very limited data on the status of this Superfamily. This study also proposes long awaited major revision of the Tettigoniid taxonomy which shows that the potential for a taxonomic revelation still remains for this Superfamily group. This possibility is indicated by the fact that a recent taxonomic study on *Austromecopoda* sp. resulted in the description of 4 different species that were previously considered as a single species (Rentz et al., 2006). Interestingly, all the four species of *Austromecopoda* sp. that look very similar to *Mecopoda* sp. appear to have lost their ability to call and their communication process remains unknown.

Mecopoda elongata is the only species of the genus *Mecopoda* reported from India (Nityananda and Balakrishnan, 2006). Hence, I refer to this species as '*Mecopoda*' throughout the thesis particularly for the populations in south India, where I did my field sampling. It is also found in eastern Asia and the presence of songtypes of this species has been reported from Cambodia, Java, Sumatra and Malaysia (Hartbauer et al., 2004; Korsunovskaya, 2009; Sismondo, 1990). The genus *Mecopoda* is widely distributed all over the Indian subcontinent, although a systematic study of its distribution has not been carried out. *Mecopoda elongata* has been studied previously mostly as a model system for understanding the mechanisms of sound production and reception and for the study of acoustic synchrony (Hartbauer et al., 2004; Krüger et al., 2010; Nityananda and Balakrishnan, 2007; Nowotny et al., 2010; Römer et al., 2002; Sismondo, 1990). *Mecopoda* males call in choruses at night and synchronise their broadband calls. During synchronization, the calls of individuals do not superimpose completely although they have significant overlap; rather the call onsets of one male (the leader) consistently leads the other in a calling bout. In this situation, when given a choice, *Mecopoda*

Songtypes	Chirper	Double Chirper	Two Part	Helicopter	Train
Chirper	-	N	N	Y	Y
Double Chirper	N	-	Y	Y	Y
Two Part	N	Y	-	Y	Y
Helicopter	Y	Y	Y	-	Y
Train	Y	Y	Y	Y	-

Table 1.1: Sympatric combinations of Chirper, Double Chirper, Two Part, Helicopter and Train recorded in south India; Y represents evidence of sympatry and N represents absence of such evidence (Nityananda and Balakrishnan, 2006)

females have been found to approach leaders over followers among the group of synchronizing *Mecopoda* (Fertschai et al., 2007). Although these studies involved *Mecopoda* whose calls consisted of a simple repeated identical chirp structure, various other *Mecopoda* sp. songtypes with complex song structures have been described from various part of its range (Korsunovskaya, 2009). The occurrence of songtype divergence in *Mecopoda* from south India (Nityananda and Balakrishnan, 2006) makes it an interesting system to study speciation. *Mecopoda elongata* has not been used as a model system to study speciation until now.

The five south Indian songtypes, namely Chirper, Double Chirper, Two part, Helicopter and Train, occur in different combinations of sympatry with one another [see figure 1.3 and table 1.1] (although their distribution is not extensively studied) and the different songtypes have overlapping mating seasons (Nityananda and Balakrishnan, 2006). Currently known combinations of sympatry of Indian *Mecopoda* songtypes are shown in table 1.1. The only two dyadic combinations that have yet to be observed are Chirper-Double Chirper sympatry and Chirper-Two Part sympatry whose occurrence is, however, not unlikely. Therefore, based on the present information, we can conclude that *Mecopoda* songtypes successfully co-exist in eight out of ten combinations within a relatively small geographical area in south India. There is a general trend of existence of stronger discriminating mechanisms found among the divergent sympatric groups of a species than the divergent populations of a species that are found in allopatry (Noor, 1999). Following this trend, therefore, we sould expect to find a stronger isolation among the eight known sympatric combinations of *Mecopoda* songtypes while relatively weaker reproductive isolation between Chirper and Double Chirper population pair and Chirper and Two Part population pair.

The different *Mecopoda* songtypes also occupy similar microhabitats including when in sympatry and are to be found in the same vertical strata in forests (close

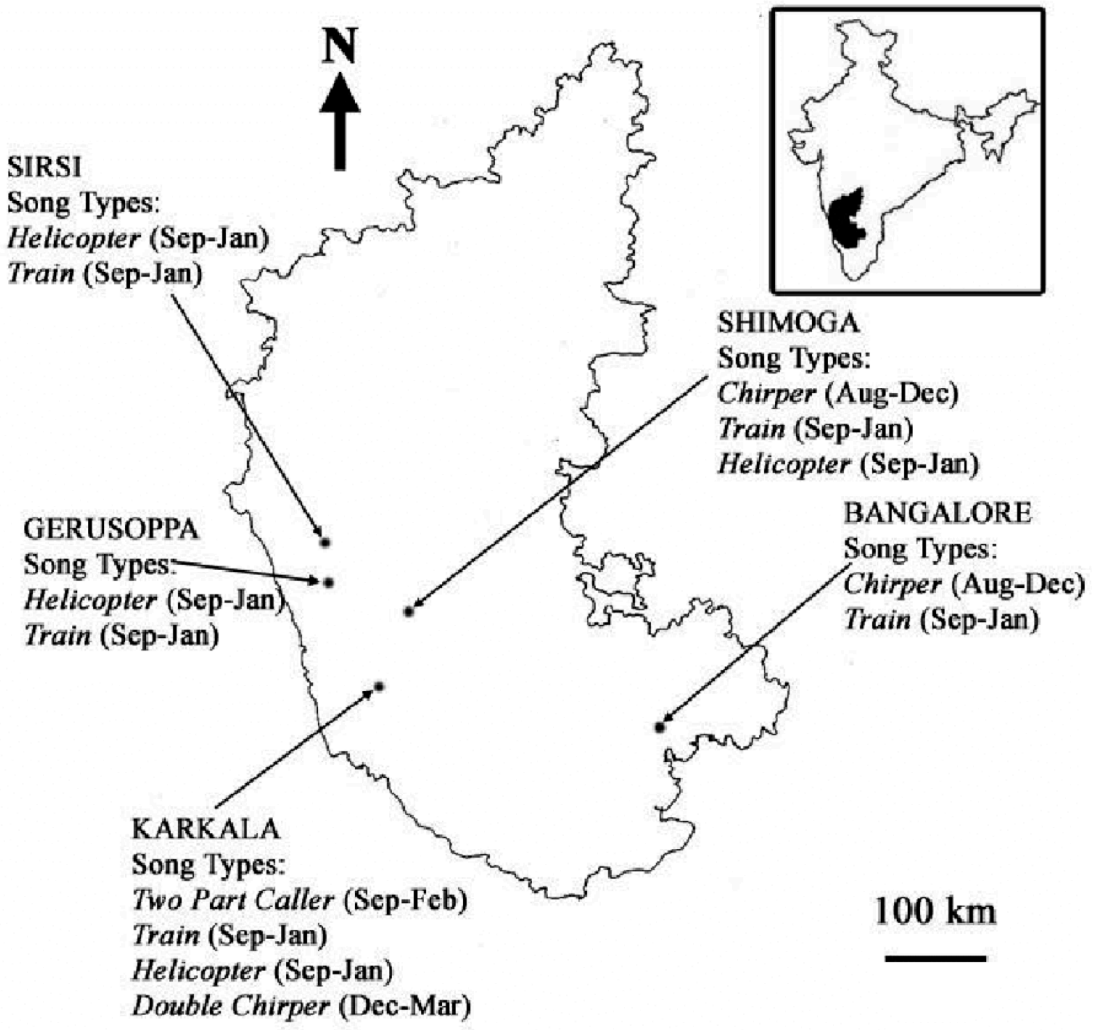


Figure 1.3: Evidence of co-occurance of *Mecopoda* songtypes at different sampling locations in Karnataka, south India (Nityananda and Balakrishnan, 2006)

to the ground) (Diwakar and Balakrishnan, 2007; Jain and Balakrishnan, 2010). Males of different songtypes are often to be found calling simultaneously and interspersed with each other (R. Balakrishnan and R. Dutta, personal observations). There is no record of hybrid formation in the wild and the lack of intermediate songtypes suggests that hybridsation is very rare (Nityananda and Balakrishnan, 2006) if not absent completely. All of these observations suggest that *Mecopoda* females pay attention to the parameters of the different songtypes and preferentially mate with their 'own' songtype. The males and females of the different songtypes cannot be discriminated visually and attempts to discriminate amongst them using traditional morphological analyses (Nityananda and Balakrishnan, 2006) have

also been unsuccessful, hence females remain unidentifiable in natural conditions since they do not call. south India had been a part of the African Gondwanaland that separated from the African plate in the early Cretaceous period and collided with the Asian plate around 50 to 55 mya resulting in the creation of the Himalayas. Apart from experiencing profound climatic change after creation of the Himalayas, peninsular India had been mostly isolated from rest of the the Eurasian land mass (Karanth, 2006, 2015). There have been no further break-ups or massive geological changes to the Indian peninsular land mass in recent times. Hence, we may conclude that *Mecopoda* have not gone through a vicariance process in recent times. Thus, *Mecopoda* satisfies all the criteria (Coyne and Orr, 2004) to be considered as a potential study system for sympatric speciation due to its unique songtypes, morphologically cryptic nature and co-existence of different songtype combinations in sympatry in a relatively small and geographically stable region.

1.9 Divergence in the bushcricket Genus *Mecopoda*

Many Tettigoniid species have shown great divergence in genital morphology as well as calling songs (Heller, 2005). There are multiple examples of song divergence in a species that is otherwise morphologically similar or vice versa. There are also many examples of species that have subpopulations that differ in both calls and genital morphology to varying degree (Heller, 2005). One of the interesting features of *Mecopoda* is that it consists of five distinct songtypes that differ in the temporal features of the male calls [see figure 1.4] while their spectral features appear similar (Nityananda and Balakrishnan, 2006). It has been observed in Tettigoniids in general that although sympatric species differ in frequency composition and amplitude modulation, closely related species could not be differentiated spectrally; rather studies have shown that temporal features can differentiate between such groups (Heller, 2005). Interestingly, *Mecopoda* call types from Indo-China region differ in both spectral component and temporal parameters (Korsunovskaya, 2009). Call of Chirper songtype consists of simple chirps with a mean chirp period of 0.48 seconds. Double Chirper has doublet chirps whose chirp period is of 0.38 seconds (Nityananda and Balakrishnan, 2006). These two songtypes have similar calls when compared to other songtypes and distribution of temporal parameters of Chirper and Double Chirper overlap. Chirper and Double Chirper calls do not have trills and have a simpler song structure than the other three songtypes (Nityananda and Balakrishnan, 2006). Interestingly, the

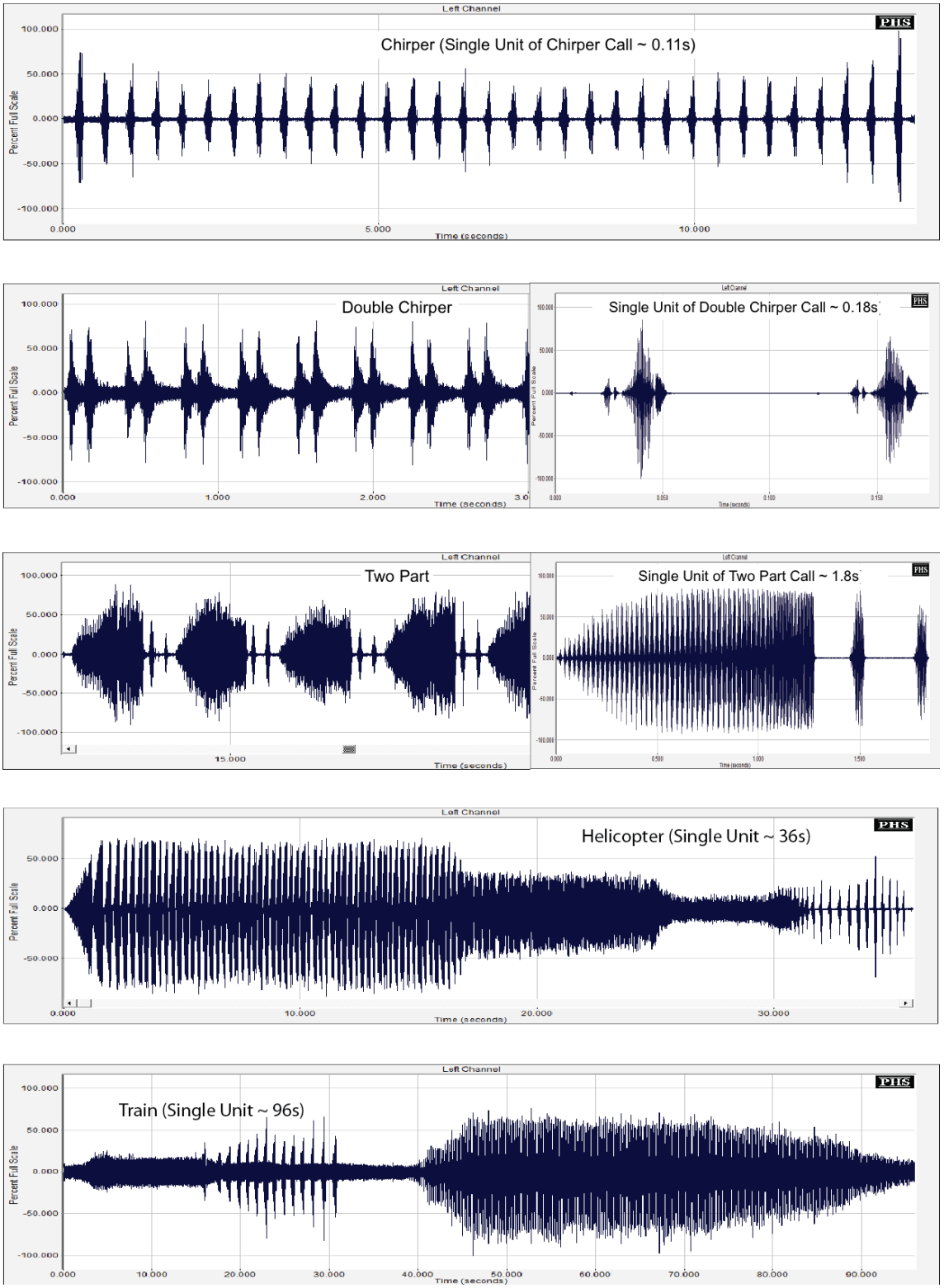


Figure 1.4: Representative oscillograms of five *Mecopoda elongata* songtypes found in south India (x-axis represents time in seconds and y-axis represents relative amplitude). The songtype to which each oscillogram belongs and the mean call duration of a single unit of each call are mentioned within each oscillogram

Chirper songtype of *Mecopoda* from south India is acoustically closest to a songtype found in Malaysia though these two songtypes differ in call duration and call period (Malaysian Chirper has a chirp duration ranging from 200 to 300 ms, a mean chirp period ranging from 1.6 s to 2.3 s while Indian Chirper has a mean chirp duration of 109 s and four times the pulse rate of the Malaysian chirper songtype) (Fertschai et al., 2007; Nityananda and Balakrishnan, 2006). All other songtypes (Two Part, Helicopter and Train) have trill components along with that of chirp with increasing call period and complexity. The Two Part call period is 1.9 s while Helicopter mean call duration is 36 s and Train mean call duration is 95 s (Nityananda and Balakrishnan, 2006).

Mecopoda appears to form a cryptic species complex since the songtypes remain morphologically identical and as a study system becomes even more interesting since strong divergence in calls is not accompanied by equivalent degree of divergence in morphology. The prevalence of this phenomenon of morphological differences not following divergence pattern in songs, thus forming a potential complex of cryptic species was predicted by Walker (1964) and observed in the sympatrically occurring Tettigoniid, *Amblycorypha rotundifolia* (Walker et al., 2003). Like *Mecopoda*, two species of *Hexacentrus* (*H. japonica* and *H. unicolor*) that are found in Asia were found to differ in calls but remained morphologically similar (except in the structure of file and small differences in genitalia). They had a geographical overlap over a small area but were separated by habitat in the overlapping areas in Japan (Inagaki et al., 1986, 1990). A similar phenomenon is also found in *Phyllomimus inversus* (of Malayan peninsula) where colouration on hind legs appeared to distinguish otherwise two identical *Phyllomimus* songtypes (Heller, 1995). Similar divergence in songs with respect to negligible divergence in genital morphology of certain populations is found in the European species of *Decticus* and *Poecilimon* (Heller, 2005). As early as 1958, it was found that species specific calls of otherwise morphologically similar grasshopper species of the Genus *Chorthippus* acted as the main isolating barrier while other premating and postmating barriers seemed to have smaller effects (Perdeck, 1958). Calling song acting as an agent of reproductive isolation has been speculated in Rotundifolia complex of *Amblycorypha* as well (Walker et al., 2003). Heller (2005) has observed that different song patterns generally indicates completion of the speciation process between the two different songtypes.

The unique species-specific calls of most ensiferans including the Tettigoniids are usually produced by males (Alexander, 1967; Heller, 1990). The Tettigoniids like

other orthopterans mostly use their calls for advertising and attracting the opposite sex for mating (Heller, 2005) and these calls can be considered as secondary sexual characters. These sexual signals can also be used by unintended listeners in nature (Zuk and Kolluru, 1998). Thus, the song structure may evolve under sexual selection and can also be driven by selection pressure imposed by exploiters. Most of the times the evolutionary forces act in synergy and determine a particular trajectory. Song evolution in Orthopterans in general and Tettigoniids in particular, is thought to follow a pattern where subtle change in temporal parameters of simple signals (series of syllables) and continuous repeats and combinations of these signals produce highly complex song structure (Heller, 1990). The call structure of the five songtypes of *Mecopoda* is an example of such diversification, which ranges from simple chirps to a highly complex mix of chirps and trills. It has been found that Malaysian *Mecopoda* females respond to the calls of *Mecopoda* males when in chorus (Fertschai et al., 2007). This indicates that *Mecopoda* calls are actual sexual signals. Habitat is an unlikely cause for *Mecopoda* songtype to diversify as they are found to occupy similar niches. They also overlap temporally, indicating minimal effect of abiotic ecology on diversification and evolution of *Mecopoda* songs. However, other ecological factors may have affected *Mecopoda* song divergence.

Divergence in many species of hosts or prey is thought to be caused by exploiters although examples to support this are limited (Buckling and Rainey, 2002). There is a continuous antagonism between host or prey species to avoid exploiters and exploiters to find novel ways to detect the hosts or preys. Through this antagonistic interaction, selection pressure by exploiters on exploited species to evolve mechanisms to remain inconspicuous, may lead to speciation of host or prey species. Indeed, there are examples of Orthopterans using non-calling (silent) strategies to communicate. Bats are known to be major predators of Tettigoniids (Morris et al., 1994) and has been confirmed again recently (Raghuram et al., 2015). Diet analysis of *Megaderma spasma* from locations around our field station (N13° 13', E75° 05') near Kudremukh National Park has found that orthopterans cover almost 60 % of its diet. These 60 % of Orthoptera consisted of 98 % Tettigoniid remnants out of which 10 % were that of *Mecopoda*. *M. spasma* has also been found to locate Tettigoniids by their calls in the same study. On the other hand, 'hearing' parasitoids may also track *Mecopoda* calls and infect them. Many Tachinid flies, which are parasitoids of many Orthopteran including Tettigoniids, are not extensively studied and little is known about their evolution, ecology and behavior (Stireman et al., 2006). Tachinid flies have been found to locate nocturnal

calling host species through specialised orthopteroid sound receiving structures (Lakes-Harlan and Heller, 1992; Robert et al., 1992).

The songtypes of the bushcricket genus *Mecopoda* have been suggested to represent a cryptic species complex and in spite of being mostly sympatric form distinct groups based on their calls but not morphology (Nityananda and Balakrishnan, 2006). This pattern of sympatric songtypes in *Mecopoda* suggests that songtypes are being maintained consistently over generations and that there is restricted or no gene flow between them. To study potential reproductive isolation of the different *Mecopoda* songtypes, it is likely to be informative to examine patterns of trait divergence and potential isolating mechanisms among them. Balakrishnan (2005) has argued that genital and behavioural characters may be sufficient indicator to reproductive isolation in orthopterans. This is supported in the review by Heller (2005) which deals with variation of genital morphology and calling songs that are very conspicuous among various divergent populations in different Tettigoniid species. While morphology of *Mecopoda* has been considerably dealt with, genital morphology needs more detailed study using more recent techniques such as geometric morphometrics. Other studies should include molecular genetic and chromosomal studies, cuticular hydrocarbon profilling, and most importantly behavioural studies of possible isolating mechanisms using phonotaxis and mating experiments. In a study examining karyotypes of two different species (possibly songtypes) of *Mecopoda* found in south India, differences in chromosome number were recorded (Aswathanarayana and Ashwath, 1994). Although there is no call record for these two apparent species of *Mecopoda*, there is an indication of genetic divergence and possible cryptic species. In another unpublished work, (Nityananda et al) conducted a phylogenetic study of the five songtypes of Indian *Mecopoda elongata*, one *Mecopoda elongata* individual from Sulawesi and two other *Mecopoda elongata* from Malaysia, based on the analysis of a part of cytochrome oxidase subunit I (COI) gene. *Mecopoda* from India, Malaysia and Sulawesi were found to form separate clades but songtypes within India appeared to have low genetic divergence as well as low haplotype diversity among them. There was considerable sharing of haplotypes among the songtypes with 4 songtypes sharing the most frequent haplotype while two other haplotypes were mostly restricted to two songtypes. These findings underline the fact that *Mecopoda* may still be essentially a possible cryptic species with recent divergence in only songs till further studies on more rapidly diverging traits are performed or more robust analytical methods are used. However, the genetic study mentioned above may have had a very different result had multiple markers been used since it is often

useful to consider as many characters (here, genetic markers) as possible as it is more likely to capture more variation. Since this study, there has been some advancement in availability of genetic markers, most notably, the complete mitochondrial genome (GenBank Accession No: JQ917910.1) in 2013 (Zhi Jun et al., 2013). Difference in *Mecopoda* songtypes based on cuticular hydrocarbon, which may play an important role in species identification prior to mating has not been investigated, nor is the songtype preference by females of each songtypes. These differences may act as a basis for behavioural isolation since females can choose to ignore irrelevant signals. Behavioural studies are thus necessary and direct tests to establish reproductive isolation.

1.10 Taxonomic status of *Mecopoda elongata*

Mecopoda elongata (Linnaeus, 1758) belongs to the Order Orthoptera under Class Insecta. Orthoptera, which falls in the midrange (more than 25000 described species) in terms of number of species compared to other insect Orders, is derived from Neoptera that appeared in the early Late Carboniferous (Bidau, 2014). Although orthopterans are a highly diversified group of organisms, it is now accepted that the two Suborders Ensifera and Caelifera are monophyletic groups with Ensifera evolving earlier than Caelifera (Bidau, 2014; Song et al., 2015). The ensiferans are represented by a tremendous variety of crickets such as true crickets, mole crickets, scaly crickets, ant crickets, grigs, camel crickets, cave crickets, cave wetas, dune crickets, wetas, king crickets, cooloola monsters, leaf-rolling crickets, raspy crickets, Jerusalem crickets and Tettigoniids (Bidau, 2014).

The taxonomic status of *Mecopoda elongata* has not changed drastically in almost a hundred years. Previously, *Mecopoda elongata* was classified under Subfamily Mecopodinae that was in turn divided into 4 sections, namely Phrictae, Segestini, Mecopodi and Phyllophori. Section Mecopodi consisted of 6 Species distributed among 4 Genus, out of which *Mecopoda elongata* was one (Hebard, 1922). The Subfamily Mecopodinae, under Superfamily Tettigonioidea and Family Phaneropteridae is now classified into six tribes including the Tribe Mecopodini (equivalent to Section Mecopodi in previous scheme) and 12 unassigned genera. While Tettigoniids are widely distributed all over the world, members of Tribe Mecopodini are found mostly in the old world regions in Asia, Africa and Australia (Eades et al., 2014; Gwynne and Morris, 2002). Out of 34 Species classified

into 8 genera belonging to Tribe Mecopodini, Genus Arachnacris, Genus Characta and Genus *Mecopoda* are only found in Asia. Genus *Mecopoda* consists of five reported Species with *Mecopoda elongata* having two subspecies (Eades et al., 2014). The subspecies *Mecopoda elongata elongata* is found over a large area covering India, China, Myanmar, Malaysia, Indonesia and surrounding areas. This subspecies is quite well studied with acoustic records, images and fourteen specimens listed (Eades et al., 2014). The other subspecies *Mecopoda elongata pallida* is represented by only one female specimen from India (Eades et al., 2014) with no other records. To summarise, the taxonomic status of *Mecopoda elongata* (Eades et al., 2014) is as follows:

Class Insecta
Subclass Pterygota
Infraclass Neoptera
Order Orthoptera
Suborder Ensifera
Superfamily Tettigonioidea
Family Phaneropteridae
Subfamily Mecopodinae
Tribe Mecopodini
Genus *Mecopoda*
Species *Mecopoda elongata*

1.11 Thesis outline

Genital morphology is often a good diagnostic character for species level identification in insects (Mutanen et al., 2006) and is particularly helpful in differentiating species when external morphology is similar (Song, 2009). External genital characters used in a traditional morphometric study by Nityananda and Balakrishnan (2006) were not studied in detail. With more robust techniques such as geometric morphometrics it is possible to study shapes of such characters in quantitative detail. The external genital characters such as sub-genital plate and cerci have a sensory role in orthopterans and take part in mating (Faucheux, 2012) and thus, may have a potential role in tactile reproductive isolation in *Mecopoda*. Thus,

genital morphology was studied in the first chapter of the thesis to test morphological divergence among *Mecopoda* songtypes; the relative success of different statistical methods applied was also assessed.

The second chapter describes the study of cuticular lipids in *Mecopoda*. Among all chemicals found on insects, CHCs are suitable for studying differences among species since they have been found to be species-specific (Blomquist and Bagnères, 2010). This is of significance as a successful mating takes place only if the mating pair is satisfied with mate recognition cues during antennation events (Nagamoto et al., 2005). *Mecopoda* CHCs have not yet been profiled although it has the potential to also show divergence among the songtypes which in turn may reflect reproductive isolation among songtypes based on cuticular chemicals as shown in other insects (Coyne et al., 1994; Grula et al., 1980; Mullen et al., 2007; Peterson et al., 2007). Additionally, CHCs have been shown to evolve very rapidly (Mullen et al., 2007) and have been suggested to show differences even when morphology or genetics fail to show differences among groups of animals (Kather and Martin, 2012).

The third chapter examines behavioural isolation in *Mecopoda* by asking whether Chirper females prefer their own call with respect to calls of other songtypes. A successful phonotaxis reveals that the female of a cricket has recognised the call of a same-songtype male and can localise it (Schul, 1998). The temporal features are used singly or in combination by many species for conspecific recognition and heterospecific discrimination (Deily and Schul, 2004). A mating experiment would also show the strength of this discrimination in *Mecopoda*. Irrespective of the processes by which *Mecopoda* songtypes may have come to exist, their ability to exist as distinct groups in sympatry indicate reproductive isolation through female preference for calls of their own songtype.

The fourth chapter introduces a Tachinid parasitoid fly found to infect songtypes of *Mecopoda*. Intraspecific auditory communication (advertising presence, location and potentially fitness information (Gerhardt and Huber, 2002)) is predominantly found in orthopteran and hemipteran species and dipteran parasitoids have evolved to take advantage of it (Lakes-Harlan and Lehmann, 2014). A very recent discovery of many Malayasian *Mecopoda* infected by larvae of parasitoid fly belonging to family Tachinidae is reported (Hartbauer et al., 2011). With ear tuned to frequency range of the hosts, Tachinid parasitoids open up a new angle to examine the divergence of *Mecopoda* songtypes.

Chapter 2

Acoustically divergent groups of *Mecopoda* separated by geometric morphometrics of external genital characters

2.1 Abstract

The unique biology of the five acoustically divergent populations of *Mecopoda* found in sympatry in south India gives us an opportunity to study reproductive isolation among these songtypes. Traditional morphometrics were used in a previous study but failed to yield evidence for a distinct morphological identity of any of the songtypes, leading to speculation that the songtypes may be a cryptic species complex. In the wild, it is impossible to differentiate females into songtypes in zones of sympatry as they do not call and males can be positively identified only by their calls. The advent of landmark based geometric morphometrics allowed me to compare the shape of cerci and the subgenital plate of all the *Mecopoda* songtypes. My approach was successful in distinguishing among songtypes of *Mecopoda* with 89 % correct assignment of songtypes based on genitalic characters analysed using random forest analysis. I speculate that since these two external genitalic characters are involved in mating and have sensory roles, the genitalic characters themselves might be involved in assortative mating. The differences I have observed may be enough to cause each songtype to reject a

different songtype during copulation. The discrimination will most likely be of tactile in nature. Further work will be needed to establish whether the morphological distinctiveness I have identified drives reproductive isolation or has evolved subsequently as a result of restricted gene flow and rapid evolution of secondary sexual morphological traits within songtypes.

2.2 Introduction

Although there has been a long standing appreciation of the role of isolating barriers in speciation and the persistence of divergent species, the study of the evolution of these isolating barriers has only recently become a major research area (Coyne and Orr, 2004). Mechanical isolation is generally regarded as one of the less important of these isolating barriers (Coyne and Orr, 2004; Masly, 2012; Shapiro and Porter, 1989) since it is either rare or less detectable (Coyne and Orr, 2004).

Mechanical isolations are barriers that prevent reproduction due to incompatibility of the reproductive structures of males and females from divergent groups (Coyne and Orr, 2004). They can be identified as responsible for reproductive isolation only when isolation resulting from other differences is incomplete (Coyne and Orr, 2004). Studies in a range of species have identified both structural and tactile forms of mechanical isolation leading to speciation (Coyne and Orr, 2004; Masly, 2012). Structural isolation, which is more common in plants (Linder and Midgley, 1996; Niklas, 1982; Niklas and Buchmann, 1987) has also been found to reduce gene flow by 65% in Japanese Carabid beetles (Sota and Kubota, 1998). Apart from Japanese Carabid beetles (Coleoptera), structural isolation has also been found in Lepidoptera, Araneae, Polydesmida, Zygoptera and Diptera (Masly, 2012).

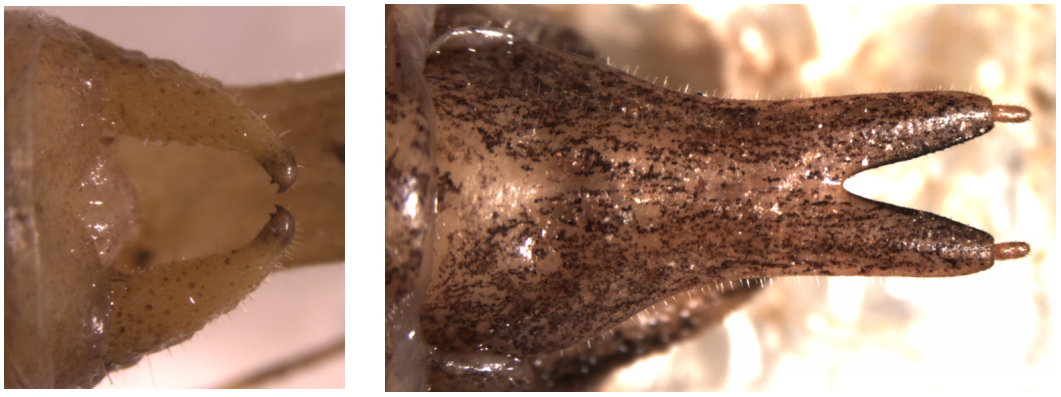
Tactile isolation, on the other hand, may not involve any physical incompatibilities of reproductive structures between males and females but rather, involves the ability to detect conspecific or non-conspecific mating structures and behaviour (Coyne and Orr, 2004; Masly, 2012). This kind of isolation may be expected in situations where one of the mating partners is able to sense aberrant copulatory organs or behaviour of a heterospecific individual and subsequently avoid mating. There are very few studies attributing speciation solely to tactile isolation (Masly, 2012). In these few cases (Coyne, 1993; Eberhard, 1992; Lorković, 1958), tactile

isolation seems to act where males have different copulatory structures while females do not but females seem to be able to control the mating events and prevent a successful heterospecific matings.

There might be a role of mechanical isolation in the case of five songtypes of *Mecopoda elongata* that have been identified based on the temporal features of their calls in Karnataka, India. Nityananda and Balakrishnan (2006) studied the morphology of five *Mecopoda* songtypes based on 75 quantitative characters and 61 qualitative characters. In this traditional morphometric analysis, Nityananda and Balakrishnan (2006) found that Chirper was the only songtype to cluster separately from the other songtypes in spite of the large number of qualitative or quantitative characters used in the analysis. The difference between Chirper and other songtypes turned out to be the combined result of many characters as even after the removal of certain characters (such as body size, file length, peg number) thought to play important roles in song production, the Chirper individuals did not cluster with other songtypes. They were also unable to assign any diagnostic morphological features that could help differentiate among the songtypes.

The external genital characters of the *Mecopoda* songtypes, including cerci and subgenital plate were not, however, examined in detail by Nityananda and Balakrishnan (2006). Genitalic morphology often acts as good diagnostic characters that help in differentiating species when external morphology seems to be similar (Song, 2009). It is possible that significant differences between external genital structures do exist between males of the different songtypes. The length and width of the secondary sexual characters such as cerci [see figure 2.1a] and subgenital plate [see figure 2.1b] were considered for quantitative analysis by Nityananda and Balakrishnan (2006), but they may have failed to show up as diagnostic characters in the multivariate analysis as a result of the large number of characters examined which necessitated a conservative approach to reduce the potential for type 1 error. Although the study by Nityananda and Balakrishnan (2006) included the shape of the subgenital plate and cerci of the *Mecopoda* in the qualitative analysis, the study did not compare the actual shapes of these two structures due to limitations in the ability of traditional morphometric techniques to quantify and retain original shapes (Rohlf and Marcus, 1993).

Genital morphology particularly male genitalia has been commonly used in taxonomic studies to identify species and is considered as one of the most important characters for species level identification in insects (Mikkola, 2008; Mutanen et al.,



(a) Cerci (b) Subgenital plate

Figure 2.1: The two external genital structures of *Mecopoda*, cerci and subgenital plate, that have been used in morphometric analysis to differentiate the songtypes

2006; Schilthuizen, 2003; Shapiro and Porter, 1989). Until recently, use of genital characters in morphometric analysis were predominantly limited to external structures, which usually play a role in the early stages of mating (Mikkola, 2008). Rentz et al. (2006) used external genital structures such as cerci and the subgenital plate in delimitation of the Austromecopoda species complex. Cerci [see figure 2.1a], hook-like structures at the end of the 10th tergite, are mostly a sensory organ acting as proprioceptors, sensitive to air movement (Chapman et al., 2012; Faucheux, 2012; Snell and Killian, 2000); apart from being vibroreceptors, they also host a small number of chemoreceptors and olfactory receptors (Chapman et al., 2012; Faucheux, 2012). Cerci have also been found to work as claspers (Faucheux, 2012) during mating in crickets and successful mating depends on its movement during mating (Sakai and Ootsubo, 1988; Snell and Killian, 2000). The subgenital plate [see figure 2.1b], which is an elongated structure attached to the 9th sternite and bifurcated at the posterior end (Faucheux, 2012) acts as a protective organ for the male genitalia (Chapman et al., 2012) as well as a sensory organ in bushcrickets (Faucheux, 2012). It also helps in the articulation of male genitalia onto female genitalia in crickets and bushcrickets (Faucheux, 2012). Both cerci and subgenital plate actively take part in the mating process and have tactile functions during mating (Faucheux, 2012) and thus, appear to be secondary sexual characters in bushcrickets. This indicates a good possibility for both these characters having a role as participatory organs in tactile isolation in *Mecopoda*. Mechanical isolation, thus, may play an important role in the reproductive isolation of *Mecopoda* songtypes.

The evolution of mechanical isolation revolves around divergence and evolution

of genital characters within a population. If sexual selection can bring about divergence in other traits such as mating signals in sympatric populations, there is no reason why sexual selection cannot bring about divergence in reproductive structures (Coyne and Orr, 2004). However, studies on Carabid beetles and spiders failed to find any evidence of enhanced divergence of reproductive structures where mechanical isolation may be present in sympatric species (Coyne and Orr, 2004; Eberhard, 2009). This trend is also seen in the *Mecopoda* songtype complex, many of whose members are sympatric and overlap temporally in their breeding season. In general, sexual selection, more specifically female choice, is considered a major factor in the evolution of male genitalia (Eberhard, 1985, 2009; Schilthuizen, 2003). In *Mecopoda*, where reproductive isolation among five songtypes could be due to female mate choice, the presence of mechanical isolation may strengthen the claim of sexual selection in driving their speciation.

We use landmark-based geometric morphometric tools to study the difference in shapes of the subgenital plate and the cerci of the five different songtypes of *Mecopoda* to examine the possibility of mechanical isolation between them. Landmark-based geometric morphometry is hardly three decades old but it has already established itself as the most accurate method available to analyse shape of an object. The shape is included in the analysis as Cartesian coordinates, which are compared among the individuals being studied (Rohlf, 1993). The effects of size, position, and orientation are resolved using shape as the main source of variation between specimens (Adams et al., 2004; Rohlf, 1993).

The objectives of the study are as follows:

1. To apply landmark-based geometric morphometrics to the subgenital plate and cerci to study differences among the five visually cryptic songtypes of *Mecopoda*.

2. To compare the results of traditional morphometrics from a previous study and landmark-based geometric morphometrics in differentiating the *Mecopoda* songtypes.

3. To assess the accuracy and efficiency of the new method in its ability to assign *Mecopoda* individuals to their respective songtypes.

2.3 Materials and methods

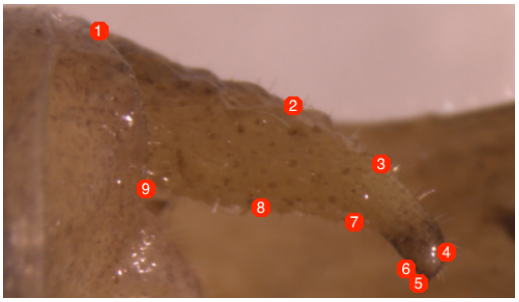
2.3.1 Specimen collection

Mecopoda elongata L. (Orthoptera, Tettigoniidae, Mecopodinae) males were collected near the field station (N13 13', E75 05') at Kadari, Dakshin Kannada District, at Ullodu (N13 38', E77 42') and at the Indian Institute of Science Campus (N13 01', E77 34'), Bangalore, India. Males were tracked by listening to their calling songs or by following their movement when ground vegetation was disturbed. The songs of all these males were recorded to ascertain the songtype of the individuals before the study was conducted. All the specimens were chosen such that the genital structures to be studied were intact and undamaged. The pictures (in TIFF format) of the subgenital plate (SGP) and the cerci of *Mecopoda* specimens were taken using a digital camera (Leica DFC290) at 96 pixels/inch resolution connected to a microscope (Leica M165C). The subgenital plates were photographed by placing each individual *Mecopoda* ventral side up. Nineteen anatomical landmarks were used in each picture to define the coordinates for the study of the shape. The cerci were photographed similarly with the dorsal side of the *Mecopoda* facing the camera while 9 landmarks were used in this case to obtain the landmark coordinates. The total number of specimens analysed for subgenital plate and cercal morphology was 130 (30 each of *Mecopoda* Chirper, Two Part and Double Chirper; 21 Helicopter and 19 Train).

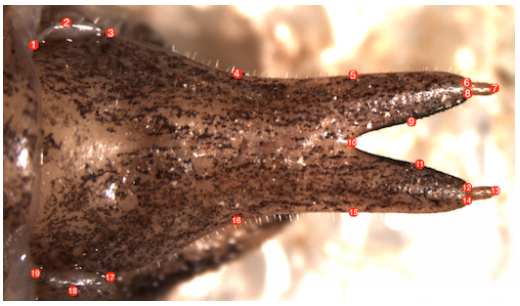
2.3.2 Geometric morphometric analysis

The general steps for the analysis involve identifying specific landmarks in all samples. The landmarks should be homologous, lie in the same plane, adequately cover the structure and be found reliably and repeatedly at similar positions with respect to adjacent landmarks (Zelditch et al., 2012). The landmarks in this study were chosen according to these criteria and their classification tabulated according to (Bookstein, 1997) [see figure 2.2 and table 2.1].

A suitable function (Thin Plate Spline function) was fitted to the space between two landmarks to define the space and hence the relationship between the landmarks (Rohlf, 1993). The Thin Plate Spline function has been found to be effective in displaying overall differences in shape and is implemented in the TPS software



(a) Landmarks on right cercus



(b) Landmarks on subgenital plate

Figure 2.2: Location of landmarks denoting the boundary of the right cercus and the subgenital plate used in the landmark-based geometric morphometrics. 9 landmarks were plotted on the right cercus while 19 landmarks were plotted on the subgenital plate

LM No.	Type (for SGP)	Description	Type (for Cerci)	Description
1	Type 1	Intersection of SGP and 10th sternum	Type 1	Intersection of cerci and 10th tergum
2	Type 2	Maxima of convex curvature on SGP	Type 2	Maxima of convex curvature on outer cercus boundary
3	Type 2	End of the convex curvature on SGP	Type 2	Beginning of outer apical narrowing of cercus
4	Type 2	Minima of the concave curvature on SGP	Type 2	Posterior most point on cercus
5	Type 3	Point corresponding to LM 10	Type 1	1st subapical spine
6	Type 1	Base of apical projection	Type 1	2nd subapical spine
7	Type 2	Maxima of the curvature of apical projection	Type 2	Beginning of inner apical narrowing of cercus
8	Type 1	Base of apical projection	Type 2	Minima of concave curvature on inner cercus boundary
9	Type 2	Point midway between LM 8 and LM 10	Type 1	Intersection of cerci and 10th tergum
10	Type 1	Mid-junction of posterior bifurcation of SGP	-	-
11	Type 2	Point midway between LM 10 and LM 12	-	-
12	Type 1	Base of apical projection	-	-
13	Type 2	Maxima of the curvature of apical projection	-	-
14	Type 1	Base of apical projection	-	-
15	Type 3	Point corresponding to LM 10	-	-
16	Type 2	Minima of the concave curvature on SGP	-	-
17	Type 2	End of the convex curvature on SGP	-	-
18	Type 2	Maxima of convex curvature on SGP	-	-
19	Type 1	Intersection of SGP and 10th sternum	-	-

Table 2.1: Description of the landmarks used in the landmark-based geometric morphometric analysis of right cercus and subgenital plate based on the criteria formulated in (Bookstein, 1997) and summarised in (Zelditch et al., 2012)

package (Rohlf, 1999) which was used in the current analysis. The parameters of this function vary between the specimens when compared to a reference shape which is usually the mean configuration of landmarks (Rohlf, 1993). These variables may be then analysed using Relative Warp Analysis (using the 'TPS' series programme), which is a Principal Component Analysis in which the relative warps are the principal components of a set of thin-plate spline transformations (Rohlf, 1994; Zelditch et al., 2012). Relative Warp Analysis tests intra-population variation with respect to inter-population variation.

The utility software, tpsUtil (Rohlf, 2013c) was used to list the previously taken photographs in a .tps file. The landmarks were digitised on the photographs with tpsdig2 software (Rohlf, 2013a) and coordinate values were stored in the same .tps file. We used tpsrelw software for further relative warp analysis. This program produces a consensus shape also called the reference shape through the generalised procrustes method (GPA), which produces the most unbiased mean configuration, closest to the true shape (Rohlf, 2003).

Following the definition of shape we have to filter out location, scale and rotational effects from each configuration of landmarks. The translation is achieved when each configuration of landmarks is aligned with its centroid at the origin. The shape of each specimen is then transformed to a unit centroid size, thus teasing out the effect of size on the shape. The effect of rotation is also removed from the data when one configuration of landmarks is rotated with respect to another such that the summed squared distance between the corresponding landmarks is at its minimum. When there are more than two configurations, all are rotated to optimal alignment with the first and a mean shape is calculated. The reference shape is formed after iterating the optimal alignment of the entire configuration on this mean shape and repeating the process such that the difference is minimised. Typically, two iterations are sufficient to get the reference shape. The specimens are then aligned to this reference shape for the computation of their tangent space projections (Rohlf, 2000). The disadvantage of the thin plate spline function is that the statistical analysis based on the tangent space approximation cannot be used if there is a large variation in shape among the samples (Rohlf, 1999). With the tpsSmall software (Rohlf, 2013b), I confirmed that the variation within the population was small enough to satisfactorily use the tangent space approximation during analysis after thin plate spline function interpolation (Querino et al., 2002).

The TPS interpolation allows us to derive a bending energy matrix such that the energy required to deform the consensus shape of the structure to match the shape

of each specimen is at a minimum. This bending energy matrix is then decomposed to get the Principal Warps. The vectors of Principal Warps can be explained in terms of Partial Warp vectors (non-affine) and Uniform (affine) Components. The relative warps are then calculated from these non-affine components (Rohlf, 1993). A parameter, Alpha introduced by Bookstein (1997) in the Relative Warp analysis biases the analysis in favour of large-scale variation if it is not set at zero (Rohlf, 1993). Thus, for exploratory studies where we do not have an a priori expectation that variation at particular scale will be of greater importance, Rohlf (1993, 1994) suggests that the value of alpha should be set at zero. Relative warp analysis was performed based on landmarks on subgenital plate and cerci for this study [see figure 2.2]. The photographs of the subgenital plate and cerci of each sample were taken in no predetermined order and they were digitised randomly. For 25 randomly selected individuals spread equally among five songtypes, 2 photographs were taken at different times and analysed twice separately to quantify within individual variability and to check the repeatability of the measurements. We performed linear regression analysis between corresponding relative warps derived separately on two different photographs as well as between corresponding relative warps from two different analysis of the same photographs following method used in House et al. (2013). Based on Relative Warp data, the number of relative warp components and the landmarks that explain most of the variation were identified. We can also visualize graphical representations of relative warps (that are equivalent to principal components) against each other to find out how the individuals group with respect to each other. Further statistical analyses were performed with the individual relative warp scores of each individual to see how *Mecopoda* individuals form groups (similar to analysis as done by Richmond et al. (2012)).

2.3.3 Random forest analysis

Statistical data of biological origin often tends to be highly variable and complex. The variables under study may differ in nature or in scale, may be related to each other and may have unequal sizes. We are often required to study the interactions among such complex groups of numerous variables. Random forest analysis is a technique designed to allow complex data to be used to place individuals into groups. Random forest analysis (RFA) arranges (classifies) data based on multiple schemes of data categorisation (also called classification trees) which it builds

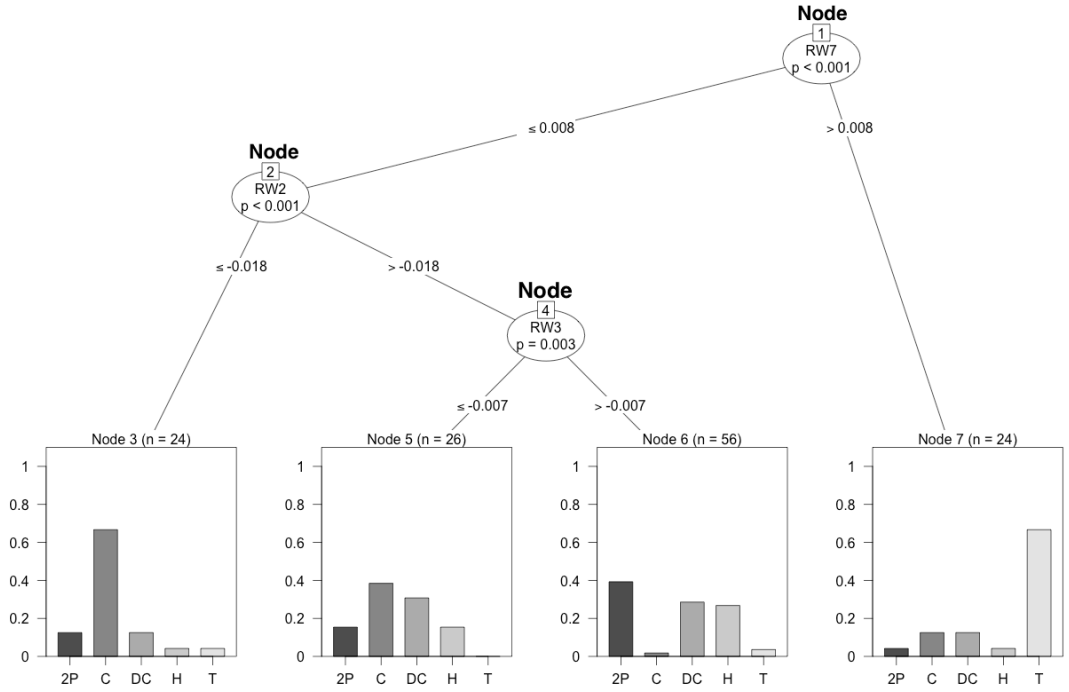


Figure 2.3: A representative classification tree formed using 'party' package in R through random forest analysis

using classification and regression tree algorithms such as CART or C4.5. A representation of a classification tree formed using random forest analysis is shown in figure 2.3.

The formation of each classification tree is initiated by creating a new set of data, also called bootstrap set. The bootstrap set is formed by including a random subset consisting of about 63% of the original data points under study. The remaining 37% is filled by randomly picking data from within the already chosen 63% representation of original data. In figure 2.3, the bootstrap set consists of 130 data points (sum of all 'n' shown in brackets) that is also equal to the number of original data points. This bootstrap set is then arranged into groups based on a subset of variables (also called predictor variables). Predictor variables are also chosen randomly out of all variables. For example, in figure 2.3, the number of predictor variables is 3, namely RW7, RW2 and RW3. The bootstrap set of 130 individuals were first divided into 2 groups containing 24 individuals and 106 individuals respectively based on a value (here, 0.008) of the predictor variable RW7 that serves as node 1. The 106 individuals were further divided into 24 individuals and 82 individuals based on whether RW2 (node 2 in figure 2.3) value was more or less than equal to 0.018. Further bifurcation of 82 data points was based on

RW3. This process of grouping similar individuals and sorting out dissimilar individuals is called impurity reduction. The reliability of the impurity reduction process is measured by the p-value (shown within the circle of a node in figure 2.3). As shown in figure 2.3, impurity reduction is repeated at each node to create a single classification tree. The number of classification trees that are formed as a part of random forest analysis is decided a priori and the resultant set of classification schemes (also called base learners) serves as the basis for classification of the entire data and for predicting group affinity of individuals within a data set (Strobl et al., 2009b; Touw et al., 2013). The classification schemes produced by the method mentioned above (eg. analysis by "randomForest" algorithm in R) may be biased in case the variables are correlated or differ in scale as is often the case with biological data (Strobl et al., 2008, 2009a; Touw et al., 2013). The "cforest" algorithm from the "party" package (R Core Team, 2014) is an implementation of random forest analysis in R that uses a conditional inference forest algorithm to produce conditional inference trees (a type of classification tree) as base learners that overcome the bias among correlated variables in the analysis (Hothorn et al., 2006; Strobl et al., 2008, 2007). Hence the "cforest" algorithm was used in this study. The number of trees in the forest (ntree) and number of predictor variables used at each node (mtry) (also called hyper parameters) were fixed before the analysis was performed. The stability of the random forest analysis can be checked by increasing the number of iterations for classification trees (ntree) from a smaller number to a larger number. Increasing the ntree parameter should also be done when increasing the number of predictor variables or increasing the sample size, as this will make the analysis more stable and robust. The number of predictor variables used for assessing subsequent branching off at each node should be equal to the square root of the total number of variables as suggested by Strobl et al. (2009b).

Random forest analysis produces an error estimate for the classification of data under study. This is done by comparing the value of the known independent variables with the predicted value of the same independent variables from the random forest analysis. I also performed a proximity analysis to find out how the individual data points group together. Proximity is the measure of similarity between any two individual observations from a data set and the proportion of times these two individuals occurred at the same node of the classification tree during random forest analysis. A multi-dimensional scaling (MDS) of the calculated proximity matrix produces a scatterplot, which can help visualize how the individuals group together. This is equivalent to plotting multivariate data to detect clustering and is

similar to principal component analysis. Therefore, I also visually compared the relative ability of the random forest analysis and principal components analysis (done prior to RFA in my study) to group similar individuals from the same data set. For this, the scores of two most important principal component and the scores of two MDS components were plotted against each other.

It is also helpful to measure the significance of each variable under study to explain its role for some particular arrangement of data. Random forest analysis can be used to calculate the relative contribution of the different predictor variables to classification of data (also called the variable importance measure). In cforest analysis of data, the variable importance measure is derived from permutation based 'mean decrease in accuracy' (Hothorn et al., 2015). This process involves calculating the mean difference in prediction accuracy of the classification trees before and after random permutation of variable values (Touw et al., 2013). The absolute estimates of variable importance should be used only to understand and interpret the relative importance of different variables under study. They cannot be used for comparing variable estimates from two different studies (Strobl et al., 2009b). In interpreting the results of variable importance analysis, the variables that are more than the magnitude of the lowest negative variable score (also called the significance threshold) are considered significant (Strobl et al., 2009b). Distance from the significant threshold forms the basis of the relative importance measure, with the variable that is farthest from the significant threshold being considered the highest contributing variable. This means the variables that are contributing to decision-making during classification tree formation are significantly different from zero as irrelevant variables tend to occupy random values around zero (Strobl et al., 2009b).

Random forest analysis is being increasingly used in exploratory studies with complex data (Ranganathan and Borges, 2011; Strobl et al., 2007) where the number of variables are more than the sample size (Strobl et al., 2007). These complex data tend to vary in scale, contain missing data and zeroes, data points in them can be highly correlated and the structure for these data is usually unknown (Cutler et al., 2007; Ranganathan and Borges, 2011). For classification of these kinds of data and predicting important variables accurately without having prior assumptions of relation between the two individual data points, we can use random forest analysis (Cutler et al., 2007). Random forest analysis is suitable for the present study mainly because:

1. The number of variables in the form of relative warps is more than the sample size for each group (i.e. songtypes). For robust multivariate analysis, we need to have a sample size that is 3 to 4 times the number of variables as a rule of thumb. This is not possible in the present study.

2. Morphometric data points are not independent of one another especially when the landmarks themselves are not independent of each other.

3. This test provides an index for error in classification as well as predicting the classification with similar novel data based on the computed classification trees.

All the 48 relative warps from the previous relative warp analysis were used in the random forest analysis. This was possible because relative warp scores (a continuous variable) can be used as a substitute for the original discontinuous Cartesian coordinates (Rohlf, 1993; Zelditch et al., 2012).

2.4 Results

2.4.1 Geometric morphometric analysis

The regression of tangent space onto procrustes distance (done in tpsSmall software (Rohlf, 2013b)) produced a slope of 1 for both cerci and subgenital plate, which indicates that the variation in the shape of all specimens is small enough and that the approximation does not exclude too much information. This allowed the use of the 'TPS' package for further analysis.

In regression analyses to quantify within individual variation due to measurement error, the 3 components (explaining 70 % variation) from relative warp analysis of subgenital plate and 2 components (explaining 88 % variation) of relative warp analysis of right cercus were used. For right cercus, regression analyses of relative warp 1 from different repeat analyses produced r-square values from 41% to 92% and regression analyses of relative warp 2 showed r-square value from 26% to 91%. Similarly for the subgenital plate, regression analyses of relative warp 1 from different repeat analyses produced r-square values from 45% to 98%, regression analyses of relative warp 2 showed r-square values from 35% to 97% and regression analyses of relative warp 3 produced r-square values from 33% to

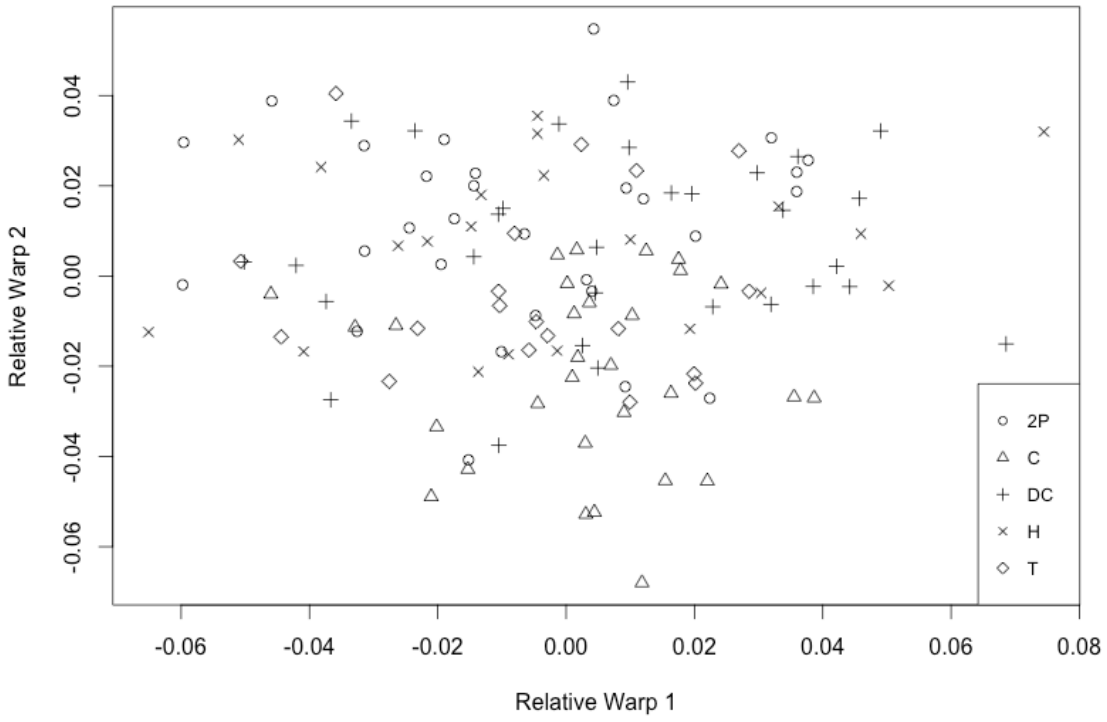


Figure 2.4: Landmark-based relative warp analysis of the subgenital plate of *Mecopoda* songtypes. The scores of relative warp 1 are plotted against the scores of relative warp 2 to examine grouping of 130 *Mecopoda* individuals under study

88%. This shows that the method followed in digitising the samples were mostly repeatable and there was moderate to low within individual variation.

In relative warp analysis, 34 relative warp components were obtained for the SGP and 14 relative warp components were obtained for the cerci for each individual. The first 3 components explained 70 % of the variation for the SGP while the first 2 components explained 88 % of the variation for the right cercus. The test also confirmed that the landmarks 6, 8, 12 and 14 [see figure 2.2b] out of 19 landmarks on the subgenital plate actually contribute more to variation in shape whereas landmarks 5 and 6 [see figure 2.2a] out of 9 landmarks contribute most to the shape variation in cerci. The relative warp analysis failed to differentiate between the songtypes adequately when subgenital plate and cerci were analysed and visualised in graphs separately although they indicated some grouping in the case of Chirper songtype with respect to other songtypes [see figure 2.4, figure 2.5].

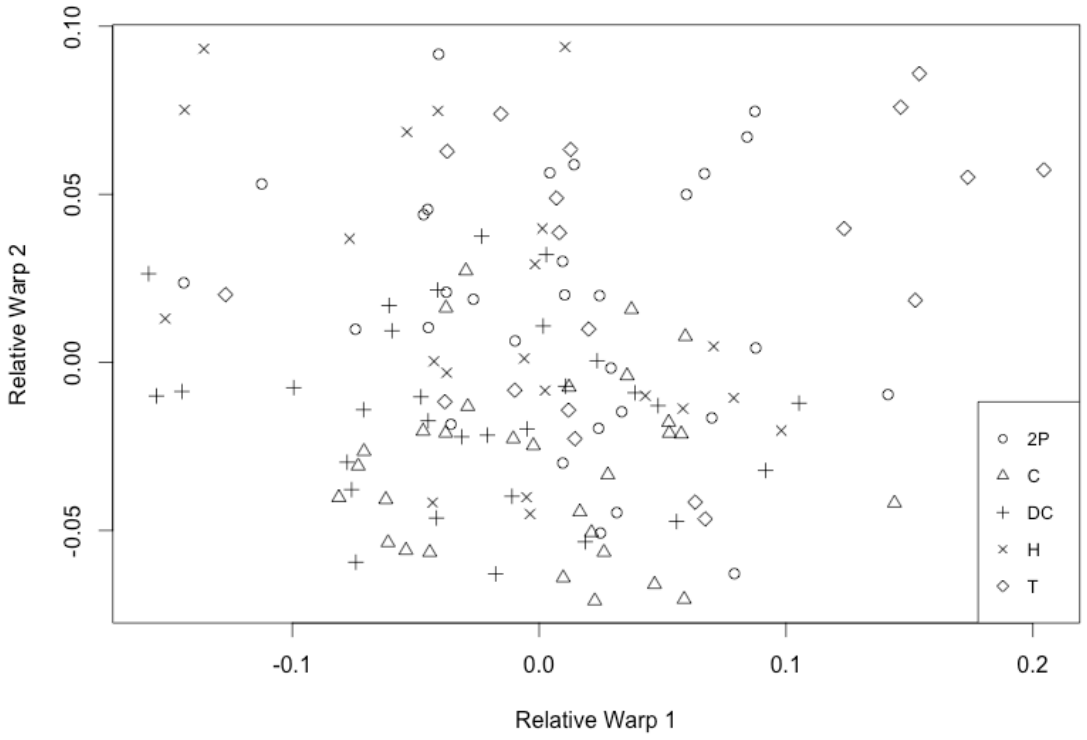


Figure 2.5: Landmark-based relative warp analysis of the right cercus of *Mecopoda* songtypes. The scores of relative warp 1 are plotted against the scores of relative warp 2 to examine grouping of 130 *Mecopoda* individuals under study

MANOVA with Pillai's Test on the first three relative warp components for the subgenital plate (covering 70 % of variation in its shape) and first two relative warp components of the right cerci (covering 88 % of variation in its shape) when considered together showed a significant (Pillai statistic = 0.856, F (20, 496) = 6.75, $p < 0.0001$) difference between the five songtypes and also indicated that between-group variability is more than the within-group variability (approximate F = 6.75). However, four out of five relative warp components showed significant p-values ($p < 0.005$ at 4 degree of freedom) when the components were checked individually. Interestingly, the first relative warp component, which explained about 33 % of variation in relative warp analysis of the 3 components of subgenital plate turned out to be non-significant. Its F value of 1.39 indicates that between-group variability for this relative warp is not much different from its within-group variability.

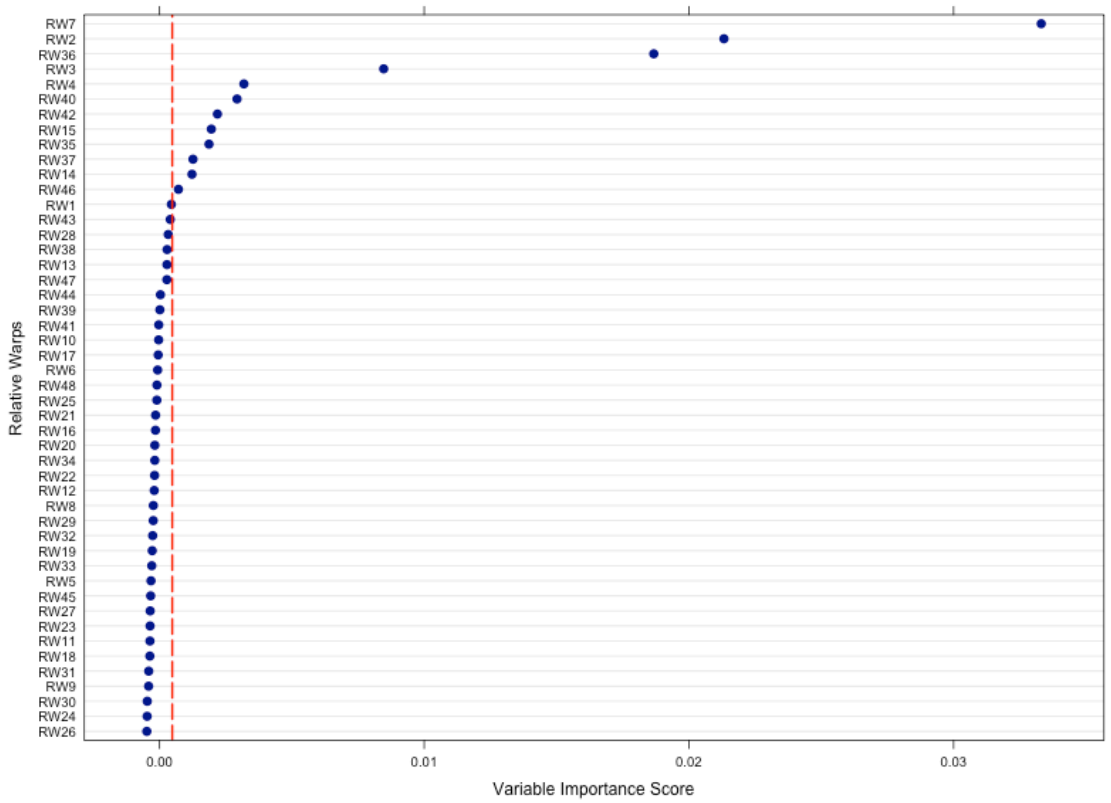


Figure 2.6: RFA based variable importance analysis of the 48 relative warps scores derived from the relative warp analysis of the right cercus and the subgenital plate of *Mecopoda* individuals. 1-34 relative warps belong to subgenital plate and 35-48 relative warps belong to right cerci. Relative warps (predictor variables) to the right of dashed red vertical line have significant role in explaining the variation in the sample under study

2.4.2 Random forest analysis

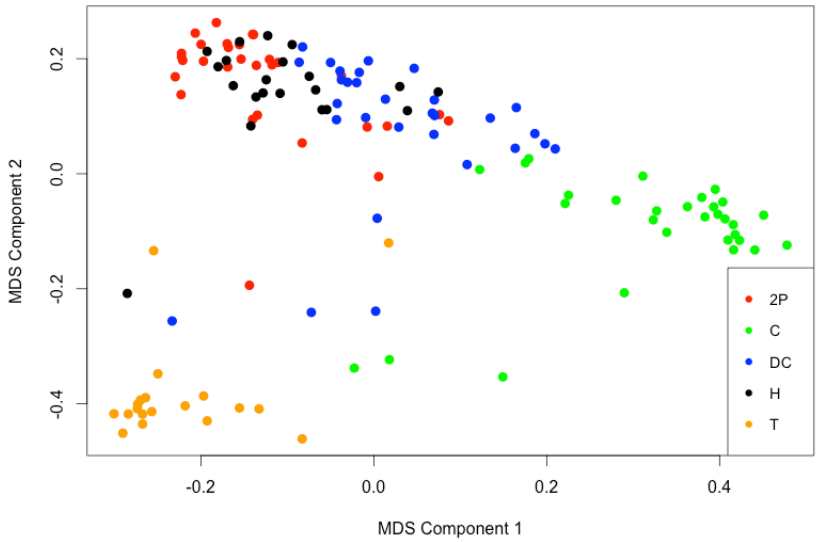
The hyper parameters for the random forest analysis in this study were fixed at ntree = 5000 and mtry = 7 (approximate square root of 48, the number of relative warps), in the cforest algorithm. The variable importance measure derived [see figure 2.6] using the cforest analysis showed that the 7th relative warp is the most significant of all the relative warps by being farthest from the threshold. The next three relative warps, 2nd, 36th, 3rd in decreasing order of importance, were also quite far away from the threshold of significance and thus more important than other significant relative warps [see figure 2.6]. Other variables that were marginally significant (since they were quite close to the significance threshold) in descending order were 4th, 40th, 42nd, 15th, 35th, 37th, 14th and 46th relative

warps [see figure 2.6]. Thus only 12 out of 48 relative warps contributed significantly more to the variation in shape of the subgenital plate and the cerci than the other relative warps [see figure 2.6].

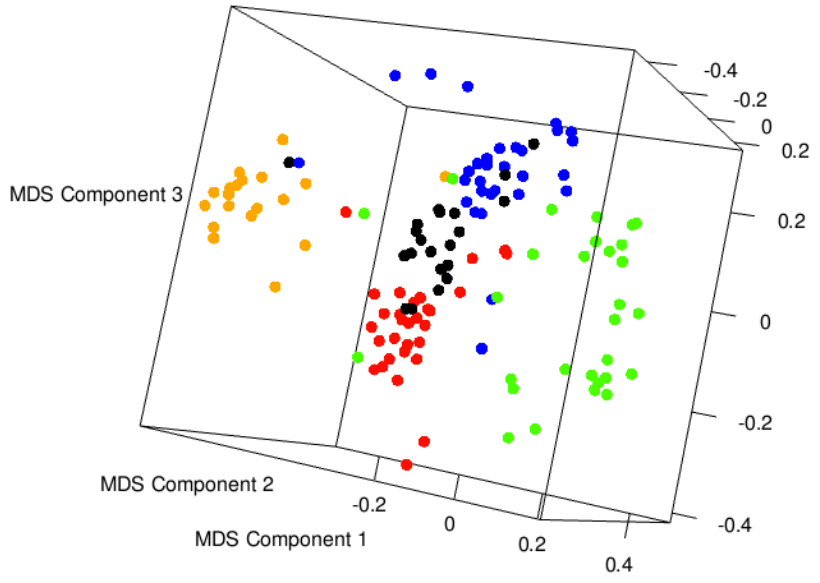
The prediction of the songtypes to which each individual *Mecopoda* (whose songtypes were already known) belonged was derived based on the classification scheme from the “cforest” analysis of the relative warps. When this analysis predicted songtypes of the same *Mecopoda* individuals used for the ‘cforest’ analysis, it was found that the prediction from the “cforest” analysis differed in only 15 individuals out of the total 130 individuals. The error estimate in predicting the songtype of the each *Mecopoda* individual from this random forest analysis was 11.5 % (15 out of 130), which was much less than expected, particularly since other multivariate analyses failed to differentiate the *Mecopoda* songtypes. This means almost 9 out of 10 times the algorithm was able to correctly predict the songtype an individual *Mecopoda* belongs to. It is also interesting to note that greatest number of errors (10 out of the 15 wrong predictions) occurred while predicting if an individual belonged to the *Mecopoda* Helicopter songtype.

The proximity matrix was derived from the random forest analysis of the relative warps. Multi-dimensional scaling of the proximity data produced component scores that could be plotted together to visualise group formation of the *Mecopoda* individuals. The first two components of multi-dimensional scaling of the proximity matrix were able to substantially segregate the songtypes [see figure 2.7a]. This is expected given the ability of the random forest analysis to predict songtypes of *Mecopoda* individual with high accuracy. A 3D scatterplot from the first three components of multi-dimensional scaling of the proximity matrix was able to differentiate clearly among all the *Mecopoda* songtypes and form distinct clusters of each of the songtypes [see figure 2.7b].

MANOVA analysis showed that the first relative warp, which explained about 33 % of the variation in relative warp analysis of the subgenital plate was not capable of differentiating the songtypes. The variable importance measure derived from RFA corroborated this fact. It indicated that relative warp 7 from the relative warp analysis of subgenital plate and relative warp 36 from relative warp analysis of the right cerci were the best predictor variables for songtype clustering. When the two relative warps, 7 and 36, were plotted against the two components from multi-dimensional scaling of proximity matrix, the relative success of the random forest analysis based prediction over the Relative Warp analysis in differentiating the songtypes could be visualised [see figure 2.8]. In figure 2.8, plots between the



(a) First 2 components of the multi dimensional scaling of proximity matrix plotted to show grouping of *Mecopoda* individuals under study



(b) First 3 components of the multi dimensional scaling of proximity matrix plotted to show grouping of *Mecopoda* individuals under study

Figure 2.7: Clustering of *Mecopoda* individuals based on multi dimensional scaling of the proximity matrix. The proximity matrix itself is derived from the random forest analysis of 48 relative warps from the relative warp analysis of the right cercus and the subgenital plate of *Mecopoda* individuals. Red represents Two Part, green represents Chirper, blue represents Double Chirper, black represents Helicopter and orange represents Train

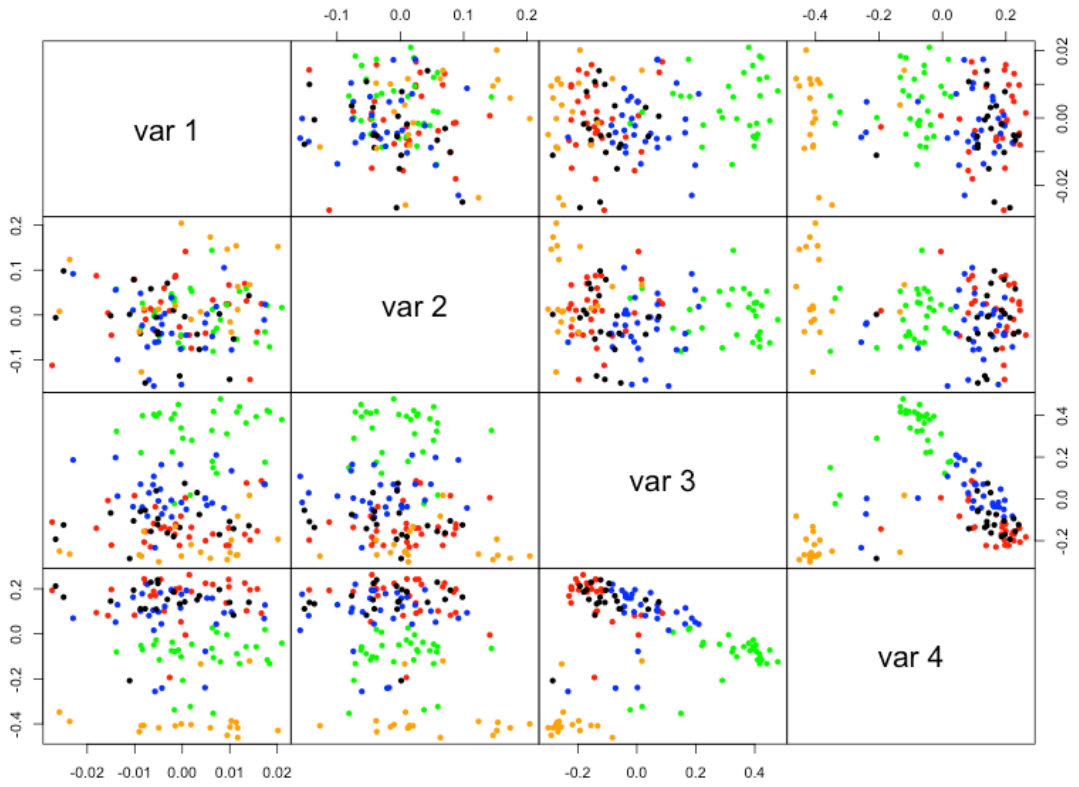


Figure 2.8: Comparison between relative warp analysis and multidimensional scaling of proximity matrix in their ability to differentiate the 5 *Mecopoda* songtypes. var 1 represents scores of relative warp 7 from the relative warp analysis of subgenital plate and var 2 represents scores of relative warp 36 from relative warp analysis of right cercus. var 3 represents scores of 1st component of multidimensional scaling of proximity matrix and var 4 represents scores of 2nd component of multidimensional scaling of proximity matrix. The graphs in each row represents plots between the mentioned variable with three other variables. Red represents Two Part, green represents Chirper, blue represents Double Chirper, black represents Helicopter and orange represents Train.

two principal components (var1 vs var 2) show that there is no clustering of the songtypes based on these components while plots between first two MDS scores (var 3 vs var 4) are able to distinguish the songtypes with much more clarity than the PCA. Comparison between PCA scores (var 1 and var 2) with first MDS score start to show the underlying structure of the data and there is some indication of segregation of the songtypes than the PCA alone. Similarly, comparison between var 1 and var 2 with var 4 also shows similar trend to the previous comparison where the 5 songtypes starts grouping better than PCA alone.

2.5 Discussion

2.5.1 Are *Mecopoda* songtypes morphologically distinct?

Morphometric studies involving genital characters to delimit species have been conducted since times pre-dating Darwin's seminal work but this field of study still appears to be far from being saturated (Eberhard, 2011; Masly, 2012). It is generally considered that there should be strong divergence in genital structures among species due to stabilizing selection (Eberhard, 1985; Mutanen et al., 2006; Richmond et al., 2012; Schilthuizen, 2003) and less variation within species when the species is sympatric (Mutanen et al., 2006; Schilthuizen, 2003). On the other hand, sexual selection should increase intra-population divergence (Richmond et al., 2012) such that there may be formation of cryptic species complexes if female choice of genital structure coevolves with such genital divergence. However, there is a dearth of studies regarding variation in genital shapes in closely related sympatric species or cryptic species that appear to belong to a single species morphologically but may be isolated. In a study of moths of three closely related *Euxoa* sp. (Mutanen et al., 2006) (which are reproductively isolated species and distinguishable based on wing pattern and genitalia) that occur in sympatry in Finland, multivariate analysis showed that there is a consistent significant overlap of external genital characters; however, the males of three species could be successfully differentiated with an error of only 4.5 %, using a discriminant function analysis based on ten characters of the external genital morphology. Also the external morphology varied least intraspecifically as against internal genital morphology and non-genital morphology. This might be true for the acoustically divergent groups of *Mecopoda* although their internal genitalic morphology remains to be studied. In another study on the subspecies of *Drosophila mojavensis* of the North American desert it was found that shape analysis of the aedeagus (male genitalia) made it possible to differentiate between three subspecies but the fourth subspecies overlapped with all the other three subspecies in a PCA analysis (Richmond et al., 2012). A discriminant function analysis using significant PC scores in the same study found that Drosophila mojavensis individuals were correctly classified as subspecies with 86 % accuracy. Most of the misclassification also belonged to the subspecies, which overlaps with other three subspecies. A similar study on the grasshopper genus *Schistocerca* (Song, 2009) using landmark based geometric morphometrics found that male genitalia (n=60) cannot be a diagnostic character to differentiate among the four species in the genus. The two genital structures

(basal eminence of cingulum and epiphallus) used in the study did not allow differentiation of the four species and there was overlap among the already established species based on genital characters. Only when the size and shape of these two characters were considered together, were separate groups identified. In another landmark-based geometric morphometric study on parasitic *Trichogramma pretiosum* (Querino et al., 2002) obtained from 10 different host Lepidopteran hosts, it was found that the anterior region of the genital capsule was able to differentiate *T. pretiosum* into 4 distinct groups, showing that landmark-based geometric morphometric study was able to detect even small intraspecific differences, although the small sample size of this study requires us to be cautious about its findings.

The previous study (Nityananda and Balakrishnan, 2006) on the same songtypes speculated that the five different songtypes of *Mecopoda* might be cryptic species based on the fact that four out of five songtypes were morphologically indistinguishable although all five songtypes could be distinguished based on the temporal features of male calls. The study extensively covered all the major external morphological characters (75 quantitative characters and 61 qualitative characters) of *Mecopoda*. The use of so many characters captures as much variation as possible in the external morphology of *Mecopoda*, especially when there is no obvious diagnostic feature. However, the use of traditional multivariate morphometrics has limitations when compared to landmark-based geometric morphometrics; these include:

1. Arbitrary distances, angles and ratios surrogate for the measure of shape, which impart only partial information when compared to landmarks in geometric morphometrics (Adams et al., 2004; Rohlf, 1999) and a lot of data for other dimensions are lost (Rohlf and Marcus, 1993).

2. It is impossible to recover and use the original shape of the character after the multivariate analysis especially when the ultimate motive is to compare the shapes (Rohlf and Marcus, 1993).

3. This kind of analysis depends on some prior knowledge of what predictor variables might be important for the analysis or we have to choose the variables intuitively before the analysis. This often leads to comparison of many more characters than required, resulting in possible statistical artefacts.

4. When there is no prior knowledge of predictor variable(s), the studies tend to include a large number of characters, running the risk of diluting the singular effects of variables and increasing the risk of type 1 error.

5. While traditional morphometrics only allow us to view the change in numerical descriptors of shape numerically or graphically, landmark-based geometric morphometry produces excellent visual graphics to see changes at particular position in the shape (Adams et al., 2004; Rohlf, 1999; Rohlf and Marcus, 1993).

All these general limitations had a bearing on the previous morphometric study on *Mecopoda* (Nityananda and Balakrishnan, 2006). In this study, an improved version of shape analysis, namely, landmark-based geometric morphometrics was followed. As the results suggest, the relative warp analysis, too, failed to distinguish between the songtypes although grouping trends similar to traditional morphometrics study was noticeable as Chirper tended to group together distinct from other songtypes. However, two improvements over the previous study were that only two characters (subgenital plates and cerci) were used in the relative warp analysis and the present study involved comparison of the shape of these two characters. In fact, the maximum contribution to the variation of the shape of the subgenital plate came from the posterior apical projection on the subgenital plates. Similarly, the maximum contribution to the variation of the shape of cerci involved the two subapical hook-like structures on posterior projection of cerci. Nevertheless, our landmark-based geometric morphometrics failed to identify any morphological difference between the *Mecopoda* songtypes although MANOVA indicated significant differences among songtypes. This result can be explained by the fact that although these characters caused significant difference in shape, they failed to capture all the variables leading to morphological difference in songtypes or that the statistical method used (e.g. principal component analysis) might not be fully sensitive to this particular type of configuration of the landmarks. Indeed, limitations of landmark-based geometric morphometrics can be categorized as follows (Richtsmeier et al., 2002):

1. Although landmarks capture the overall shape of a character, they do not capture all the information regarding the shape (say, the region between two landmarks).

2. The ultimate result of a particular study depends on the choice of landmarks. There may sometimes be an over-representation of a certain portion of a structure due to conglomeration of biological features when compared to other parts. Additionally the accuracy with which landmarks can be placed may make a difference in the final results.

With these general drawbacks, the only obvious limitation of the present landmark-based geometric morphometric study was our relatively modest sample size. One way to ameliorate this problem, other than to increase sample size is to use bootstrap-based data mining statistical analysis. Thus, random forest analysis was used on the relative warp score from the relative warp analysis of subgenital plate and cerci for this purpose. Interestingly, the random forest algorithm was able to classify the *Mecopoda* songtype individual with almost 90 % probability using the same relative warp score that was used in the PCA-equivalent relative warp analysis. This high percentage might also be due to the use of random predictor variables with random subsets of data such that any correlated variables will break down to truly act as independent ones. The random forest was also able to denote the important relative warps, those that were significantly affecting the formation of groups. It is expected that these relative warps may, in turn, be derived from the landmarks representing the posterior apical projection on the subgenital plates and the two subapical hook-like structures on posterior projection of cerci: the structures that produced more variation than other landmarks in relative warp analysis. The comparison of relative warps and principal components from multi-dimensional study also indicates the superior accuracy of random forest analysis over classical multivariate analysis when sample sizes are small.

Given the result, we can conclude that the *Mecopoda* songtypes may also be morphologically distinguishable into songtypes based on the subgenital plate and cerci. It is interesting to note, however, that the difference in shape of these two characters among the songtypes are likely to be subtle given that both the convenient methods of differentiating shape failed to capture the true variability of the shape of the two characters. This suggests that songtypes may still remain cryptic species, as it is possible that instead of any particular feature in the characters used for the analysis, the overall shape of the two characters contributes to the difference among the songtypes. However, even this subtle change of morphology may be significant enough for the females of *Mecopoda* songtypes to discriminate against heterospecific males when they confront each other in the act of mating. That *Mecopoda* songtypes are not identical, at least, with respect to two possible secondary sexual characters, makes it an interesting case for sexual selection driven maintenance of isolating barriers with potential implications for speciation.

2.5.2 Is mechanical isolation contributing to reproductive isolation in *Mecopoda*?

There is overwhelming evidence in the literature that the evolution of genital structures may be driven by sexual selection (Eberhard, 1985, 2009; Hosken and Stockley, 2004; Masly, 2012; Schilthuizen, 2003). It is, thus interesting to examine if sexual selection, also helps in maintenance of mechanical isolation between potentially speciating populations. The acoustically divergent group of *Mecopoda* provides opportunities to study this kind of isolation. However, in cryptic divergent populations, it is quite challenging to demonstrate divergence of reproductive characters and more often differences in genital structures are quite subtle and small.

Studies on structural isolation (a form of mechanical isolation) in animals have been few because the cases of structural incompatibility between two potentially hybridizing species are quite rare (Masly, 2012). On the other hand, tactile isolation may be more common than previously thought (Coyne and Orr, 2004; Masly, 2012). Male genitalia have been shown to evolve faster than other morphological features and may lead to subtle changes in the form of the reproductive structures (Mutanen et al., 2006). This, in turn, may lead to the neurological changes associated with the response of females to the detection of male genitalia (Coyne and Orr, 2004).

In conclusion, the *Mecopoda* songtypes appears to be a good model to examine mechanical isolation as a reproductive barrier. Firstly, it may be seen as a truly cryptic species complex as two well-established techniques failed to distinguish between the songtypes morphologically. The subgenital plate and cerci are good candidates for being secondary sexual characters. The difference in shape of the two genital characters appears to be subtle as seen in some speciating closely related species (Song, 2009; Sota and Kubota, 1998; Ware and Opell, 1989). Given that random forest analysis was able to distinguish between the five songtypes of this Orthopteran species, we can assume that there is a potential for structural isolation. However, it is more likely that the isolation is tactile in nature in *Mecopoda*. The mating behaviour in *Mecopoda* suggests that there exists a small possibility of matings between two songtypes, Chirper and Double Chirper (see chapter 4). However, we need to study the mating behaviour in detail before we can conclude for sure whether mechanical isolation is present between *Mecopoda* populations of different songtypes.

Although there is the potential for mechanical isolation, the relatively small divergence in genital structures (that we have also identified in *Mecopoda*) suggests that there may be other isolating barriers involved in premating isolation (Shapiro and Porter, 1989) . It will be important to examine behavioural isolation between songtypes based on acoustic and chemical signals. This might seem obvious since *Mecopoda* songtypes are defined based on their acoustic signals, which may restrict female approach even before actual mating takes place. Similarly, chemical signals such as cuticular hydrocarbons, which most likely play a role in conspecific recognition, could also deter *Mecopoda* females from mating with heterospecific males. Further studies following these aspects may throw light on the reproductive isolation and speciation in *Mecopoda*.

Chapter 3

Cuticular lipid profiles differentiate acoustically divergent *Mecopoda* populations

3.1 Abstract

Chemical signals are known to play an important role in the reproductive biology of numerous insect species, including many orthopterans. As a result, divergence in the composition of chemicals found on the cuticle where they can potentially be detected by would-be mates might represent a reproductive isolating mechanism among populations. In the bushcricket *Mecopoda elongata* females are attracted to males through phonotaxis and subsequently come into direct contact with the male via their antennae, at which point the potential for receiving information about the male's cuticular chemistry is apparent. *Mecopoda* females may prefer a cuticular lipid profile belonging to their own songtype and reject mating with that of other songtypes. This has the potential to lead to isolation based on cuticular chemicals. However, to investigate the possibility of such isolation, it is necessary to establish differences in chemical profile with respect to songtypes. With this aim, cuticular lipids were extracted from known *Mecopoda* songtypes and analysed using gas chromatography and mass spectrometry. Statistical analyses were able to differentiate between all the songtypes with high probability. Differences between the songtypes were due to differences in the relative quantities of cuticular chemicals rather than absolute presence or absence of specific components. The

result indicates that *Mecopoda* are truly divergent populations based on their cuticular profile and that some degree of premating isolation based on preference for chemical profile may be contributing to gene flow restriction between the songtype populations.

3.2 Introduction

The acoustic mode of communication in Orthopteran insects has for a long time received more attention than their chemical signals (Tregenza and Wedell, 1997). This is presumably because their species specific acoustic signals (Alexander, 1967; Honda-Sumi, 2005) are more conspicuous and readily recorded. Nevertheless, the role of chemicals in sex recognition and species identification in Diptera, Coleoptera and Lepidoptera is now well recognised (Blomquist and Bagnères, 2010; Howard and Blomquist, 2005). Here, I investigate whether there are differences in the profile of such chemical signals between acoustically divergent orthopteran Tettigoniid populations of *Mecopoda* and discuss the potential role of these differences in reproductive isolation between songtypes.

Following the typical pattern of Orthopteran acoustic signals getting prior attention over other modes of communication, the *Mecopoda* population in southern India has been differentiated into five songtypes that differ in the temporal features of the male calls. Initial study (Nityananda and Balakrishnan, 2006) suggested these populations could be cryptic species in the absence of any diagnostic morphological characters capable of morphologically differentiating the songtypes. Besides, these songtypes overlap in distribution and reproductive season in various combinations. Along with the fact that *Mecopoda* females do not call, it is virtually impossible to identify an individual of a songtype in the wild except when the males call at night. The strong acoustic differences in the *Mecopoda* population despite sympatry suggest that there are reproductive barriers between songtypes that are preventing gene flow. Apart from experiments to investigate *Mecopoda* females' preference for their own songtype, studies involving molecular analysis of the *Mecopoda* genome or chemotaxonomic studies on *Mecopoda*, cuticular hydrocarbons (CHCs) could also unravel the differences between the songtypes and indicate isolating mechanisms maintaining them. An unpublished previous work by Nityanand et al found low genetic divergence among Indian *Mecopoda*

songtypes based on cytochrome oxidase sequence. Although use of more genetic markers could have made a difference to the findings of the molecular genetic study, study on the difference in CHC profiles could provide us additional avenues to study reproductive isolation among *Mecopoda* songtypes. This is because CHCs have been shown to evolve very rapidly (Mullen et al., 2007) and have been speculated to show differences even when morphology or genetics fail to show differences among individuals in a number of animal species (Kather and Martin, 2012).

While there are many sources of chemical signals in insects, cuticular hydrocarbons have received most of the attention (Blomquist and Bagnères, 2010). This is presumably because they are abundant in insect cuticle (Blomquist and Bagnères, 2010; Kather and Martin, 2012; Lockey, 1991), are known to have species-specific patterns of composition and because of their potential role as contact pheromones. (Howard and Blomquist, 2005; Kather and Martin, 2012). To add to this, CHCs in two species of leaf beetles (Coleoptera) have recently been found to be involved in male mate choice that is also thought to be reinforcing behavioural isolation between these two species (Peterson et al., 2007). Most of the CHCs in insects are biosynthesized by the insect rather than acquired from the environment (Blomquist and Bagnères, 2010) and thus, they may reflect the insect's genotype (Blomquist and Bagnères, 2010; Lockey, 1991; Page et al., 1997) and have been shown to be potentially heritable (Kather and Martin, 2012). The composition of CHCs shows a greater divergence and complexity among species than within species both in terms of presence-absence and relative abundance of different CHC components (Howard and Blomquist, 2005; Kather and Martin, 2012).

Cuticular lipids acts as sex pheromones in crickets and are a crucial part of the mate recognition process (Nagamoto et al., 2005; Tregenza and Wedell, 1997). They have also been shown to influence mate choice during the orthopteran mating process (Steiger et al., 2013; Thomas and Simmons, 2009). In insects, mating occurs in a series of behavioural stages leading to actual sperm transfer (Heller, 2005; Nagamoto et al., 2005). *Mecopoda* males and females antennate [see chapter 4] after the female has successfully localized a male through phonotaxis to a long-range acoustic signal from the male. A successful mating commences only after a mating pair is satisfied with the mate recognition cue (most likely cuticular lipids since they are found over the external surface of an insect) during this antennation event (as discussed in Nagamoto et al. (2005)). Assuming that the ancestral population of *Mecopoda* in southern India lacked differentiation into

distinct CHC types, the potential existence of differentiated CHC types within the current distribution, which coincides with acoustic signal types would be further evidence for reproductive isolation among these songtypes. The question would then be whether one of the two, or both of these potential mate choice/recognition signals is responsible for reproductive isolation among songtypes. It is possible for instance that substantial reproductive isolation could be created by either acoustic or chemical signals, and that once isolated these new populations could diverge in the other signal through neutral processes. However, given the very low divergence in morphology between songtypes, if there is significant divergence in CHC profiles among songtypes this would suggest that they are under selection, and likely have a role in mate recognition or choice.

CHC profiling has been accomplished in many insect species (Blomquist and Bagnères, 2010). They have proved to be particularly helpful at identifying cryptic species that occur commonly in insects and are morphologically similar due to their shared ecology (Kather and Martin, 2012). In morphologically and ecologically cryptic species of *Laupala* (Mullen et al., 2007), it was found that differences in CHC profiles among eight closely related species matched that of the phylogenetic tree and the songtypes. Maroja et al. (2014) showed that despite a lack of differentiation in male CHC profiles, the behaviourally and morphologically similar species *Gryllus firmus* and *Gryllus pennsylvanicus*, males from a hybrid zone, use differences in CHC composition of females to identify conspecific relative to heterospecific females. This has been interpreted as indicating a possible premating barrier to gene exchange. In sympatric European corn borers, *Ostrinia nubilalis*, strong isolation between two races is based on trans and cis forms of the female pheromone (not CHC, however) that attracts conspecific males (Linn et al., 1997). Similar studies of CHC profiles to distinguish sympatric species have been achieved mainly in ants (Akino et al., 2002; Lucas et al., 2002; Martin et al., 2008; Schlick-Steiner et al., 2006), termites (Haverty et al., 1990), beetles (Page et al., 1997; Peterson et al., 2007) and butterflies (Guillem et al., 2012). Further, the difference in CHC profiles leading to chemical isolation has been studied in sulphur butterflies (Grula et al., 1980) and Drosophila (Coyne, 1996; Coyne et al., 1994).

The objectives of the study are as follows:

1. To acquire CHC profiles for the *Mecopoda* songtypes.
2. To test whether the CHC profiles can differentiate between the songtypes.

3.3 Materials and methods

3.3.1 Specimen collection

Adult *Mecopoda elongata* L. (Orthoptera, Tettigoniidae, Mecopodinae) males (n=166) were collected from 8 different locations:

Location	GPS Location	Songtypes					Total
		C	DC	2P	H	T	
Kervashe	N13° 14', E75° 04'	0	17	8	5	0	30
Hurabi	N13° 10', E75° 07'	0	11	3	0	0	14
Heringe	N13° 15', E75° 08'	0	0	0	9	0	9
Kudremukh Field Station	N13° 13', E75° 05'	0	10	32	0	0	42
Shimoga	N13° 55', E75° 35'	11	0	0	0	1	12
Ullodu	N13° 38', E77° 42'	11	0	0	0	17	28
Peresandra	N13° 58', E77° 79'	0	0	0	0	1	1
IISc	N13° 01', E77° 34'	11	0	0	0	1	12
Lab Bred	N13° 01', E77° 34'	8	7	2	0	1	18
Total		41	45	45	14	21	166

Table 3.1: Locationwise sampling of *Mecopoda* songtypes that were subsequently used for cuticular lipid analysis. C represents Chirper, DC represents Double Chirper, 2P represents Two Part, H represents Helicopter and T represents Train songtype respectively

Kervashe, Hurabi and Heringe locality are situated within six kilometres of the field station at Kadari, Peresandra is situated at around twelve kilometres from Ullodu while laboratory raised individuals are mostly progeny of an allopatric Chirper population found on the Indian Institute of Science Campus, Bangalore. Males were localised by listening to their calling songs or by following their movement when ground vegetation was disturbed. The songs of all these males were recorded to ascertain the songtype of the individuals before the study was conducted.

3.3.2 CHC extraction and GCMS

Insect cuticle is covered by a thin layer of lipid that contains a variety of chemical substances (Lockey, 1980, 1988, 1991). The free lipid component of this lipid layer primarily protects the insect from desiccation (Lockey, 1988) and is composed of alcohols, aldehydes, ketones, esters, glycerides, long chain fatty acids, sterols

and CHCs (Lockey, 1980, 1988, 1991). The CHCs in turn can be broadly classified into 3 types, namely, n-alkanes, olefins and methyl alkanes.

Any study involving CHC profiling follows a common analytical procedure (Lockey, 1991):

1. Organic solvents are used in extraction of CHCs.
2. The extracted samples are analysed using gas chromatography to separate out different hydrocarbons and create a CHC profile.
3. The hydrocarbons are identified by mass spectrometry (GCMS).

Before extraction, both containers and forceps were washed in Hexane to remove any contamination. Initially, I tried extracting CHC from the hind leg by dipping the legs completely in Hexane for five minutes. This process failed to display any peaks in gas chromatography presumably as a result of their being only a low concentration of CHCs that is available for extraction on the hind legs. Another method, in which a cotton bud dipped in Hexane was used for scraping the surface of different *Mecopoda* body parts also failed to show any presence of CHC in gas chromatography. Thus, although *Mecopoda* males are of very large body size compared to some other insects in which extraction from the whole body has been used to sample hydrocarbons (Kaib et al., 1991; Lockey and Metcalfe, 1988; Martin et al., 2008; Mullen et al., 2007), a whole body CHC extraction method was followed.

A *Mecopoda* male was put in a 10 ml glass vial, which was then filled completely with Hexane such that the *Mecopoda* specimen was totally submerged. After five minutes, the male was pulled out with the forceps and the remaining hexane was left to evaporate to about 1 ml. Using a pipette, this 1 ml of Hexane and dissolved CHCs was transferred to 1.5 ml GC vial. Hexane was again allowed to evaporate completely so that the content of the vial was dry: the CHC extracts would have been deposited on the wall of the GC vial. After this, the GC vial was capped and stored in refrigerator until GCMS was conducted.

The mass spectrometer (Agilent 5975B MSD) enabled gas chromatography (Agilent 7890A) instrument with Agilent J&W DB-1MS capillary column (30 m x 250 µm x 0.25 µm) was used in the CHC profiling. Before the GCMS run, the air-dried sample was returned to solution by adding 500 ml of Hexane. A known volume (1

ml of 10 ng/µl Pentadecane) of internal standard was also added to this solution and this sample solution was vortexed for 10 seconds. The amount of sample injected into the capillary column was 2 µl via an autosampler (G6500-CTC). Highly pure Helium was used in the analysis and its flow in the column was maintained at 3 ml/min. The 27 minute oven programme used was set to rise from 50°C to 280°C at 30°C/min continuing its rise from 280°C to 350°C at 5°C/min and then maintained at 350°C for 5 minutes (the DB-1 capillary column used here can reach up to 360°C). The peaks detected by the mass spectrometer were analysed using Agilent ChemStation software.

A chromatogram represents the retention time of individual compounds on the x-axis and ion abundance of CHC on the y-axis. Molecules with greater molecular weight and (to a lesser extent) more branching structures have higher retention times and appear later in the chromatogram than simpler molecules. Those molecules that are abundant are represented by peaks that enclose a greater area underneath them than less abundant components. Sometimes the molecular weight of two molecules may be so similar that the two peaks overlap and merge with each other, making a broader peak. A sharp peak, on the other hand, denotes purity of the peak i.e. a peak corresponding to a single molecule. In many biological solutions, there are a lot of trace elements and impurities that appear as background noise and make the baseline of an abundance-retention time graph appear rough. By choosing an abundance threshold baseline for the graph this problem can be overcome. The peaks that appeared consistently in chromatograms over the abundance threshold were integrated using ChemStation integrator. The integration events involve identifying the start time, end time and apex of the peak, constructing the baseline and then calculating the area, height and width of each peak. An integration event was calibrated by determining the retention time for each of the identified peaks from a sample and the method was saved. This saved calibrated integration event was then used to integrate all samples since retention time for all identified peaks remained constant as GC parameters were constant. Changing the basic parameters of the integration process would change the absolute values of the area under the peaks. However, it would not affect my analyses since I used the relative abundance of each peak in each sample. The relative abundance was calculated by standardising the area under each peak relative to the area under the internal standard peak.

3.3.3 Statistical analysis

Since the actual concentrations of the CHCs in each sample were unknown we standardised the 25 target peak values by using the value of the internal standard (Pentadecane) to control for variation in the exact quantity of sample extracted and injected. This was done by dividing the area under the peak value of each of the 25 peaks for each sample with the area under the peak value of Pentadecane in the same sample. The values of area under the 25 target peaks of 166 samples were initially used in a principal component analysis (PCA). However, a lot of structure was observed in the PCA plots where the two plotted principal components [see figure 2] had a strongly wedge-shaped distribution. Because PCA is not expected to produce relationships between individual components, such distributions are often indications of violations of assumptions. Therefore we log transformed the peak values and repeated the PCA. We log transformed 19 out of the 25 peak values using 10 as base (Peaks 13, 20, 23, 26, 27, 28 were not used in PCA as they have some 0 values in the sample that cannot be log transformed, and even adding a small positive number can be problematic because it creates a series of peak areas with no variance). For further analysis (DFA and RFA) too, we used the log transformed data set of 19 target peaks of the 166 Mecopoda samples. The excluded peaks are likely to be less informative than the remaining peaks as they were the smaller peaks found in the profile. The principal component analysis was used to reduce the number of variables (here, 19) to a few components that explain the majority of the variation within the sample. The first two principal components were used to plot a 2D scatterplots to visualize the extent of grouping within our *Mecopoda* samples.

The DFA produces a linear function that produces the maximum differences among the groups that are being investigated. The first two components of the PCA were also used to visualise songtype grouping in a Discriminant Function Analysis (DFA). We were obliged to use our PCA scores in the DFA rather than the raw peak areas because the number of samples we had for helicopter (n=14) and train (n=21) songtypes was smaller than the number of peaks identified in our chromatograms. The loading value of the first component of PCA indicated the relative importance of the different peaks. The first 5 important peaks (peaks contributing most to PC 1) were identified. Then 3 separate analyses were done using the two, three and five best peak scores from PCA and log transformed area under the CHC peaks values to compare the percentage correct prediction of the songtypes by DFA.

Next, a relatively new statistical tool called Random Forest Analysis (RFA) was used with the same 19 log transformed peaks of the 166 samples. RFA essentially derives classification trees based on repeated random subsampling of random subsets of data and based on random choice of the predictor variable. The analysis then uses these classification trees to determine how efficiently all the data points group together (Strobl et al., 2009b). The “cforest” algorithm from the “party” package was used in the present study (Hothorn et al., 2006; Strobl et al., 2008, 2007). For more details on the analysis refer to the subsection 2.3.3 in the section 2.3 in chapter 2. Random Forest analysis was suitable for our study mainly because:

1. In traditional multivariate analysis, we need to have a sample size that is at least 3 to 4 times the number of samples in each constituent group as a rule of thumb. The groups with maximum sample size in this study had only 45 individuals.
2. The independence of the data points cannot be controlled in gas chromatography and the presence of links between CHC components may be expected since they appear to be produced in similar biochemical pathways.

The error in predicting the identity of the samples was calculated along with the relative importance of the different CHC peaks (predictor variables) based on RFA. Proximity analysis was also performed to find out the proportion of times two individuals occurred at the same node of the classification trees during Random Forest analysis. A multi-dimensional scaling (MDS) of the calculated proximity matrix of the RFA was done to visualise group membership of the data points. Additionally, we compared the results of principal component analysis and MDS of the proximity scores to show better efficacy of RFA over PCA in classifying the data set.

3.4 Results

3.4.1 GCMS

A total of 28 peaks including that of the Pentadecane standard (Peak 1) appeared consistently in all five *Mecopoda* songtypes. However, Peak 15 and Peak 16 were

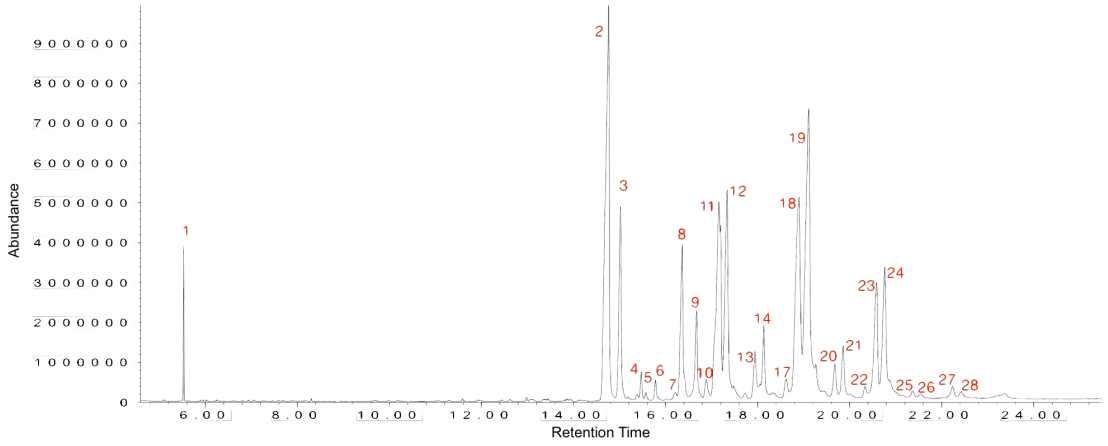


Figure 3.1: A representative chromatogram showing 25 consistent CHC peaks along with a peak for internal standard (Pentadecane as Peak 1). The X-axis represents retention time and the Y-axis represents ion abundance.

later dropped from further analysis since Peak 16 seemed to merge with Peak 15 in some cases [see figure 3.1]. The details of the remaining 25 peaks, all of which appear to be CHCs rather than other types of compound, are shown in table 3.2. The peaks were quantified using a similar analytical technique to that followed in Steiger et al. (2007) and the results are summarized in table 3.2 & table 3.3. Values of zero percent in some peaks in table 3.3 are because of the presence of those chemicals in negligible quantities rather than complete absence.

Out of 25 consistent peaks, 14 peaks were determined to be methyl alkanes [see table 3.2], and were positively identified as hydrocarbons by their mass spectra and retention index (following the references in Steiger et al. (2007)). The rest of the peaks are also very likely to be hydrocarbons following the trends in results from searching probable peak identity in the database of the NIST 08 library (blast results predicted hydrocarbons ranging from C27 to C44).

Peak No.	Retention Index	CHC Identity	Diagnostic Ions
2	3473	4-MeC34	492, 449
3	3506	Undetermined Hydrocarbon	-
4	3568	4-MeC35	506, 463, 71
5	3580	3-MeC35	506, 477
6	3600	Undetermined Hydrocarbon	-
7	3679	4-MeC36	520, 477
8	3705	7-MeC37	113, 449
9	3757	6,14-diMeC37	447, 99, 351, 225
10	3790	4,14,18-triMeC37	519, 71, 365, 225, 295
11	3807	5,9,13-triMeC37	505, 85, 435, 155, 365, 225
12	3853	3-MeC38	519, 57
13	3875	4-MeC38	505, 71
14	3884	4,24-diMeC38	519, 71, 225, 365
17	3952	Undetermined Hydrocarbon	197, 435
18	3964	Undetermined Hydrocarbon	197, 435
19	3989	15,19,23-triMeC39	393, 225, 323, 295, 253, 365
20	4004	7,17,27-triMeC39	590, 505, 113, 267, 197
21	4057	Undetermined Hydrocarbon	-
22	4078	x,x-diMeC40	590
23	4096	Undetermined Hydrocarbon	-
24	4145	Undetermined Hydrocarbon	225, 435
25	4177	Undetermined Hydrocarbon	-
26	4195	Undetermined Hydrocarbon	-
27	4212	Undetermined Hydrocarbon	-
28	4341	Undetermined Hydrocarbon	225, 435

Table 3.2: List of cuticular hydrocarbons consistently found in the *Mecopoda* samples through gas chromatography-mass spectrometry along with their retention indices

Peak No.	Chirper			Double Chirper			Two Part			Train			Helicopter		
	M	SD	%0	M	SD	%0	M	SD	%0	M	SD	%0	M	SD	%0
2	5.95	4.11	0	5.88	3.95	0	7.16	4.37	0	10.1	3.40	0	8.65	5.54	0
3	1.23	1.09	0	0.44	0.23	0	0.79	0.64	0	3.37	1.25	0	0.94	0.71	0
4	0.29	0.21	0	0.60	0.35	0	0.10	0.05	0	0.34	0.15	0	0.15	0.12	0
5	0.33	0.24	0	0.15	0.08	0	0.04	0.02	0	0.15	0.05	0	0.04	0.03	0
6	0.16	0.24	0	0.29	0.20	0	0.44	0.37	0	0.31	0.14	0	0.65	0.58	0
7	1.87	2.02	0	11.74	6.11	0	1.46	0.87	0	1.70	1.30	0	1.92	1.55	0
8	0.55	0.54	0	0.69	0.36	0	0.26	0.21	0	1.36	0.53	0	0.34	0.30	0
9	2.23	1.37	0	1.98	1.08	0	0.12	0.10	0	2.05	1.85	0	0.21	0.27	0
10	4.62	2.61	0	8.07	4.29	0	0.18	0.14	0	2.75	1.47	0	0.26	0.31	0
11	0.27	0.23	0	1.17	0.68	0	0.12	0.10	0	0.23	0.56	0	0.19	0.18	0
12	0.25	0.29	0	0.59	0.30	0	0.15	0.07	0	0.99	0.42	0	0.17	0.16	0
13	0.20	0.52	**4.8**	1.02	0.84	0	0.33	0.24	0	0.29	0.51	0	0.36	0.33	0
14	0.56	0.50	0	2.18	1.10	0	0.20	0.17	0	0.94	0.50	0	0.20	0.17	0
17	0.20	0.22	0	0.58	0.75	0	0.41	0.56	0	0.66	0.53	0	0.39	0.52	0
18	1.66	0.91	0	2.83	1.40	0	2.51	1.55	0	5.51	2.77	0	2.92	2.79	0
19	2.79	1.77	0	7.99	3.86	0	2.81	1.52	0	6.74	2.75	0	3.03	2.69	0
20	0.35	0.93	0	1.59	0.91	0	0.12	0.10	**2.2**	0.33	0.43	0	0.15	0.16	0
21	0.10	0.08	0	0.19	0.10	0	0.83	0.41	0	0.84	0.42	0	0.90	0.90	0
22	0.12	0.10	0	0.42	0.19	0	0.90	0.47	0	0.76	0.33	0	1.06	0.99	0
23	0.06	0.07	**12.1**	0.10	0.05	0	0.06	0.08	**8.8**	0.06	0.05	0	0.11	0.13	0
24	0.13	0.15	0	0.93	0.69	0	0.82	0.56	0	0.82	1.15	0	1.11	1.23	0
25	0.12	0.11	0	0.75	0.37	0	4.48	2.33	0	1.71	1.11	0	5.38	4.34	0
26	0.15	0.17	**9.75**	0.21	0.14	0	3.60	3.00	0	0.41	0.80	**4.7**	4.45	6.21	0
27	0.13	0.16	**41.5**	0.08	0.07	**6.6**	0.17	0.22	0	0.05	0.06	0	0.34	0.55	0
28	0.04	0.04	**4.8**	0.11	0.06	0	0.20	0.12	0	0.10	0.11	0	0.19	0.16	0

Table 3.3: Mean and standard deviation of each standardised area under CHC peak values of *Mecopoda* songtypes. Percentages of individuals in which a particular CHC was present in quantities below our detection threshold (% Zero) value are also provided in the third column under each songtype. The peaks numbered in bold font were ommitted during log transformation since they contained zero values in some samples

3.4.2 Principal component analysis

Initial PCA with 25 standardised CHC peaks indicated formation of groups by the *Mecopoda* songtypes but appeared to have a strong correlation between the plotted components [see figure 3.2]. Therefore, 19 of these 25 peaks were log transformed and used for PCA. The other 6 peaks had 0 values in the samples [see table 3.3] that could not be log transformed. The first 2 principal components using log transformed peak values explained 77 % of the total variation in the samples [see table 3.4]. The contribution of each of the CHC peaks to the PCA are shown in table 3.5. An indication of grouping of the songtypes became apparent when I plotted first two principal components [see figure 3.3].

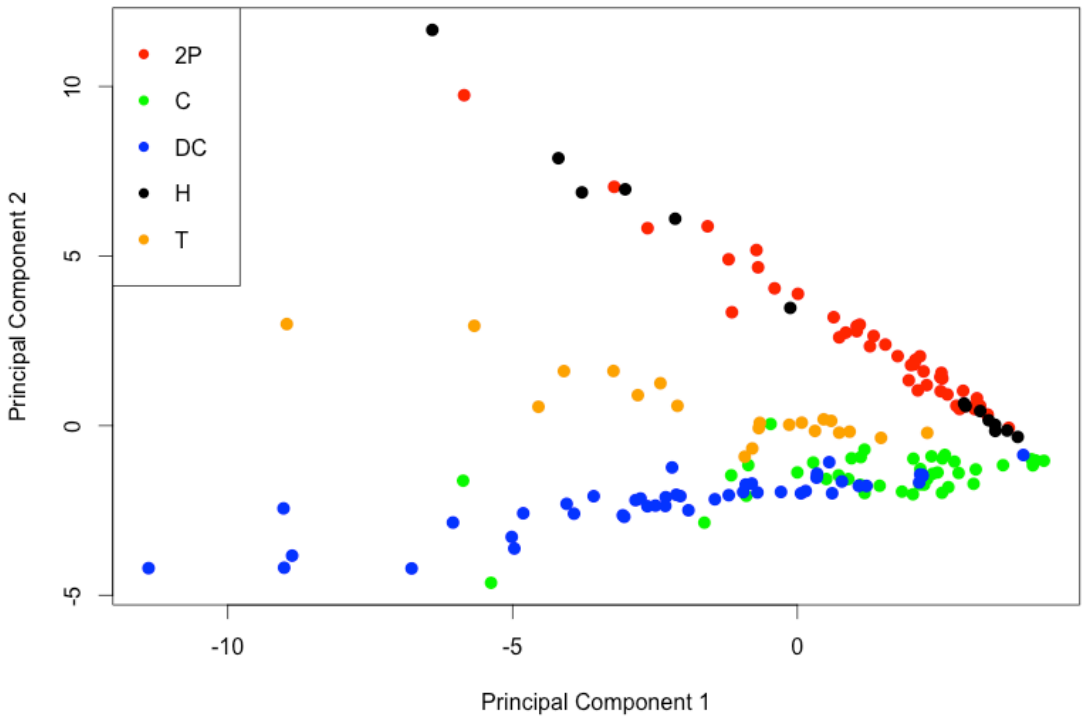


Figure 3.2: Principal component analysis using 25 standardised area under CHC peak values shows grouping of *Mecopoda* songtypes when principal component 1 and principal component 2 are plotted against each other

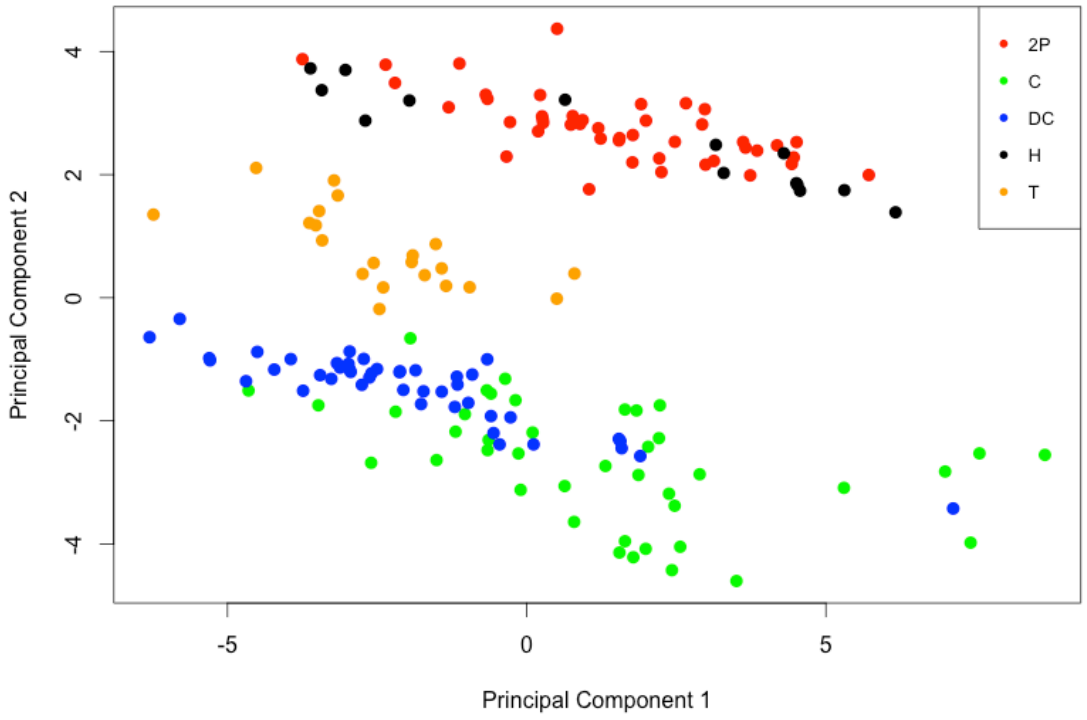


Figure 3.3: Principal component analysis using 19 log transformed area under CHC peak values shows grouping of *Mecopoda* songtypes when principal component 1 and principal component 2 are plotted against each other

Principal Components	Proportion of Variance(%)	Cumulative Proportion(%)
1	47.4	47.4
2	29.2	76.6
3	9.5	86.1
4	3.3	89.4
5	2.1	91.5

Table 3.4: List of principal components from principal component analysis and percentage variation in the sample that they explain

Sl. No.	Peak No.	CHC Identity	PC 1 Loadings	PC 2 Loadings	PC 3 Loadings
1	2	4-MeC34	-0.23	0.17	0.27
2	3	C35	-0.18	0.08	0.58
3	4	4-MeC35	-0.29	-0.18	-0.07
4	5	3-MeC35	-0.20	-0.27	0.26
5	6	C36	-0.21	0.27	-0.02
6	7	4-MeC36	-0.19	-0.12	-0.46
7	8	7-MeC37	-0.29	-0.07	0.18
8	9	6,14-diMeC37	-0.22	-0.29	0.12
9	10	4,14,18-triMeC37	-0.21	-0.32	-0.02
10	11	5,9,13-triMeC37	-0.24	-0.13	-0.30
11	12	3-MeC38	-0.28	-0.06	0.05
12	14	Undetermined HC	-0.25	-0.20	-0.18
13	17	Undetermined HC	-0.26	0.12	-0.01
14	18	4,24-diMeC38	-0.28	0.15	0.11
15	19	15,19,23-triMeC39	-0.31	0.01	-0.12
16	21	Undetermined HC	-0.13	0.38	0.07
17	22	x,x-diMeC40	-0.17	0.35	-0.11
18	24	Undetermined HC	-0.19	0.26	-0.26
19	25	Undetermined HC	-0.08	0.40	-0.13

Table 3.5: Contribution (loadings) of the 19 log transformed area under the cuticular hydrocarbon peak values in the principal components, 1, 2 and 3, of principal component analysis

3.4.3 Discriminant function analysis

First, DFA was performed on the first 2 principal components of the PCA that explained 77 % of the total variation in the samples with assumptions of equal probability of *Mecopoda* individuals belonging to the 5 songtypes. The DFA scatterplot [see figure 3.4] showed very similar clustering as shown by the PCA scatterplot [see figure 3.3]. Then DFA was used to predict the songtypes of same 166 individuals whose identity were already known. A comparison was made between DFA percentage-correct-predictions on scores of principal components explaining most variation within samples and data from most contributing log transformed peaks to principal component 1 in PCA [see table 3.6]. It is to be noted that the first 5 components of the PCA explains 92 % variation in the sample, the first 3 components of the PCA explains 86 % variation in the sample and the first 2 components of the PCA explains 77 % of the variation in the sample [see table 3.4].

Most Important Variables	DFA % Correct Prediction (using Principal Components)	DFA % Correct Prediction (using Log Transformed Area Under Peak Values)
Using best 5 variables	89.8 %	77.7 %
Using best 3 variables	85.5 %	70.5 %
Using best 2 variables	75.9 %	57.2 %

Table 3.6: Percentage correct prediction of *Mecopoda* songtypes by discriminant function analysis. First column includes the number of variables used to calculate the percentage correct prediction, second column shows the percentage correct prediction using principal components from PCA of the 19 log transformed CHC peak values and third column shows the percentage correct prediction using standardised CHC peak values as variables

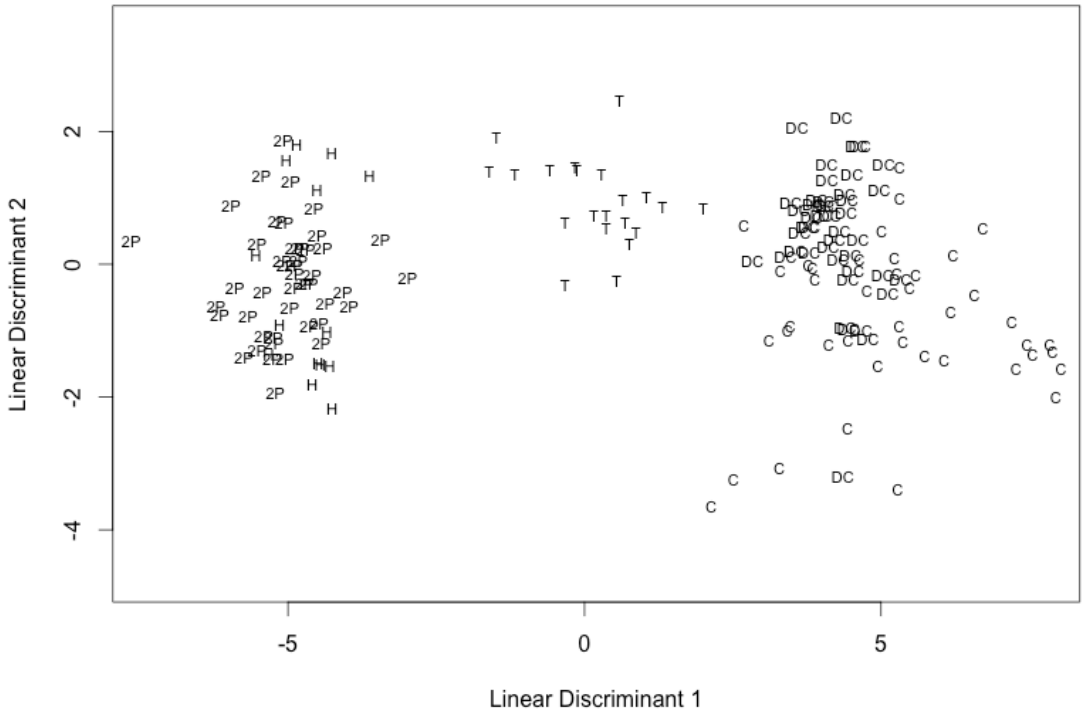


Figure 3.4: Grouping of *Mecopoda* songtypes when two linear discriminants from discriminant function analysis are plotted against each other. C represents Chirper, DC represents Double Chirper, 2P represents Two Part, H represents Helicopter and T represents Train songtype respectively.

3.4.4 Random forest analysis

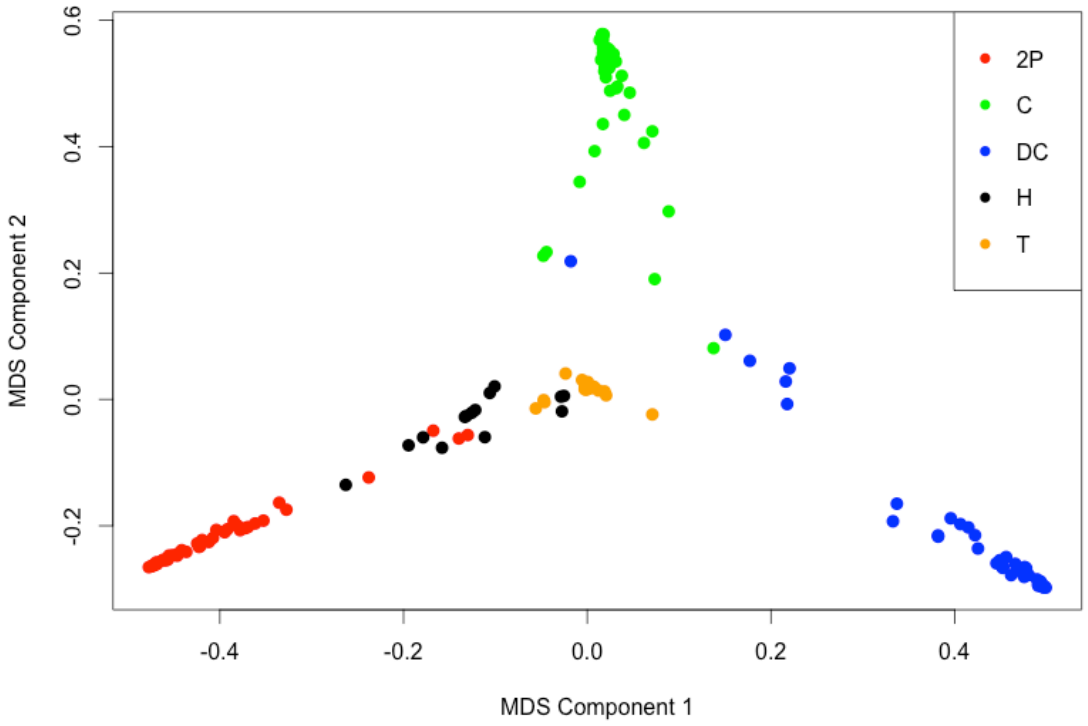


Figure 3.5: Clustering of *Mecopoda* individuals based on multi dimensional scaling of the proximity matrix derived from the random forest analysis of 19 log transformed CHC peak values. Red represents Two Part, green represents Chirper, blue represents Double Chirper, black represents Helicopter and orange represents Train

The hyper parameters to control the “cforest algorithm” during the random forest analysis were ntree = 10000 and mtry = 4 (rounded square root of 19, the number of log transformed CHC peaks). The “cforest” analysis of the transformed CHC peak data predicted songtype of the each *Mecopoda* individual (with an error estimate of 7.2 % i.e. 12 errors out of total 166). This means that in more than 9 out of 10 times the algorithm was able to correctly predict the songtype of an individual *Mecopoda*. It is also interesting to note that the greatest number of errors (8 out of the 12 wrong predictions) occurred while predicting whether an individual belonged to the *Mecopoda* Helicopter songtype; in fact, Helicopter individuals were predicted as Two Part individuals 5 times and as Train 3 times. Another 2 errors involved single error when Chirper and Double Chirper was predicted as Double

Chirper and Chirper respectively. Two Part individuals were also misclassified as Train twice. All Train individuals were predicted correctly.

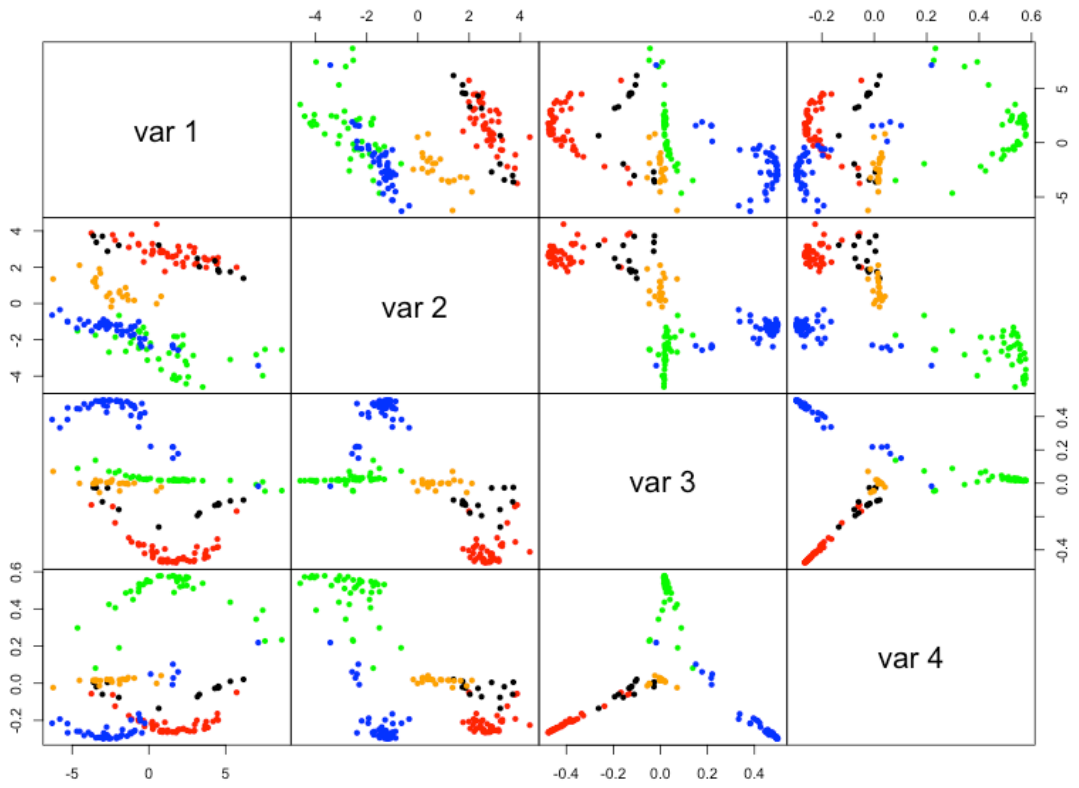


Figure 3.6: Comparison between principal component analysis and multidimensional scaling of the proximity matrix in their ability to differentiate the 5 *Mecopoda* songtypes. Var 1 represents scores of principal component 1, Var 2 represents scores of principal component 2. Var 3 represents scores of the 1st component of MDS of the proximity matrix and Var 4 represents scores of the 2nd component of MDS of the proximity matrix. The graphs in each row are plots comparing the variable named in the row against the other three variables. Red represents Two Part, green represents Chirper, blue represents Double Chirper, black represents Helicopter and orange represents Train.

After proximity analysis, multi-dimensional scaling of the proximity data was able to differentiate all the five *Mecopoda* songtypes graphically [see figure 3.5]. While Chirper and Double Chirper formed distinct clusters, Train, Two Part and Helicopter formed clusters with very few overlaps. This graphical overlap concords with the prediction from the random forest analysis where 5 Helicopter individuals were predicted as Two Part individuals and 3 Helicopter individuals were predicted as Train individuals.

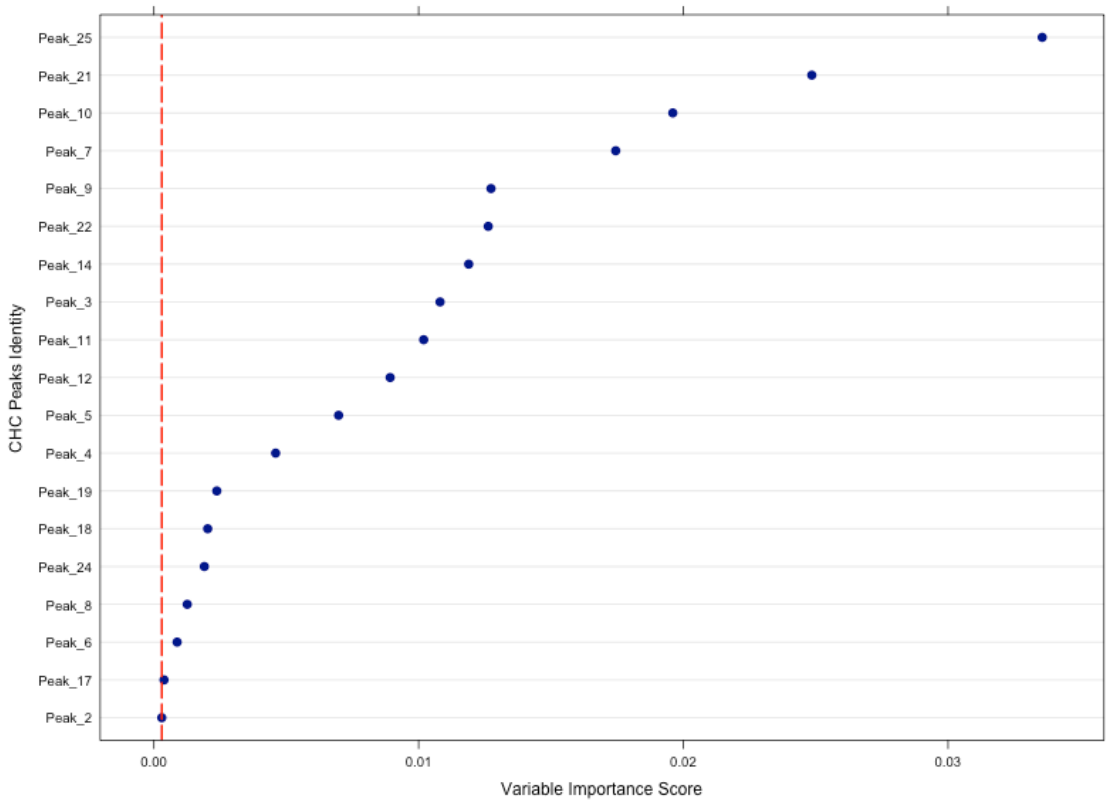


Figure 3.7: RFA based variable importance analysis of the 19 log transformed CHC peak values of *Mecopoda* individuals derived through gas chromatography-mass spectrometry. CHC peaks (predictor variables) to the right of dashed red vertical line have significant role in explaining the variation in the *Mecopoda* sample under study

Since PCA is a form of multi dimensional scaling we can compare our PCA and our MDS of the proximity matrix. When scores of the first two principal components from PCA and scores of the two components from the multi-dimensional scaling of proximity matrix are plotted against each other, we can visually compare the relative success of the Random Forest based method in differentiating Mecopoda songtypes over the principal component analysis [see figure 3.6]. In figure 3.6, plots between the two principal components (var1 vs var 2) show that there is considerable overlap in the clustering of the songtypes (especially there is an overlap between Chirper and Double Chirper as well Helicopter and Two Part) while plots between first two MDS scores (var 3 vs var 4) are able to distinguish the songtypes with much more clarity than the PCA. Comparison between PCA scores (var 1 and var 2) with first MDS score too show better grouping of the songtypes than the PCA alone. Similarly, comparison between var 1 and var 2 with var 4 also show better clustering of all the 5 songtypes than PCA alone.

The variable importance measures derived [see figure 3.7] using the cforest analysis showed that all measures were positive values. Peak 25 followed by Peak 21 were the two most important peaks of all the CHC peaks by being farthest away from the significance threshold than any other peak. All but one peak (Peak 2) had a variable importance measure that is more than the significance threshold value indicating that almost all peaks contributed to the variation in samples. Peak 2 was least important among all the peaks since it was exactly on the significance threshold. It is interesting to note here that peaks (variables) picked up by PCA were somewhat different to those indicated by variable importance analysis. For example, out of first 10 contributing peaks of both the analyses, only 4 were common and their order of significance did not match.

3.5 Discussion

3.5.1 Are *Mecopoda* songtypes chemically distinct groups?

Although the different songtypes within the *Mecopoda* species found in southern India appeared to be indistinguishable morphologically (Nityananda and Balakrishnan, 2006), robust statistical analysis of genital morphology was able to predict the songtypes of *Mecopoda* individual with almost 89 % accuracy based on just two external genital characters [see chapter 2]. This is an indication that although broadly cryptic in nature, *Mecopoda* songtypes are divergent for a range of phenotypic characters. Persistent songtype groupings based on temporal features (Nityananda and Balakrishnan, 2006) suggest that assortative mating may be taking place within the *Mecopoda* population. In this scenario, it is very likely that barriers appear in different forms and at different levels of the mating process. CHCs, which so commonly play an important role in conspecific identification (Blomquist and Bagnères, 2010; Howard and Blomquist, 2005; Kather and Martin, 2012) are a major source of discrimination among divergent populations (Linn et al., 1997; Maroja et al., 2014; Peterson et al., 2007). CHCs are known to evolve fast, such that differences may show up even when the separation between two populations is recent (Mullen et al., 2007).

In this study, we characterized the CHC profile of *Mecopoda* for the first time. Although it is time consuming and requires expertise to precisely identify novel biomolecules through gas chromatography, preliminary identification of GC peaks

indicate CHCs are present on the *Mecopoda* cuticle and that they are of a molecular weight in the range of C27 to C44 that suggest that they are unlikely to be volatile. CHCs have been found to be important as communication signals (Blomquist and Bagnères, 2010). However, in the absence of complete structural analysis of each peak, and more importantly specifically targeted behavioural experiments, it is difficult to predict which of the CHC components have any role in *Mecopoda* communication. Nevertheless, 14 out of 25 peaks turned out to be methyl alkanes that are more likely to be communicating agents because of their 3D branched structures than simple alkanes that are more likely to act primarily as waterproof (Blomquist and Bagnères, 2010; Kather and Martin, 2012). It has been shown that CHCs are prone to change due to environment and the age and history of the animals under study (Kather and Martin, 2012; Maan and Seehausen, 2011). In this study, we have tried to control for these issues by random sampling over three years and sampling from as many locations as possible. RFA, which differentiates *Mecopoda* songtypes with a very high probability, failed to differentiate Chirper populations (41.5 % error in locality assignment), collected from 4 different localities. Similarly, Double Chirper, collected from 3 different localities could not be differentiated (51.5 % error in locality assignment). This suggests that the effect of environment in our study was less significant that the genetic differences among populations, unless there are environmental differences between the songtypes, which seems unlikely given their distribution and frequent sympatry among songtypes. Assuming that the identified CHCs play an important role in *Mecopoda* communication, the key issue in relation to this study is to test if the relative amounts of these CHCs can successfully differentiate the songtypes.

The CHC profiles of each *Mecopoda* songtypes were analysed using PCA on log transformed area under the peak data followed by a DFA on the principal components. Although using 5 principal components (explaining approximately 91.5 % variation within the sample) in DFA produced much higher success in songtype prediction (approximately 89.8 % correct assignment), it is difficult to represent this success in a single 2D graph. DFA based on first two principal components (explaining 77 % variation within the sample) from PCA formed three distinct groups of *Mecopoda* individuals: The first group only containing Train, the second group of overlapping Chirper and Double Chirper and a third group of overlapping Two Part and Helicopter. This grouping was achieved with approximately 75.9 % correct prediction of individual songtypes. While Helicopter has previously been misidentified as Two Part in the RFA of morphological characters [see chapter 2], the overlap of Chirper and Double Chirper in PCA as well as DFA is interesting. Both

Chirper and Double Chirper calls are devoid of a trill component and are based on chirps that are structurally different but have overlapping temporal parameters. These two songtypes do not have a recorded sympatric distribution in the study area [see table 1.1 chapter 1]. It is possible that the two groups have diverged in allopatry and would not be completely reproductively isolated from one another in the event of secondary contact.

The failure of standard multivariate analysis to distinguish songtypes properly based on CHCs is presumably the result of either, or a combination of the following:

1. The sample size used in the study is inadequate to produce robust separation.
2. The divergence in *Mecopoda* songtypes is sufficiently recent that CHC profiles have not changed sufficiently to produce substantial divergence in the songtypes.

However the DFA result from this study gives us glimpse of differentiation among songtypes that molecular genetics based on a single genetic marker and traditional multivariate analysis based on morphology could not achieve. It appears as though song divergence in *Mecopoda* is not equally matched by similar divergence in morphology, chemical profile or genetics. Among the limitations of the study listed above, only deficiency in sample size could be taken care of statistically by using RFA that use repeated subsampling of data and variables.

RFA used log transformed area under the peak data, unlike DFA that had to be based on PCA components due to the small sample size of Helicopter individuals. DFA on log transformed area under the peak data produced much lower success in predicting the songtypes of *Mecopoda* (only 78 % using 5 variables that had most weighting in PC1). Even in a two-dimensional representation, multidimensional scaling (MDS) of the proximity matrix derived from Random Forest based components was able to group the *Mecopoda* songtypes with much better resolution than the principal components from PCA. From the RFA, it was possible to separate out all five *Mecopoda* songtypes with 93 % success. There were few overlaps with RFA misidentifying a few Helicopters as Two Part and Train. This result is consistent with RFA on genital morphology and DFA on CHC where Helicopters seemed to overlap with Two Part. Given that Helicopter and Two Part

may not have diverged as much as some other pairs of songtypes, and can be identified only based on calls, it is interesting to note that both of them have trill component in their calls, occur in sympatry and have temporally overlapping mating seasons. It will be interesting to carry out behavioural studies on these two songtypes to identify the group affinities of the females belonging to these two songtypes. However, this result should be accepted with caution since sample size for Helicopter songtype was the lowest (n=14) and probably increasing the sample size may help RFA to separate out Two Part and Helicopter.

In conclusion, RFA based on 19 CHC peaks in 166 individuals was able to separate all the songtypes with relatively fewer overlaps. The failure of traditional multivariate analysis to separate out *Mecopoda* songtypes with high accuracy suggests that the differences between CHC profiles are likely to be subtle. The relative success of RFA to separate out most of the songtypes, on the other hand, confirms that *Mecopoda* songtypes may be a true species complex (as suggested in previous study by Nityananda and Balakrishnan (2006) that is primarily cryptic.

3.5.2 Does variation in cuticular composition among *Mecopoda* songtypes create barrier to gene flow?

It is difficult to ascertain the processes that have led to the current difference in chemical profiles in divergent populations that occur in sympatry. It is expected that both selection including sexual selection and drift have contributed but their relative importance cannot be determined without additional experimental studies. Sexual selection can independently lead to behavioural isolation if natural selection does not act as constraint; however, it is more likely to act in synergy with natural selection in natural populations. These five divergent songtypes could also be maintained by reinforcement if these populations have come to coexist as a result of secondary contact between divergent types with significant selection against hybrids. However, morphological evidence (Nityananda and Balakrishnan, 2006) suggests that this is an unlikely scenario since the populations lack divergence in morphology as much as would be expected of populations in allopatry following independent evolutionary trajectories in adapting to local environment.

Chemical isolation is a behavioural isolation where differences in the composition of chemicals produced by or found on one sex leads to reduced attraction between

members of the opposite sex from divergent groups (Coyne and Orr, 2004). Although the role of cuticular hydrocarbons (CHCs) as a chemotaxonomic tool to differentiate between species was proposed quite early (Jackson and Blomquist, 1976), a role for chemical communication between the sexes in Orthoptera has only been identified more recently (Tregenza and Wedell, 1997). Since CHCs can act as mating signals in close range encounters with members of the opposite sex (Maroja et al., 2014; Simmons et al., 2013), we can speculate that the subtle differences in the chemical profile of *Mecopoda* that we have observed could provide the basis for sexual selection in *Mecopoda* as speculated in other divergent systems (Martin et al., 2008; Mullen et al., 2007; Peterson et al., 2007). Antennae have been demonstrated to detect chemical cues from the cuticle in insects (Balakrishnan and Pollack, 1997; Blomquist and Bagnères, 2010), hence the antennation that occurs immediately prior to, and during mating could be used to determine the conspecific or population identity of mating partners. It is possible that females of the five songtypes exercise mate discrimination against individuals of divergent cuticular composition, analogous to the pattern observed in two distinct populations of *D. montana* (Veltsos et al., 2011) or two sibling species of *Drosophila* (Coyne, 1996), in two species of leaf beetles in hybrid zones (Peterson et al., 2007) or in closely related sympatric population of (Grula et al., 1980). This in turn may lead to chemical isolation among closely related *Mecopoda* songtypes and may represent a species identification character that can differentiate among the *Mecopoda* songtypes.

Chapter 4

Behavioural isolation in acoustically divergent groups of *Mecopoda*

4.1 Abstract

Reproductive isolation between populations of same species, as indicated by diversification of a parent population into subpopulations, is a necessary precondition for speciation. This study explores reproductive isolation among overlapping *Mecopoda elongata* populations in south India that can be differentiated into five distinct songtypes. Calls in orthopteran insects are mostly used as sexual signals emitted by males to attract conspecific females from a distance. Divergence of these species-specific signals is likely to be associated with reproductive isolation. The divergence of calls in *Mecopoda* songtypes has created groups with several non-overlapping parameters. A direct test for potential reproductive isolation among *Mecopoda* songtypes would be phonotactic experiments investigating the relative preference of females for a call that is representative of each songtypes towards the call of other songtypes. I describe such a study in which I examined the response of females of one songtype (Chirper) to a single stereotypical male call from each of the 5 songtypes. This design relies on the assumption that differences in female preference for the stereotypical song reflect difference in preference for the range of songs within each songtype. My study shows that females

exercise preference over songtypes. However, there were instances when a female of the Chirper songtype showed phonotaxis to that of Double Chirper songtype (the temporal features of calls of these two songtypes overlap, with Double Chirper call rate being faster than Chirper) while showing complete non-response to the remaining songtypes. This indicates incomplete isolation between the two songtypes. To examine the features of the song that trigger phonotaxis in Chirper females I recorded their response to a range of Chirper calls manipulated to vary chirp rate that also included the rate typical of the Double Chirper songtype. Female responses were strongest to their own chirp rate as would be expected under stabilising selection. Therefore, their 'wrong' choices could not be attributed to the preference of higher chirp rate over that of the lower. The strong specificity of Chirper females for their own songtype provides evidence for behavioural isolation among songtypes resulting from female preference although based on the assumption that the playback calls were representative of the songtype population. This result was supported by a no choice mating experiment between Chirper and Double Chirper songtypes where too Chirper females showed preference to mate with their own songtype than with the Double Chirper males. However, a possibility remains for Chirper females to mate with Double Chirper males on rare occasions and it would be interesting to study if such mating result in any progeny. Behavioural isolation through female preference has always been recognised to play a major role in speciation of sympatric and divergent population as in the case of *Mecopoda*.

4.2 Introduction

From being a relatively peripheral aspect of research efforts, speciation research has made recent advances by focusing on the evolution of reproductive isolating barriers (Coyne and Orr, 2004). Mayr (1963) considered behavioural isolation as the most important isolating mechanism in animals with overlapping ranges. Here I examine maintenance of this isolating barrier in the songtypes of *Mecopoda* species.

Those differences in behaviour between species that lead to reductions in the frequency of mating between heterospecific individuals are considered as behavioural isolating mechanisms (Coyne and Orr, 2004). These mechanisms can

arise by selection (including sexual selection), genetic drift or random accumulation of mutations over long periods of time, but can only be detected in the wild in divergent sympatric populations, which overlap in breeding time but do not or rarely mate when they encounter each other (Coyne and Orr, 2004).

Studies that attribute behavioural isolation to the initiation of speciation in sympatric populations are very few (Boul et al., 2007; Coyne and Orr, 1989, 1997; Seehausen et al., 1997). Nevertheless, behavioural isolation is expected to play an important role in maintaining sympatric species (Coyne and Orr, 2004), at least once the divergent populations have been established. Examples of evidence for such a role have been identified in cryptic orthopteran species that are closely related and often found in sympatry (Honda-Sumi, 2005; Schul et al., 1998).

Cryptic species (two or more distinct species classified as a single species) are morphologically very difficult to distinguish and generally overlap in their distribution, which means they share similar habitats (Bickford et al., 2007). My study system is a potential cryptic species complex of tettigoniids (Order Orthoptera, Family Tettigoniidae) documented by Nityananda and Balakrishnan (2006) consisting of 5 different songtypes. The complex belongs to the *Mecopoda* genus found in India. They are morphologically very similar (Nityananda and Balakrishnan, 2006) and populations of these songtypes co-occur in various sympatric combinations (Nityananda and Balakrishnan, 2006) within a relatively small geographical area. The songtypes differ in their temporal features and range from simple chirps to complex calls involving both chirps and trills (Nityananda and Balakrishnan, 2006) [see figure 4.1].

The populations of these *Mecopoda* call types may have come to exist via one of a number of different processes. Firstly, they may have evolved allopatrically, diverged under natural selection and have come in contact after the geographical barriers ceased to exist. Secondly, it has been rare to find strong evidence for the role of genetic drift in speciation (Coyne and Orr, 2004) but nevertheless, random genetic drift, and gradual accumulation of differences in allopatry could have led to the establishment of these five *Mecopoda* songtypes, which, again, have come into contact subsequent to their divergence. Distinguishing among these two possible causes of divergence in allopatry followed by secondary contact is beyond the scope of this study, but if either mechanism is responsible, it is quite extraordinary to find so much morphological similarity among the songtypes such that no diagnostic character was able to distinguish them from each other (Nityananda and Balakrishnan, 2006). Both processes raise the possibility that reinforcement

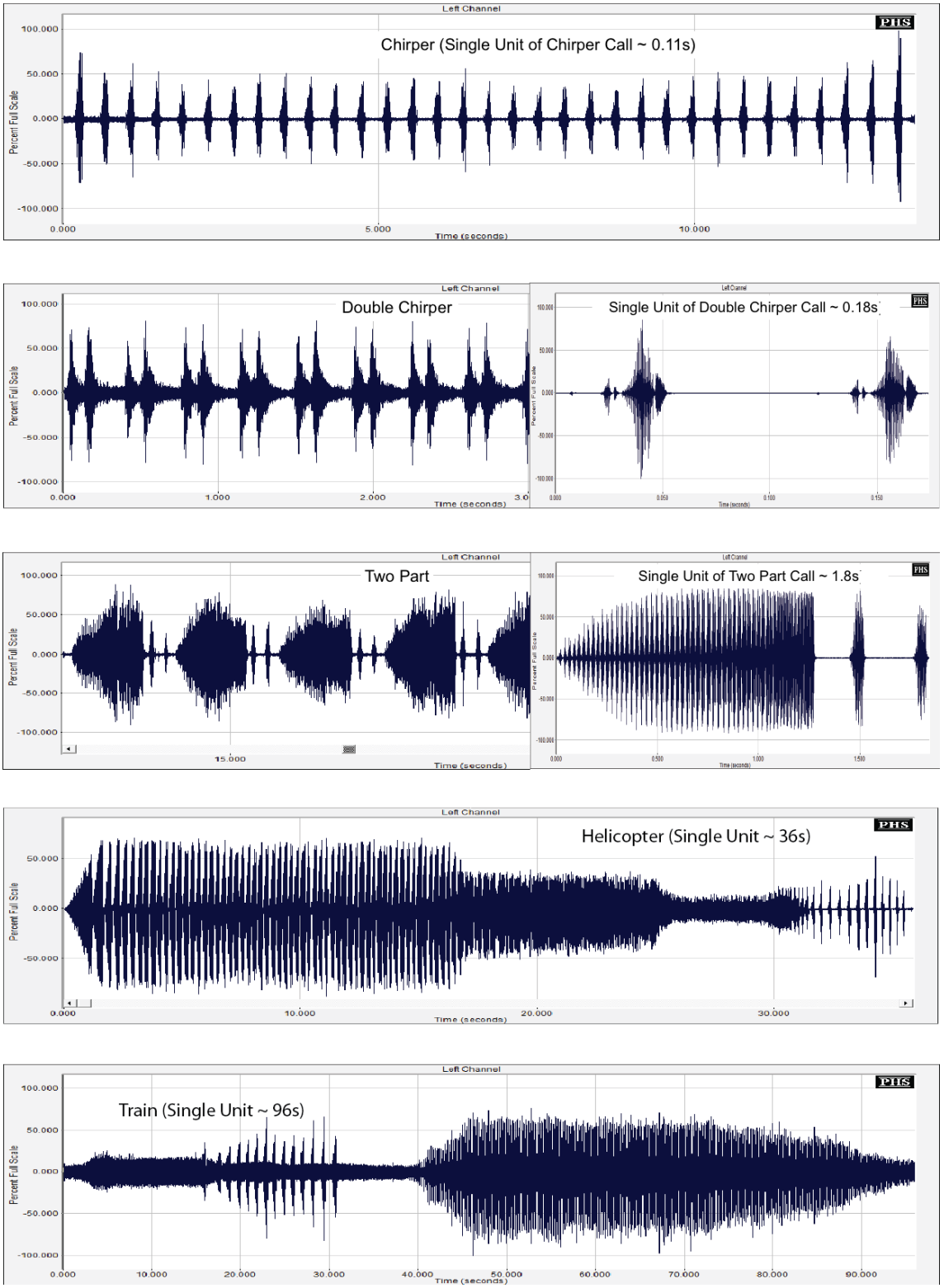


Figure 4.1: Representative oscillograms of five *Mecopoda elongata* songtypes found in south India (x-axis represents time in seconds and y-axis represents relative amplitude). The songtype to which each oscillogram belongs and the mean call duration of a single unit of each call are mentioned within each oscillogram

may have an important role to play in maintaining the isolating barriers but there is no evidence of hybrid formation in the wild (Nityananda and Balakrishnan, 2006).

Another way to look at the existence of *Mecopoda* songtypes is that they are not species in the sense that they cannot interbreed, or have distinct ecological niches, but rather that their separation is being maintained by behavioural isolation. Many ensiferans have a unique species-specific call produced only by males (Alexander, 1960, 1967; Heller, 2005; Honda-Sumi, 2005; Hoy and Paul, 1973) except for a few tettigoniid species where females also call in response to the male calls (Bailey and Rentz, 1990; Derlink et al., 2014). The calls of *Mecopoda* play an important role in their reproductive biology as they serve the purpose of allowing females to locate, and potentially to choose among potential mates (Fertschai et al., 2007). Acoustic signal divergence as simple as a change in pulse rate has been shown to have the potential to create behavioural isolation in other Orthopterans (Gray and Cade, 2000; Shaw and Herlihy, 2000). This kind of divergence has been attributed to sexual selection (Mendelson and Shaw, 2005; Wilkins et al., 2013).

Behavioural isolation may exist at different stages of the reproductive behaviour of *Mecopoda* but in the present study, behavioural isolation resulting from the production and response of acoustic cues is the focus due to the existence of acoustically divergent populations. Behavioural isolation based on acoustic signals has been studied in insects including lacewings (Wells and Henry, 1992), bushcrickets and crickets (Gray and Cade, 2000; Hoy and Paul, 1973; Maroja et al., 2014; Schul et al., 1998), frogs (Boul et al., 2007; Ryan and Rand, 1993), and birds (Irwin et al., 2001). Many of these studies speculate that sexual selection plays a role in the maintenance of behavioural isolation. However, in only very few, such as the definitive study of (Wiernasz, 1989; Wiernasz and Kingsolver, 1992) has the role of mate choice in maintaining behavioural isolation been positively verified.

Wiernasz (1989) identified that in the Peach Valley population of the butterfly *Pieris occidentalis*, females choose males based on the relative melanisation in the margin of the dorsal forewing. In the same study, females from the Alkali Flats population of *Pieris occidentalis* showed 82 % acceptance of control males against a mere 66 % acceptance of males whose marginal dorsal melanin pattern had been whitened. This suggests existence of female mate choice in the populations of *Pieris occidentalis* for the darker pattern of the males' forewing over those with the lighter pattern. Wiernasz (1989) also suggests that melanisation may form a basis for species recognition in *Pieris occidentalis* due to the fact that P. protodice

with lighter melanisation of the male dorsal forewing than *Pieris occidentalis* occur in sympatry much through its distribution. The follow-up study (Wiernasz and Kingsolver, 1992) showed that the dorsal forewing of *Pieris protodice* that was darkened to resemble *Pieris occidentalis* significantly increased the number of heterospecific matings by females of *Pieris occidentalis*. Darkened *Pieris protodice* not only had significantly greater mating success with *Pieris occidentalis* females than control *Pieris protodice*, they also did not differ significantly in terms of mating success from control *Pieris occidentalis* males. Thus, these studies indicate that what arose as female choice based on a darker melanisation in the forewings of male wing pattern within the population of *Pieris occidentalis* might have led to current existence of behavioural isolation between the species of *P. occidentalis* and *P. protodice*, which are sympatric.

No matter by what processes *Mecopoda* songtypes may have come to exist, there is clearly the potential that they are able to coexist as distinct types through behavioural isolation since they are in sympatry and still form distinct groups, at least based on their calls. Usually in closely related species of bush crickets, small changes on a basic signal pattern lead to species discrimination (Heller, 1990). In the case of *Mecopoda*, the call structure of each songtype [see figure 4.1] is drastically different from the others (Nityananda and Balakrishnan, 2006). The males of *Mecopoda* produce these calls to attract females from a distance whereas the females completely lack the ability to call. Females approach calling males and after antennation and brief calling by the male (this call does not appear to be different from the species specific call), the female mates with the male.

All *Mecopoda* populations are annual, with eggs surviving the non-breeding period and contributing to the adult population of the next year. The mating season of the different songtypes overlaps temporally as does their geographical distribution (Nityananda and Balakrishnan, 2006). The existence of five distinct songtypes, in partial sympatry with no reports of intermediate songtypes, suggests that there are five reproductively isolated populations in which females of each songtype choose to mate with males of the same songtype, rejecting males of other co-occurring songtypes. This also suggests that there may have been a co-evolution of the attracting male trait (here, male call) and corresponding female preference for the male trait (Deily and Schul, 2008; Endler, 1992; Schul, 1998). Phonotactic experiments with pure-bred females of these songtypes may show us the existence of behavioural isolation within the *Mecopoda* species in terms of acoustic signals.

If isolation in *Mecopoda* songtype populations is maintained by female choice based on acoustic cues, we expect that a female of one songtype will prefer (show a tendency to move towards) the call of its own type while ignoring (show no response) calls of other songtypes. Such a pattern would indicate the likelihood of strong reproductive isolation among songtypes. However, if a female responds to more than one call type, the reproductive isolation in terms of female choice is unlikely to be complete. Since Mecopoda songtypes differ in temporal features of their song while their spectral profiles are similar any, barrier to reproduction may lie among the temporal features of the songtypes as is common in many closely related species that use temporal features of their call for conspecific call recognition (Bush et al., 2009; Deily and Schul, 2008; Derlink et al., 2014; Schul, 1998). In this study I performed phonotaxis experiments to examine whether *Mecopoda* Chirper females preferred the calls of males of their own songtype over those of other *Mecopoda* songtypes.

If *Mecopoda* females show occasional preference for a different songtype, it is interesting to further examine the reason for this. The experiments to address this problem should involve females presented with different songtype calls with some same songtype call parameters and same songtype calls with some different songtype parameters. Before these experiments, it is also important to check the response of Chirper females to a range of chirp rates of its own call to determine whether the mean chirp rate of Chirper calls is under stabilizing selection. These experiments will give us an idea of which song trait is contributing to mate preference in female *Mecopoda* and thus, behavioural isolation in the acoustically divergent groups of *Mecopoda* species. Mating experiments, on the other hand, will be essential to further determine the strength of behavioural isolation that exists within the *Mecopoda* species.

The objectives of the study are as follows:

1. To test the existence of behavioural isolation between *Mecopoda* Chirper and other songtypes based on acoustic signal playback.
2. To test whether chirp rate in *Mecopoda* Chirper males is under stabilizing or directional selection from Chirper females.
3. In case behavioural isolation is not complete between Chirper and any other songtypes, the study aims to test if chirp rate or another aspect of call structure acts as a determinant of phonotactic behaviour.

4. To examine behavioural isolation at the level of mating between *Mecopoda* songtypes.

4.3 Materials and methods

4.3.1 Animal collection

Among the five songtypes of *Mecopoda elongata* L. (Orthoptera, Tettigoniidae, Mecopodinae) found in India, namely, Chirper, Double Chirper, Two Part, Helicopter and Train (Nityananda and Balakrishnan, 2006), the Chirper population in the campus of Indian Institute of Science, Bangalore was found to be occurring in allopatry with respect to the other four songtypes, with no other songtype known from any location within 5 km of this population during the period of this study. A pure bred population of Chirper songtype was successfully established in culture for the first time in 2011 from the 2010 collection of 47 Chirper females of unknown mating status that were collected randomly from the large population found on campus. Around five hundred nymphs from eggs distributed in six separate cages from these Chirper females were raised in together until the nymphs moulted to adult form. The number of successful rearings was low with only 48 adult Chirper including 32 females surviving to adulthood. In year of 2011, these 32 laboratory bred females were mated with 24 wild caught males en masse to continue the culture in subsequent year of 2012. Out of around 600 nymphs collected in 2012, we could only raise 28 Chirper adults including 15 females giving us an even poorer yield than previous year. These 15 Chirper lab bred females were again mated with wild caught males from the campus. These 15 lab bred females from 2012 produced 25 lab bred females out of total 33 Chirper individuals in 2013. Laboratory bred females were mostly used for the experiments as pilot phonotactic experiments with wild caught adult Chirper females of unknown mating status produced negligible phonotactic response. Because of the limited availability of lab reared females some Chirper females used in the experiment were caught as nymphs from the campus population and raised to adulthood in culture the largest number being in 2013 when 18 such females were used in the experiments. Pure bred cultures of other songtypes could not be established, as

the identity of females (that do not call) of other songtypes could not be ascertained due to their sympatric distribution with other songtypes and overlap in the mating seasons of several types.

Nymphs were fed ad libitum on oat flakes (Quaker Oats, Morten Seeds & Grains Pty. Ltd.), fish food (Taiyo Grow, Taiyo Petproducts [P] Ltd.) and dog food (Pedigree, Mars International India Pvt. Ltd.) while calcium (Calcium Sandoz, Novartis India Limited) and cabbage leaves were given as supplements. The adults were fed on fish food and oat flakes offered ad libitum. Water was also provided ad libitum. The culture room was maintained between 24˚C to 30˚C corresponding to the range of temperatures in their natural habitats. All the females were maintained singly after they moulted into adults. They had no physical contact with any male before or during the experimental period. Adult females were used in the experiments after 2 weeks from the day of last moult. The females used for experiments were acoustically separated on the experimental day. They were placed within an anechoic room (3.1m x 2.7m x 3.1m) one hour before the commencement of experiments.

4.3.2 Playback

The five songtypes of *Mecopoda* differ in temporal features to such a degree that the distribution of variation in temporal parameters within songtypes does not overlap among songtypes. This difference in temporal parameters among *Mecopoda* songtypes make them quite discrete in nature such that it is impossible to misidentify each songtype. For playback, a basic unit of a call recorded from a single representative individual of each songtype [see figure 4.1] was so chosen such that their magnitude approximated the mean of their distribution in natural populations (Nityananda and Balakrishnan, 2006). Here, we assume that the chosen segment of an individual call is representative of all the individuals of that songtype. This design does have the weakness that if there is some unknown characteristic of the song of the representative individual that we chose which we are not aware of, which is not representative of the song-type as a whole, then any conclusions about the songtype as a whole will be erroneous. However, given the stereotypical nature of orthopteran calls and the physical constraints on the system both in terms of signals and receptors this seems very unlikely. The chirp duration and chirp period of representative playback call segment of each songtype used in the acoustic playback are shown in the table 4.1.

Songtype	Call Duration	Call Period
Chirper	115 ms	0.48 s
Double Chirper	174 ms	0.38 s
Two Part	1.8 s	1.9 s
Helicopter	36 s	36 s
Train	96 s	96 s

Table 4.1: Call duration of the selected call segments for playback and the mean call period of each *Mecopoda* songtypes that were used in the playback during phonotactic experiments

The segments of calls used were collected from calls recorded previously using a Bruel and Kjaer Sound Level Meter 2231 mounted with a quarter-inch microphone (Bruel and Kjaer 4939, frequency range 4 Hz - 70 kHz) (Nityananda and Balakrishnan, 2006). The analogue signals were digitised at a sampling rate of 200 kHz using a NI-DAQ AT-MIO-16E-2 card (National Instruments) and Labview version 6.0 (Nityananda and Balakrishnan, 2006). The temperature at which all the recordings were carried out ranged from 22.9˚C to 27.7˚C (Nityananda and Balakrishnan, 2006). The mean instantaneous SPL of *Mecopoda* Chirper call measured from 29 individuals at 30 cm was 91.5 $\pm$ 5.5 dB (re 2 x 10^{-5} N/m^2). Attenuation following the power law (Y = 13.2 x $X^{0.277}$, r^2=0.56) at 1.7 m is approximately 15.3 dB (Nityananda and Balakrishnan, 2008). Therefore, the SPL for calls to be used in the playback at a distance of 1.7 m is 76.2 dB (peak). The background SPL (in the absence of playback) and temperature were noted down in each experiment. The segments of the five songs were looped in MATLAB (The Mathworks Inc., Natick, MA) to make a 10-minute call and played back from a laptop (MacBook Pro with OS X 10.9.4). Calls were played via an Avisoft power amplifier and an USB-sound card (NI USB-6215, National Instruments) using Avisoft speakers (Ultra-sound Scanspeak, frequency response: 1-120 kHz). The mean background RMS sound pressure level was 52.3 dB $\pm$ 0.6 (Mean $\pm$ S.E.M).

For many orthopterans, call parameters have been shown to vary with ambient temperature. In case of *Mecopoda*, however, the chirp period from 26 recordings of 6 Chirper individuals did not vary significantly with temperature in a regression analysis (p = 0.182, r^2 = 0.0731) between chirp period and ambient temperature [see figure 4.2, adapted from unpublished report (Agnihotri, 2007)]. Thus, change in temperature within and across nights is not likely to significantly affect the call parameters of *Mecopoda* males in a systematic fashion under natural conditions.

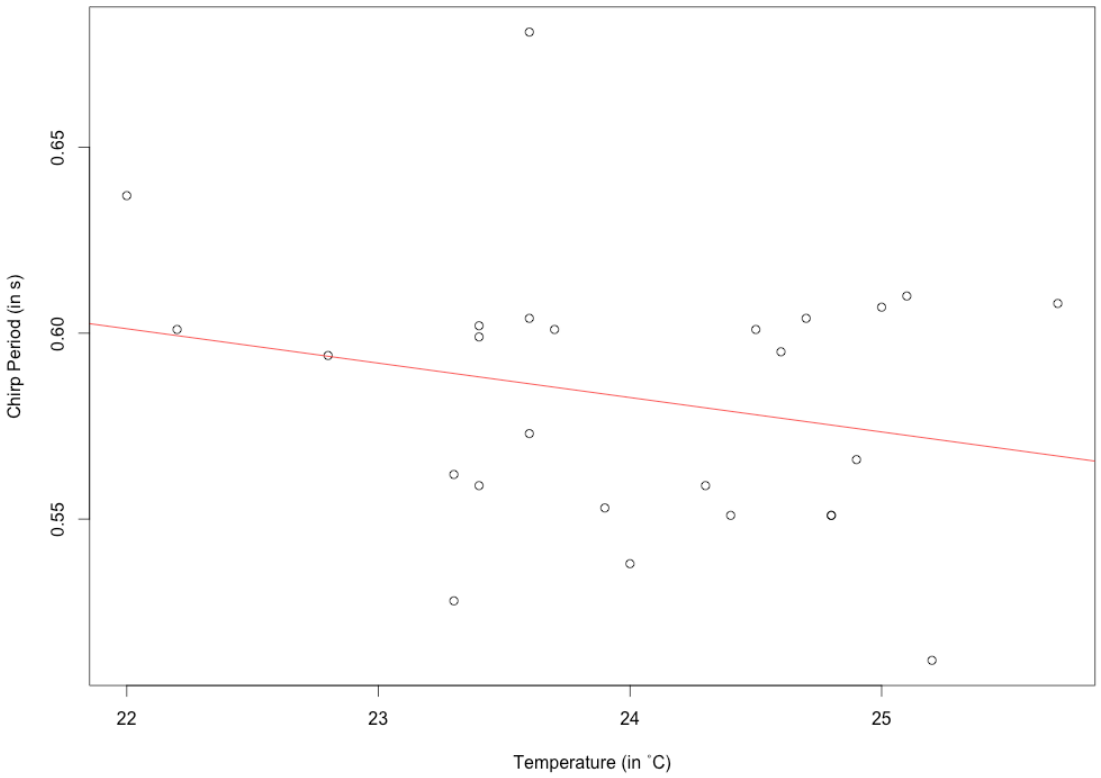


Figure 4.2: Regression analysis (r^2 value = 0.0731) between *Mecopoda* Chirper chirp period (represented in y-axis) and the ambient temperature (represented in x-axis) at which the calls were recorded

4.3.3 Experimental procedures

4.3.3.1 Phonotaxis experiments

Call preference of Chirper females

The main aim of this experiment was to study the phonotactic behaviour of Chirper females towards playback of different *Mecopoda* songtypes to establish whether behavioural isolation exists between the *Mecopoda* Chirper songtype and the rest of the songtypes. Since the number of females was limited, a repeated measures design was followed. A total of 30 females were used: 21 out of 30 females were from the laboratory bred population of the 2011 season, raised in the culture room from the eggs collected the previous year. The other 9 females were collected as nymphs from the wild in 2011 but raised to adults in the laboratory.

All the phonotaxis experiments were completed within 5 hours of the onset of the dark cycle. The trials were run in an anechoic arena that was 2 m x 2 m in area.

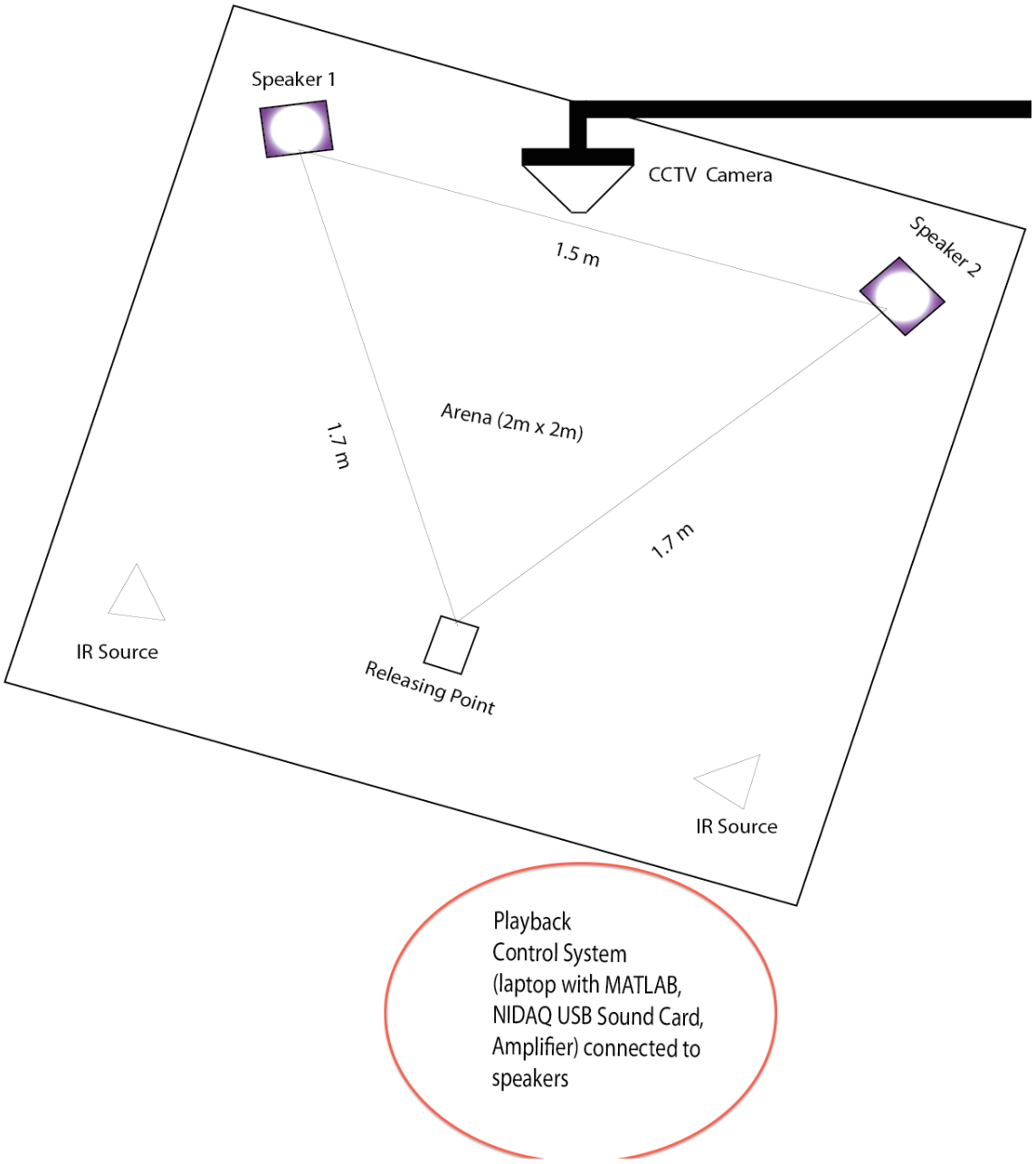


Figure 4.3: Experimental set up used for phonotactic experiments to study call preference behaviour of Chirper females

The two speakers were placed 150 cm from each other. The release point of the females was 170 cm from the speakers, the speakers and the release point forming an isosceles triangle [see figure 4.3]. Playback was carried out from a single speaker during each trial. Each trial was recorded and monitored with an infrared sensitive CCTV camera mounted above. The mean temperature throughout the experimental period was 23.6˚C $\pm$ 0.5˚C.

Each Chirper female was tested over four days for response to Double Chirper,

Two Part, Helicopter and Train calls with one stimulus trial per day. Every female used in the experiment was also tested with Chirper calls (positive control) on each day. This was done to check for motivation of the females over different days as motivation varies among days as well as individuals. All trials were carried out between the 18th and 34th day of female adulthood. The order of presentation of animals (in the case of more than one female being tested on a day) and the direction of playback were randomized within each night. The playback order for each female was randomized over four nights. The total experimental time in a day was divided into two halves. In one half, one of the four other songtypes was played to the females. In the other half, the same songtype (Chirper songs in this case) call was played to the same female as the positive control. These halves were alternated over four days. If the female did not show any response to any call in a given experiment, the female was retested on a different night. All playbacks were of 10 minutes duration with 5 minutes for acclimatization given to the animals before each trial. During acclimatization, the female was held at the release point in the arena in a mesh cage (15 cm x 10 cm x 3 cm) that had the bottom side (15 cm x 10 cm) open. After 5 minutes, playback began and the cage was removed (if females were free) or turned upside down (if females clung onto the mesh). The exact time for phonotactic response (from release from mesh cage to reaching within 10 cm of the speaker) was recorded. Only if a female showed response to the positive control was its phonotactic response used in the analysis. If a female responded to the positive control but not any other songtype, then the test was considered negative for the other songtype, and if she responded to the positive control and the other songtype then her response was considered positive for the other songtype.

Same songtype chirp rate preference by Chirper females

This experiment was conducted to study Chirper females' response to extreme chirp rates with respect to the mean chirp rate of its own call and thus, to examine whether Chirper females are tuned specifically to the chirp rate of their own call. A repeated measures design was followed. Overall 39 Chirper females were used in this experiment which was conducted in the 2013 season and included 3 wild caught adult females from the campus population, 18 pure laboratory bred adult females and 18 females which were collected as nymphs from the wild but raised to adults in the laboratory. A similar experimental set up [see figure 4.3] was used as in the study of call preference behaviour of Chirper females but with a

slight modification: only one speaker, instead of two, was used and it was placed opposite to the release point along a straight line. In this experiment, each Chirper female was tested over three different trials involving playback of mean (480 ms) chirp period of Chirper calls, mean + 2 S.D. (755 ms) chirp period of Chirper calls and mean – 2 S.D. (210 ms) chirp period of Chirper calls over three consecutive days. The order of presentation of females (in case more than one female was tested on a given night) was randomised within a night. The playback order for each female was chosen randomly over 3 nights, as was the direction of playback from among east, west, south and north. At least 5 minutes was given to the females for acclimatization in the arena before each trial. Only if a female reached within 10 cm of the speaker was it scored as a positive phonotactic response.

Call structure preference by Chirper females when played at Chirper chirp rate

This experiment examined whether Chirper females show any significant phonotactic preference between same songtype and different songtype calls when both were played back with Chirper females' preferred chirp rate (as found in the previous experiment). This allows us to test the hypothesis that Chirper females' preference is based only on the chirp-rate of the males' call, and not to the other call features. A similar repeated measures design was followed, as the numbers of females were limited to the same 39 individuals from the 2013 season used in previous experiment. The animals used in the previous experiment were used again in this experiment. The experimental set up and the files and procedure used for playbacks were same as in the previous experiment. This experiment consisted of two trials that were conducted within the same night. Where more than one female was tested on a given night, the order of presentation of females was randomised within nights. The trials involved Double Chirper calls played at mean Chirper chirp period (480 ms) and Chirper calls played back at mean Chirper chirp period. The playback directions were randomised for all females in different trials. The order of playback was alternated such that the number of trials with Double Chirper playback conducted first was approximately equal to number of trials with Chirper playback performed first. For acclimatization, the animals were put in the same mesh cage and given at least 5 minutes on the arena before each trial. Only if a female reached within 10 cm of the speaker was it scored as a positive phonotactic response.

Call structure preference by Chirper females when played at Double Chirper doublet rate

This experiment was conducted to test whether Chirper females' preference for same songtype call structure (Chirper-like call structure) over different songtype call structure (non Chirper-like call structure) could still be detected when Chirper calls were played at a faster chirp rate equivalent to the chirp rate of the Double Chirper type. The same 2013 set of 39 Chirper females was used in a repeated measures design and it also consisted of two trials for each Chirper female. The experimental set up and the experimental design were identical to the previous experiment. In this experiment, Chirper calls were played at Double Chirper doublet rate (380 ms) in one trial whereas Double Chirper calls were played at Double Chirper doublet rate (380 ms) in the second trial.

4.3.3.2 Mating experiment

The mating experiment was carried out in a no-choice paradigm since the probability of encounter in the wild is unknown. Although this does not allow us to estimate the mating rate in the wild, it allows us to examine whether there are barriers to mating between Chirper and Double Chirper songtypes that occur after phonotaxis. A plastic box 23 cm x 13.5 cm x 10 cm with an open side (23 cm x 13.5 cm) covered with a transparent plastic sheet was used for the mating experiment. The male and female of a *Mecopoda* mating pair was introduced one by one inside the box before start of the experiment. The events within the box were recorded on video for at least 1 hour for experiments involving Chirper only and 2 hours for experiments involving Double Chirper or until the spermatophore was dropped in both cases. The mating experiments were conducted for a longer time in case of different songtype matings with an assumption that the premating duration may be longer since Chirper females might not readily mate with a Double Chirper male. All the females used in the trials were unmated adults that had been bred in the laboratory from eggs to adult except for one (used in the Chirper only mating trial) that was collected as nymph in the campus and subsequently raised to adulthood. Males were chosen based on their status of calling on the experimental day before the commencement of the trials since calling serves as indication of males' eagerness to mate. An encounter (any physical contact) between a female and a male *Mecopoda* was ensured when both were put in the box for mating trials. This

was considered the beginning of the trial. Another male replaced the males that did not call within 30 minutes after the female was introduced to the box. Females were scored as mated after a successful transfer of a spermatophore, visible at the base of the ovipositor of the females after mating. If the female did not mate, it was tried again on another night with a different male. If it failed to mate the second time, it was tried one last time for experiments involving Chirper males and females. For experiments involving Double Chirper males, females were tested with a Chirper male as a positive control on the third trial. The premating duration (duration from first encounter to the start of spermatophore transfer) and mating duration (duration taken by a male to place spermatophore onto to the base of the ovipositor) of each mating event was recorded.

4.3.4 Statistical analysis

All statistical analyses were done in R (R Core Team, 2014). Various statistical tests (χ-square test, Cochran Q-test, McNemar's test and t-test) were used depending on the nature of the dependent variables used to compare two or more explanatory variables. Pearson's χ-square test with Yates' continuity correction was used for examining the response of the Chirper females to two different calls. To compare the frequency of mating of Chirper females with different-songtype and same-songtype male a special method was followed due to small sample size, where Monte Carlo simulation of p value by reiteration was done to get more robust p value. When the data points were not independent of each other due to repeated measures design, McNemar's test with continuity correction was used to compare two explanatory variables.When there were more than two non-independent explanatory variables due to repeated measures design, Cochran's Q test was used. The means of the phonotactic response time between two explanatory variables were compared using Welch Two Sample t-test when there were two levels in the data and ANOVA when there were three levels in data. Each experiment measures the number of Chirper females' that respond to the treatment. The 95% confidence interval for the proportions derived from these responses in each experiment was calculated by Wilson's interval calculation method as prescribed for small sample size (Brown et al., 2001).

4.4 Results

4.4.1 Results of phonotactic experiments

4.4.1.1 Call preference of Chirper females

Out of a total of 30 Chirper females used in the experiment, 21 females were used in the further analysis as these females showed phonotactic response in all their 4 trials, each of which also included responses to the positive control. Among the 9 Chirper females that were left out of the analysis, 5 females showed phonotactic response for less than 4 trials. The other four females did not show any response in any trials. Out of the 21 females that were included in the analysis, 16 females were bred in the culture room from egg to the adult stage. The other 5 females were caught in the wild as nymphs and raised as adults in the same culture room. The frequency distribution of the age of the 21 females is shown in figure 4.4. The age of the females with respect to each of the four playbacks is shown in figure 4.5.

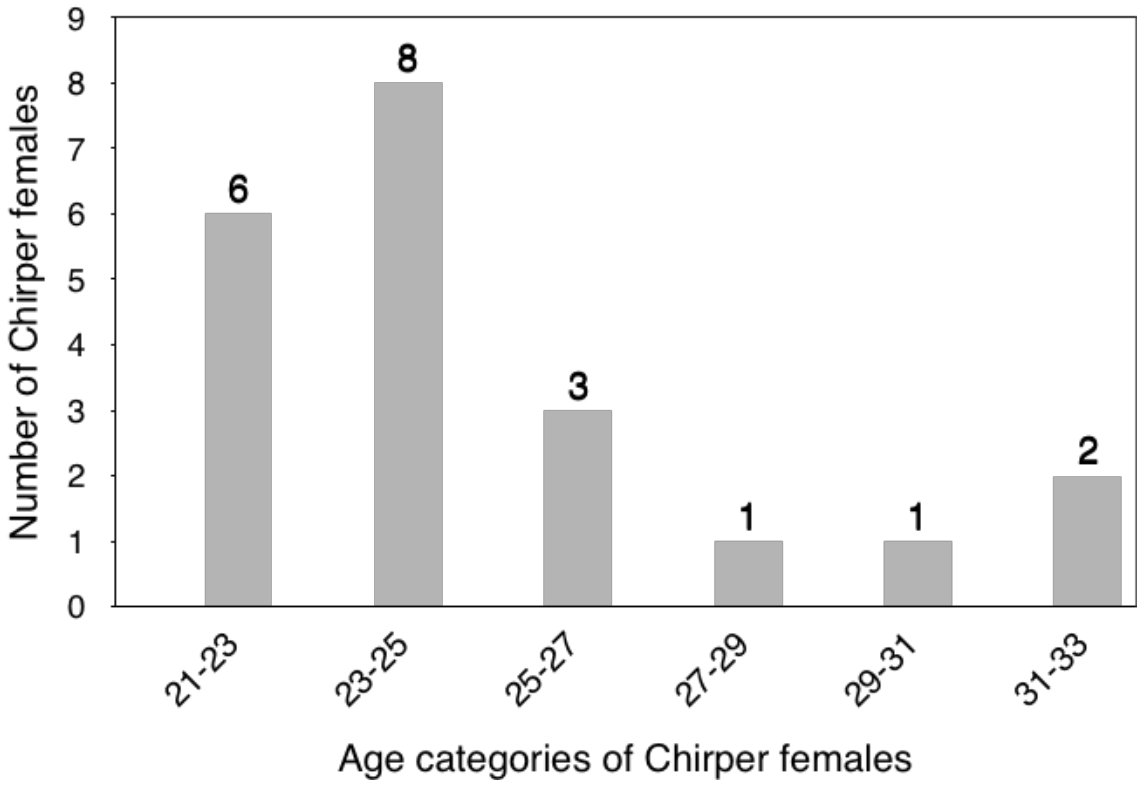


Figure 4.4: Frequency distribution of the age of the Chirper females used in the phonotactic experiments to study call preference of *Mecopoda* Chirper females

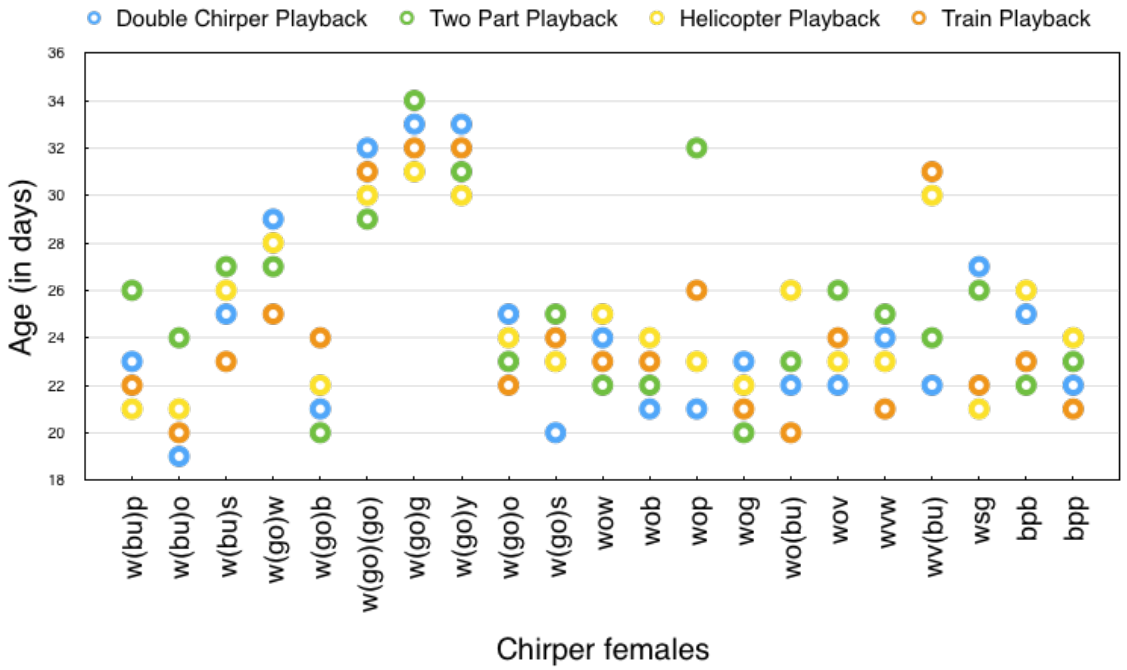
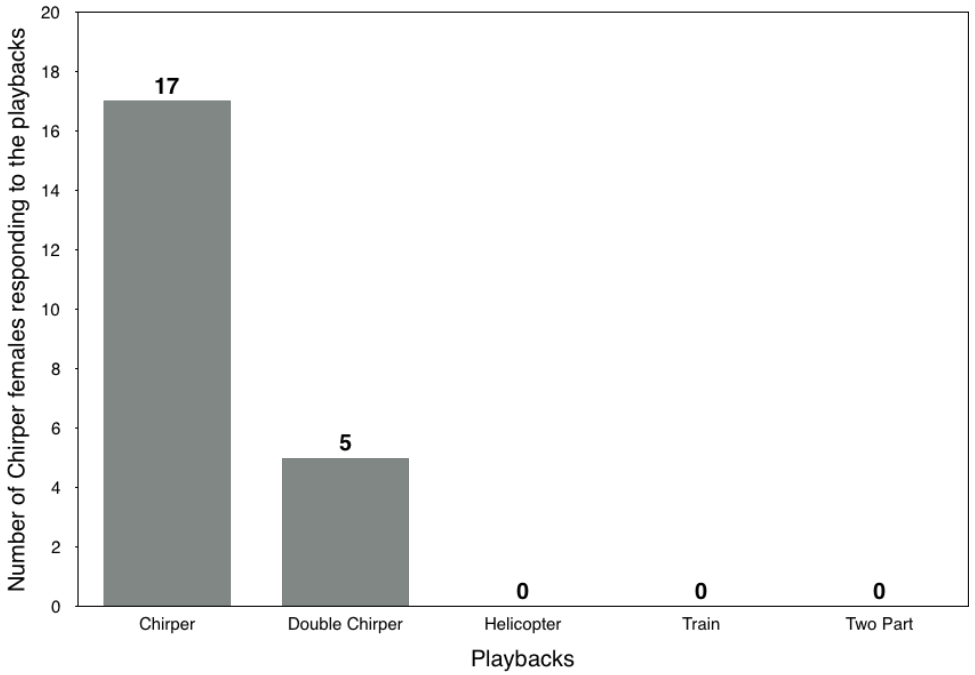


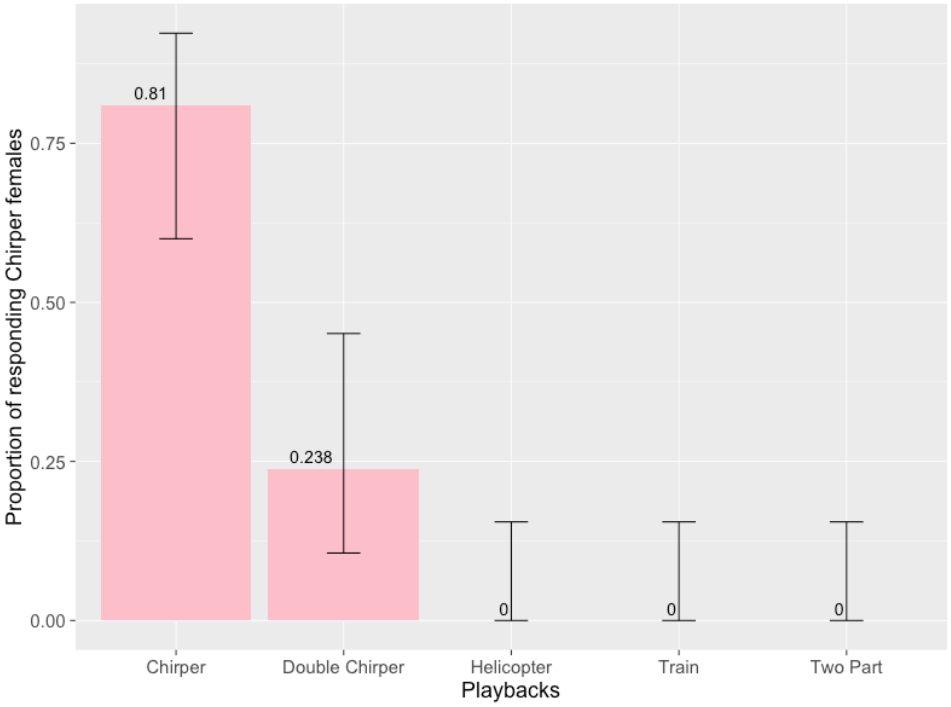
Figure 4.5: Age of each *Mecopoda* Chirper females (uniquely coded) at the time of each playback trial to study their preference for playback of other 4 songtypes

All the 21 females showed a positive phonotactic response to Chirper calls (positive control) on all the trials. Five Chirper females also responded to Double Chirper calls [see figure 4.6a]. None of the females showed any phonotactic response to any of the trilling songtypes (2-Part, Helicopter and Train) in any trial. However, due to use of Chirper calls as a control in the experimental design, the number of trials in which Chirper females were subjected to Chirper calls was four times the number of trials that Chirper females were subjected to Double Chirper calls. In order to make a statistical comparison of the two groups we used a resampling statistical approach using R. Our routine sampled 21 responses randomly out of all the trials that involved 21 Chirper females subjected to Chirper calls (including no responses and repeats). This process was reiterated 1000 times. Each time 21 randomly picked responses were generated and number of positive responses to Chirper call counted. The mean of these positive responses generated 1000 times was 17, a figure which was used in the following analysis as the true response of Chirper females towards Chirper calls [see figure 4.6a]. The 95 % confidence interval estimate of the proportional Chirper females' responses to the different playbacks are shown in figure 4.6b.

A χ-square test showed a significant (χ-square = 11.6, df = 1, p-value = 0.00068) difference between response to Chirper calls and response to Double Chirper calls. The mean response time of Chirper females towards Chirper calls was 144



(a) Phonotactic response by *Mecopoda* Chirper females to the playback of calls from all *Mecopoda* songtypes (including Chirper calls)



(b) Proportion of *Mecopoda* Chirper females responding to the playbacks of all *Mecopoda* songtypes with 95% confidence intervals

Figure 4.6: Response of *Mecopoda* Chirper females in the phonotactic experiment to study their call preference

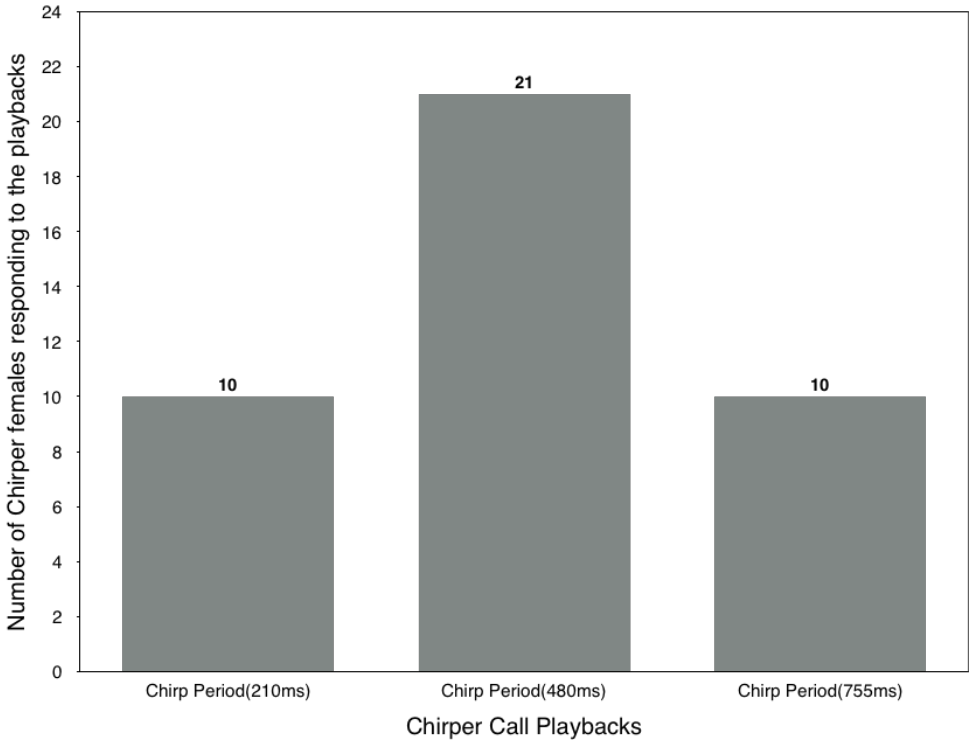
s ± 12.7 s (mean ± S.E.M). The mean response time for the 5 events when a Chirper female showed phonotaxis to the Double Chirper call was 146 s ± 48.2 s (mean ± S.E.M). A t-test (t = 0.0524, df = 4.57, p-value = 0.96) indicated that there were no significant differences between the means of the phonotactic response time of Chirper females towards Chirper call and Double Chirper call.

4.4.1.2 Same songtype chirp rate preference of Chirper females

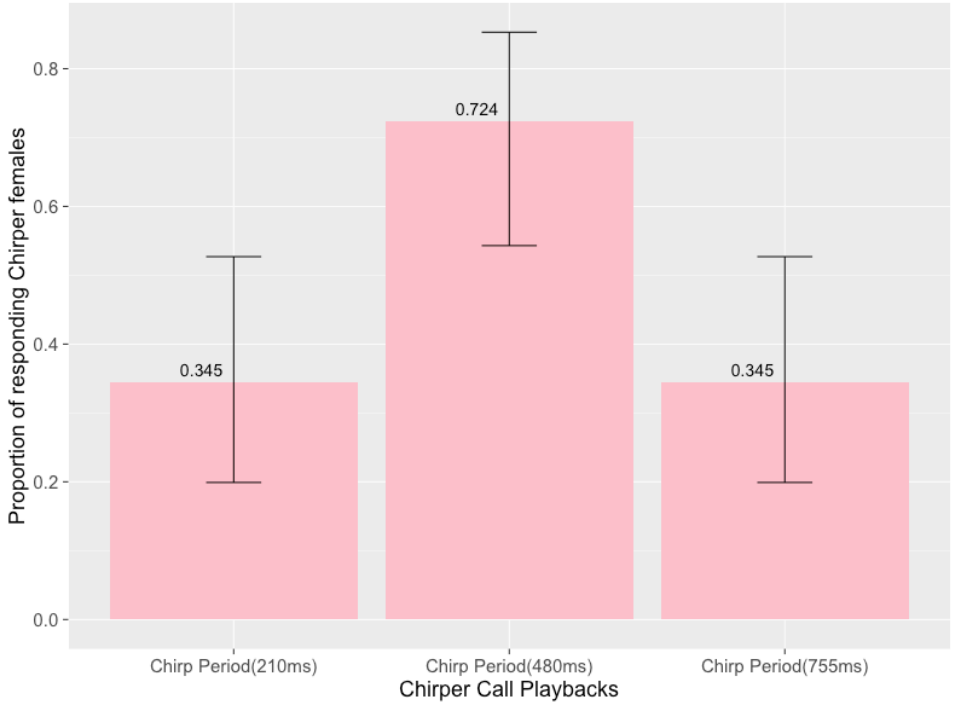
Out of 39 Chirper females was used in this experiment to study same songtype chirp rate preference in the 2013 season, 29 females responded to at least one of the trials. These 29 individuals include 13 pure laboratory bred Chirper females, 1 wild caught adult female and 15 Chirper females that were raised in laboratory after being caught as nymph from the wild. The age of each *Mecopoda* Chirper females during the three playback trials is shown in figure 4.7. Out of these 29 females, 21 responded to the mean Chirper call (480 ms), 10 each responded to mean + 2 S.D. and mean - 2 S.D. Chirper call periods [see figure 4.8a]. The frequency of responses to the 3 call periods were significantly different from each other (Cochran's Q test, Q= 8.64 and p= 0.0133). The 95 % confidence interval estimate of the proportional phonotactic responses of Chirper females to the playbacks of Chirper call at the mean chirp period of 480 ms and ± 2 standard deviation of the mean Chirper chirp period are shown in figure 4.8b.

The mean response time for 21 Chirper females to reach the speaker, when played Chirper calls at mean Chirper chirp rate was 216 s ± 30 s (mean ± S.E.M). Similarly the mean response time for 10 Chirper females that responded to high chirp rate was 242 s ± 37.5 s (Mean ± S.E.M) and that to low chirp rate was 321 s ± 48 s (Mean ± S.E.M). There were no significant differences between the means of the phonotactic response time of Chirper females towards the mean chirp rate, the higher chirp rate and the lower chirp rate (ANOVA, F= 1.98, P= 0.152).

Figure 4.7: Age of each *Mecopoda* Chirper females (uniquely coded) during the playback of Chirper calls at the mean chirp period of 480 ms and $\pm$ 2 standard deviation of the mean Chirper chirp period



(a) Phonotactic response by *Mecopoda* Chirper females to Chirper calls played at 210 ms, 480 ms and 755 ms chirp period



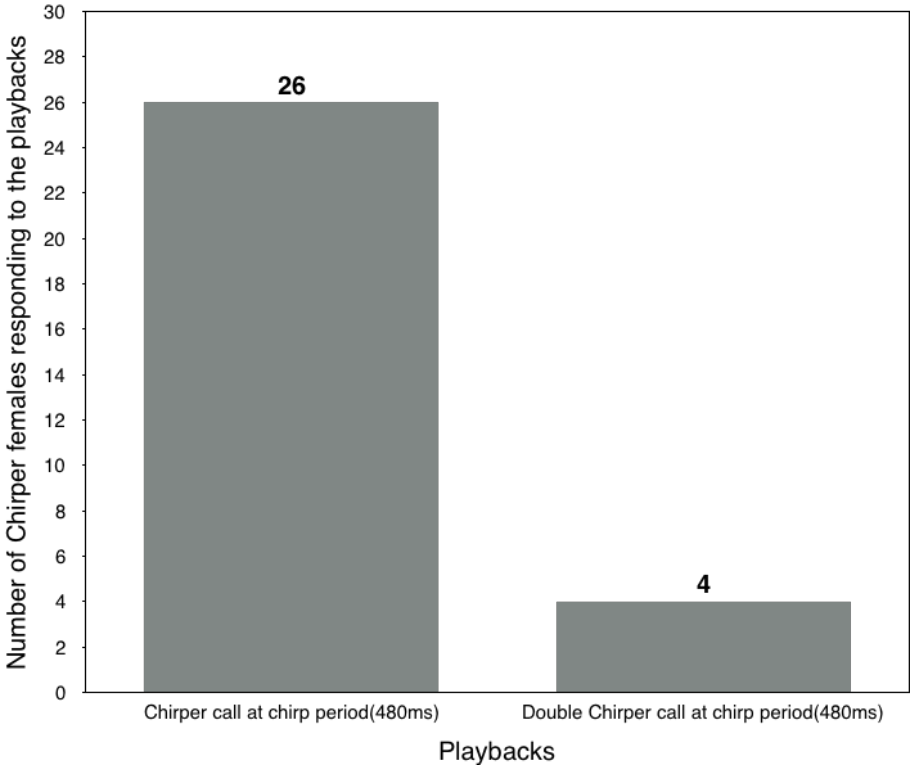
(b) Proportion of *Mecopoda* Chirper females responding to the playback of Chirper calls played at 210 ms, 480 ms and 755 ms chirp period with 95% confidence intervals

Figure 4.8: Response of *Mecopoda* Chirper females to the playback at mean chirp period and $\pm$ two standard deviations in the phonotactic experiment to study the chirp rate preference of their own call

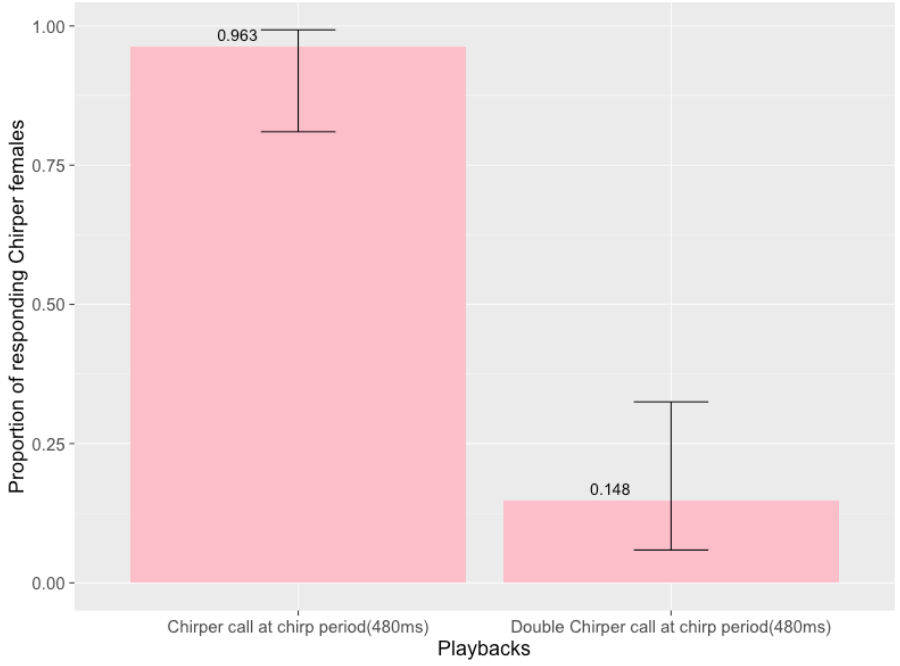
4.4.1.3 Call structure preference by Chirper females when played at Chirper chirp rate

Out of 39 females in the 2013 season, 27 (13 pure laboratory bred Chirper adults and 14 laboratory bred adults caught as nymph from the IISc campus) responded to at least one of the trials. Out of these 27 females only 4 responded to Double Chirper calls played at mean Chirper chirp period (480 ms) while 26 females went to the Chirper call played at mean Chirper chirp period (480 ms) [see figure 4.9a]. There was a significant difference between the responses to the two treatments (McNemar test, G=18.4, P= 0.000018, degrees of freedom =1). The 95 % confidence interval estimate of the proportion of Chirper females showing phonotaxis to the playbacks at 480ms chirp period are shown in figure 4.9b.

The mean response time of 4 Chirper females that showed a phonotactic response towards the Double Chirper call played at mean Chirper rate was 201 s $\pm$ 58.4 s (mean $\pm$ S.E.M) while mean response of 26 females that went to the mean Chirper call was 226 s $\pm$ 28.6 s (mean $\pm$ S.E.M). A t-test (t = -0.393, df = 4.58, p-value = 0.712) showed no significant differences between the means of the phonotactic response time of Chirper females towards the Chirper call and the Double Chirper calls, both played at a period of 480 ms.



(a) Phonotactic response of *Mecopoda* Chirper females to Chirper call and Double Chirper call played at 480 ms (mean Chirper chirp period)



(b) Confidence interval estimate based on proportional response of *Mecopoda* Chirper females to the playback of Chirper call and Double Chirper call at mean Chirper chirp period (480ms)

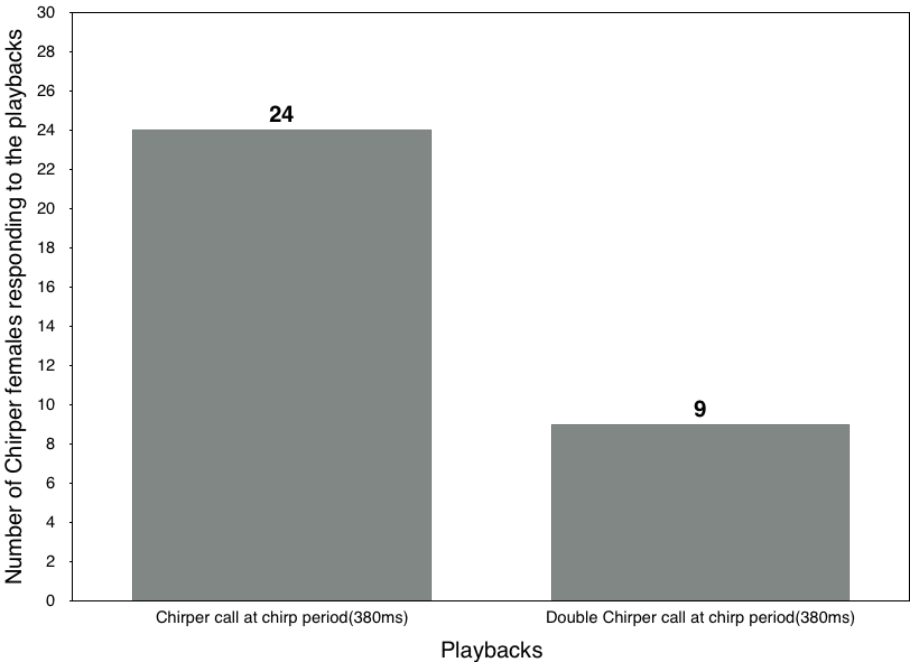
Figure 4.9: Response of *Mecopoda* Chirper females to the playback of *Mecopoda* Chirper call and Double Chirper call at mean Chirper chirp period (480ms) in the phonotactic experiment to study the chirp structure preference

4.4.1.4 Call structure preference by Chirper females when played at Double Chirper doublet rate

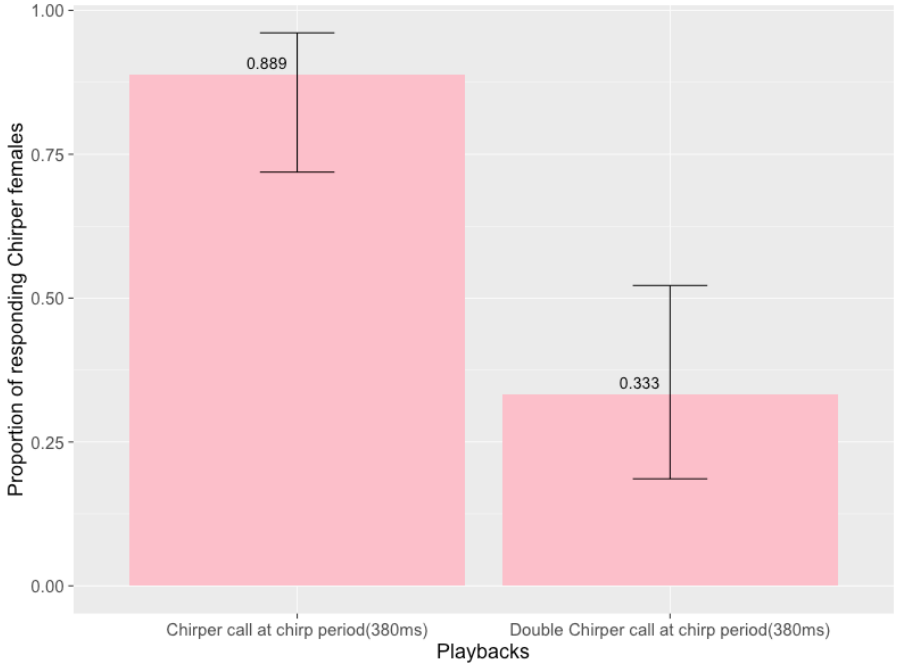
Out of the same 2013 season 39 females, 27 (1 wild caught adult, 12 nymph raised in laboratory culture to adult and 14 pure laboratory bred adults) responded to at least one of the trials. Out of 27 females, 24 responded to Chirper calls played at mean Double Chirper doublet period (380 ms) while 9 females responded to the Double Chirper call played at mean Double Chirper chirp period (380 ms) [see figure 4.10a]. There was a significant difference between the responses to the two treatments (McNemar test: G = 9.33, df =1, P = 0.00225). The 95 % confidence interval estimate of the proportional Chirper females' responses to the playbacks at 380ms chirp period are shown in figure 4.10b.

In the same experiment, the mean response time of 23 Chirper females that did phonotaxis towards the Chirper call played at mean Double Chirper rate was 183 s $\pm$ 31.6 s (Mean $\pm$ S.E.M) while the mean response time of 9 females that went to mean Double Chirper call was 191 s $\pm$ 64 s (Mean $\pm$ S.E.M).There were no significant differences between the means of the phonotactic response time of Chirper females towards Chirper call played at 380 ms and Double Chirper call played at 380 ms (t = 0.111, df = 9.12, p-value = 0.914).

When considering 16 females (8 pure laboratory bred adults and 8 caught as nymphs from the wild but raised as adults) that responded in both the third and fourth experiment, 3 females responded to Double Chirper calls played at mean Chirper rate, 14 females responded to mean Chirper calls, 13 females responded to Chirper call played at Double Chirper doublet rate and 5 females responded to mean Double Chirper calls [see figure 4.11a]. A Cochran Q-test (Q=21 and p= 0.000105 at 3 degrees of freedom) indicated that the overall difference in responses were significant. The 95 % confidence interval estimate of the proportional Chirper female responses to the playbacks are shown in figure 4.11b.

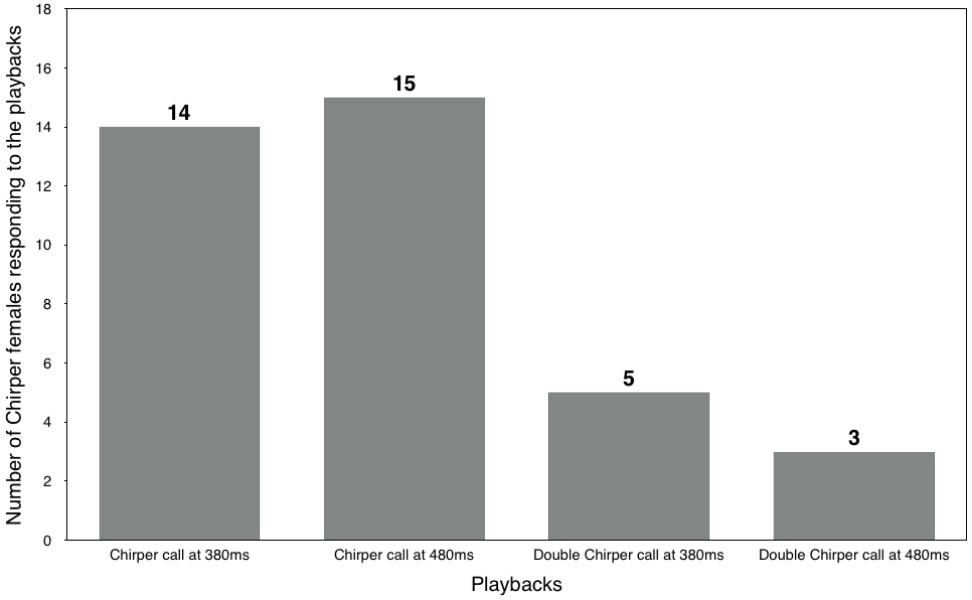


(a) Phonotactic response of *Mecopoda* Chirper females to Chirper calls played at 380 ms (mean Double Chirper doublet period) against mean Double Chirper call

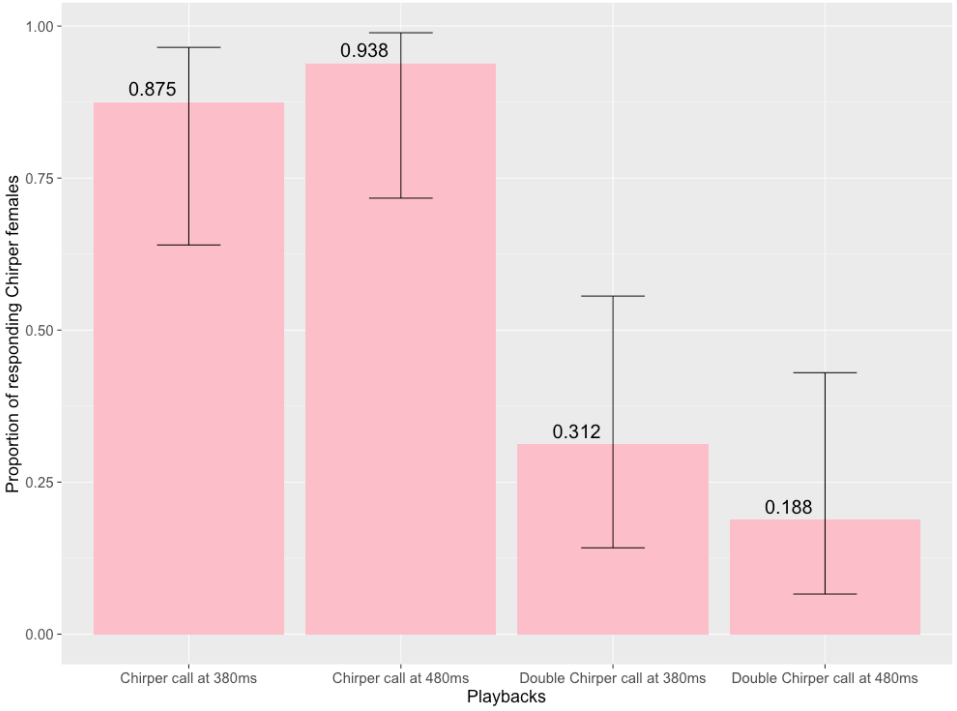


(b) Confidence interval estimate based on proportional response of *Mecopoda* Chirper females to the playback of Chirper call and Double Chirper call at mean Double Chirper doublet period (380ms)

Figure 4.10: Response of *Mecopoda* Chirper females to the playback of *Mecopoda* Chirper call and Double Chirper call at mean Double Chirper doublet period (380ms) in the phonotactic experiment to study the chirp structure preference



(a) Phonotactic response of *Mecopoda* Chirper females to Double Chirper calls played at 480 ms, mean Chirper call (480 ms), Chirper calls played at 380 ms and mean Double Chirper call (380 ms)



(b) Confidence interval estimate based on proportional response of *Mecopoda* Chirper females to the playback of Chirper call and Double Chirper call at mean Chirper chirp rate (480ms) and mean Double Chirper doublet rate (380ms)

Figure 4.11: Phonotactic response of all *Mecopoda* Chirper females that responded to both call structure preference experiments with playbacks of Chirper call and Double Chirper call at mean Chirper chirp rate (480ms) and mean Double Chirper doublet rate (380ms)

4.4.2 Results of the mating experiment

In the 12 mating trials involving Chirper males and females, 9 (all pure laboratory bred adults) out of 12 females (11 pure laboratory bred adults + 1 caught as nymph but raised as adult in laboratory) mated in the first trial and they were not tested further, 2 mated in the second trial after they failed in the first trial and 1 mated in the third trial after it failed in the first two attempts. Among the 8 trials where Double Chirper males and Chirper females were put together, only one instance of different songtype mating occurred during the first trial while no mating occurred when the females were used in the trials for the second time [see figure 4.12]. Out of these 8 Chirper females (all pure laboratory bred adults) used in the different songtype mating, 7 females mated with Chirper males on the control trial (third trial) while 1 could not be tested for the positive control due to unavailability of Chirper males. Although the sample size for this experiment is low, Pearson's χ-square test with simulated p-value based on 1000 replicates (χ-square = 7.5, p= 0.0129) indicates that the difference in frequency of same songtype and different songtype mating by Chirper females is significant. The 95 % confidence interval estimate of the proportion of Chirper females mating with Chirper and Double Chirper males are shown in figure 4.13.

The mean mating duration for same songtype Chirper matings was 101 $\pm$ 32 s (mean $\pm$ S.E.M). The mean pre-mating duration for these 12 mating trials was 1337 $\pm$ 308 s (mean $\pm$ S.E.M). In all cases, mating was preceded by brief Chirper calls sometimes continuing till the attachment of the male genitalia to that of the female. Calls used during mating could not be distinguished from long distance calling songs. The mean number of calling bouts and the mating attempts before a successful mating in these 12 trials was 3 $\pm$ 1.4 and 3 $\pm$ 1.7 respectively. Even after a successful mating, Chirper males broke into occasional calling bouts. From the same songtype mating experiments between Chirper males and females and one occurrence of different songtype mating, it was quite clear that there is no involvement of spermatophylax offering in the Chirper songtype, which is quite common in tettigoniids (Boggs, 1995; Gwynne, 1997; McCartney et al., 2012; Vahed, 1998). In bushcrickets, del Castillo and Gwynne (2007) has discussed that less calling effort leads to more investment in spermatophylax offerings. In case of *Mecopoda*, it appears that the opposite (more calling but no spermatophylax) is true. None of the females removed the spermatophore attached or fed on them within the trial time period although attempts were made by them to drop the spermatophore by rubbing it against the wall of the plastic box. Although the mating

usually occurred in less than 1 hour if both female and male were motivated, the spermatophore was retained for an astonishingly long time. In five of the Chirper females that were observed beyond their trial period, the mean time for which the spermatophore was retained was 5 hours $\pm$ 0.7 after the trial period but spermatophore retention time was also never more than 12 hours. The mating duration in the single case of different songtype mating was 9 s and the pre-mating duration for the trial was 1 hour 23 minutes. The attached spermatophore was, however, not dropped within the trial period. This mating occurred after 10 approaches by the Chirper female towards the Double Chirper male. The male did not approach the female even once before the mating and did not call either before or after the mating but only approached the female twice after the mating and within the trial period of 2 hours. Apart from this, 2 trials involving laboratory bred Double Chirper females and Chirper males were conducted. These trials between Double Chirper females and Chirper males in which they did not mate in the first attempt, were not repeated the second time.

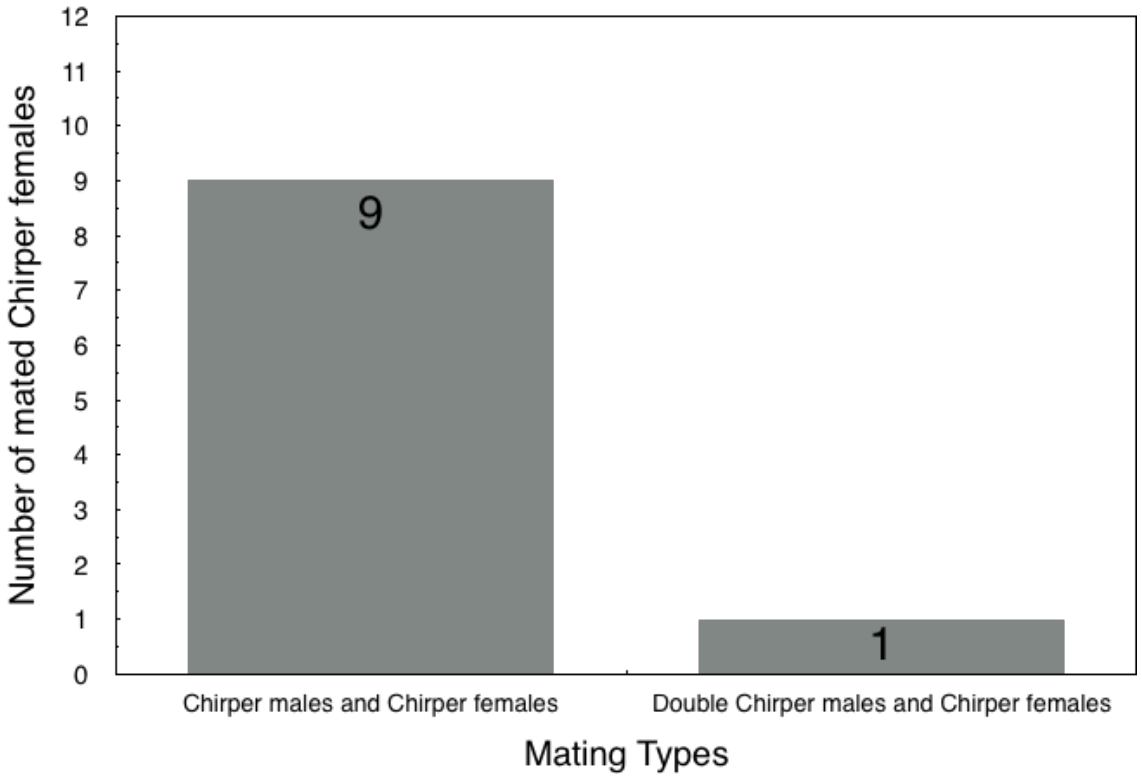


Figure 4.12: Number of *Mecopoda* Chirper females mating with *Mecopoda* Chirper male and *Mecopoda* Double Chirper male

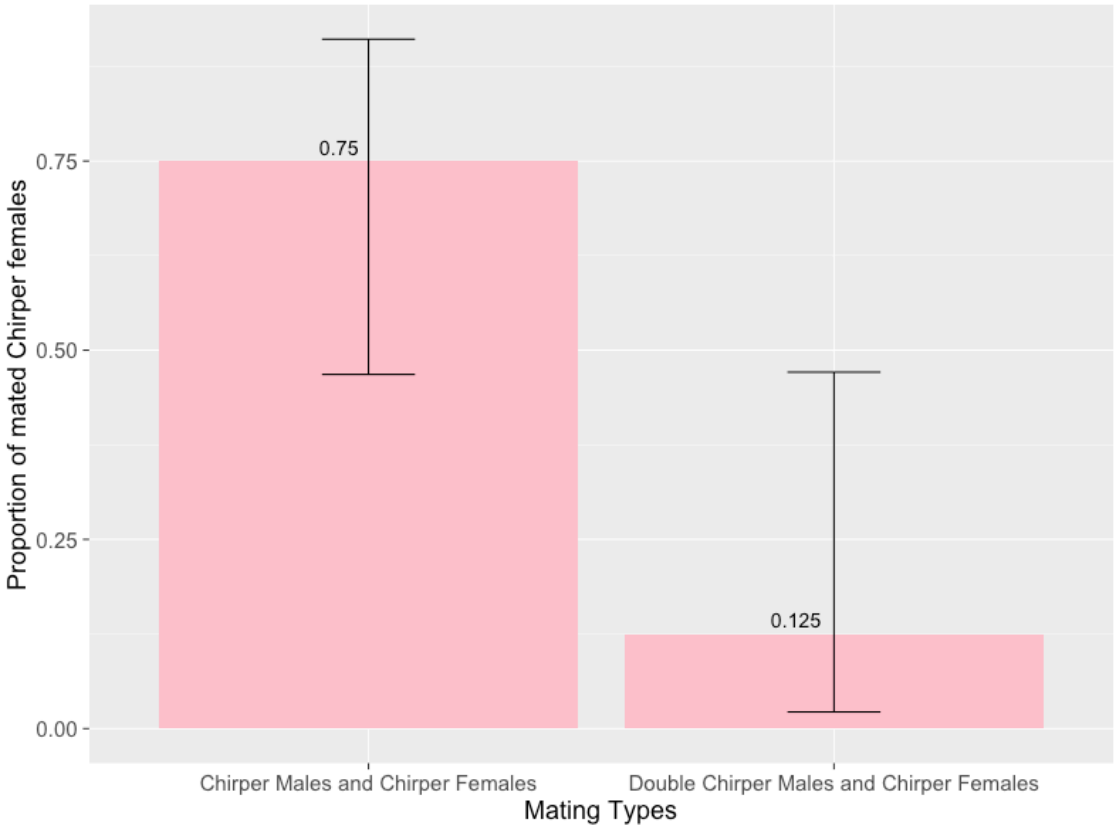


Figure 4.13: Confidence interval estimate based on proportional mating of *Mecopoda* Chirper females with *Mecopoda* Chirper males and *Mecopoda* Double Chirper males

4.5 Discussion

4.5.1 Does behavioural isolation exist between the songtypes of *Mecopoda*?

A successful phonotaxis reveals that the female of a cricket has recognised the call of a conspecific male and could localize it (Schul, 1998). The absence of a response to Two Part, Helicopter and Train playbacks in phonotaxis experiments shows that Chirper females strongly discriminate against calls of Two Part, Helicopter and Train songtypes. We can thus conclude that there is strong behavioural isolation between populations of these three songtypes and populations of Chirper. One common feature among these three songtypes is that they have a trill component in their calls while the Chirper call is only composed of chirps. Given that the spectral profiles of all the *Mecopoda* songtypes are similar, this suggests that temporal feature divergence alone is responsible for the observed

Songtype	Chirper	Double Chirper	Two Part	Helicopter	Train
Chirper	-	N	N	Y	Y
Double Chirper	N	-	Y	Y	Y
Two Part	N	Y	-	Y	Y
Helicopter	Y	Y	Y	-	Y
Train	Y	Y	Y	Y	-

Table 4.2: Sympatric combinations of Chirper, Double Chirper, Two Part, Helicopter and Train recorded in south India; Y represents evidence of sympatry and N represents absence of such evidence (Nityananda and Balakrishnan, 2006)

behavioural isolation. This has been found in other studies of speciation in other crickets (Gray and Cade, 2000; Hoy and Paul, 1973; Maroja et al., 2014; Shaw and Herlihy, 2000). It is noteworthy that Helicopter and Train songtype have a documented co-occurrence with the Chirper population [see table 4.2] in their distribution.

Chirper females showed a significantly higher preference for Chirper calls over Double Chirper calls. The occasional phonotaxis of Chirper females towards Double Chirper calls however indicates incomplete behavioural isolation between these two songtypes.This could be because Chirper and the Double Chirper calls are more similar to each other temporally in being composed of chirps only in contrast to the other three songtypes that are also composed of trills. This indicates that a proportion of Chirper females will respond to the temporal features of Double Chirper calls. Since these two songtypes are largely allopatric, these preferences of Chirper females for Double Chirper calls may not have been selected against. This will need to be tested in future studies examining more closely the geographic distributions of these two songtypes together with phonotaxis experiments on Chirper females from different populations. Although not tested, similar behavioural isolation is likely among other songtypes of *Mecopoda*; otherwise we would not expect to see such distinct populations of call types and no evidence so far of hybrid songs in the wild.

Behavioural isolation between Chirper and Double Chirper was further examined at the level of mating between the two songtypes. Mating between the two songtypes when forced into close proximity was rare. The single instance of a Double Chirper male mating with a Chirper female may represent an anomalous event that will only occur when animals are put in a very small closed enclosure in a no choice experiment (Coyne and Orr, 2004). Alternatively, this mating could be interpreted as evidence that reproductive isolation at the point of mating is not

complete, in much the same way that phonotaxis occasionally occurs in response to calls of male from different songtypes. Although our sample size is too small to form the basis of reliable calculations, if we take the probability of mating between Chirper females and Double Chirper in our study (0.125) and multiply this with the probability of Chirper females showing phonotaxis towards Double Chirper males (which is 0.24) we get a low probability (0.03) of Chirper females actually mating with Double Chirper males. This estimate ignores the fact that the confined proximity of our mating experiment may lead to overestimates of mating probability. While it is difficult to draw conclusions about the ecological significance of this mating probability, it is certainly not so low that the possibility of gene flow can be rejected.

My results suggest that there is complete pre-mating behavioural isolation between Chirper and the three trilling songtypes, namely, Two Part, Train and Helicopter and substantial, but incomplete behavioural isolation between Chirper and Double Chirper. A weakness in the experimental design is that I am making an assumption that the playback segment of the call of a single individual used in the phonotactic experiment is representative of the songtype as a whole. It is possible that any of the individual call segment that I chose is in fact not representative. In this case, my conclusions would be restricted to demonstrating that there are differences in female responses to songs among males. But given the canalisation of call structure within each *Mecopoda* songtype, the limited degrees of freedom within which a call can vary, and the simplicity of the female receiver system in insects, (Chirper females show preference for mean chirp rate, see figure 4.8a), call segments not being representative of each songtype seems unlikely. Future experiments with reciprocal combinations of Chirper males and females with males and females of all the other songtypes need to be carried out to confirm this conclusion. The sympatry of several of these songtypes together with the morphological similarity of the females made it difficult to carry out these reciprocal combinations in the current study. Attempts to produce true breeding lines of the other songtypes were unsuccessful due to high mortality during the nymphal stages.

4.5.2 Traits involved in behavioural isolation between Chirper and Double Chirper songtypes

Temporal features of calls contribute to species recognition in many insects (Deily and Schul, 2004, 2008; Schul, 1998). The temporal features used singly or in combination by many species for conspecific recognition and heterospecific discrimination includes pulse rate, duty cycle, pulse duration and gaps (Deily and Schul, 2004). Given this, there are two possible proximate explanations for Chirper females occasionally showing phonotactic behaviour towards Double Chirper calls.

Firstly, Chirper females might be influenced by the increased chirp rate of the Double Chirper call in relation to the Chirper call since higher chirp rate is generally a preferred trait (Gerhardt and Huber, 2002). If call rate in Chirper is under directional selection, with females preferring higher same songtype call rates, this could provide one possible explanation for approach towards Double Chirper calls, which are produced at higher rates. For example, individual Chirper females that weighted chirp rate more than other aspects of call structure may tend to approach Double Chirper calls. The experiment examining the preferences of *Mecopoda* Chirper females for chirp rates of their own call type (spanning the natural distribution of Chirper male calls) however indicates stabilizing selection as is commonly found in many crickets rather than directional selection on chirp rates (Gerhardt and Huber, 2002). The preference of females for chirp rate is broad but corresponds closely to the mean value of chirp rate. The reduced preference for chirp rate at both ends of the chirp rate distribution is also symmetrical giving a perfect unimodal curve [see figure 4.8]. This supports the hypothesis that chirp rates (or fine temporal features) are strongly associated with (sub) species recognition (Gerhardt and Huber, 2002). This female preference for temporal parameters (particularly mean chirp rate) may be acting as the basis of assortative mating between songtype populations. Alternatively, preference for mean temporal parameters by Chirper females may be associated with direct or indirect benefit for females, although the lack of any obvious cost to males of producing chirps at an intermediate rate makes this seem unlikely. From the study we also find that there is no significant Chirper female preference for the Double Chirper chirp rate over Chirper chirp rates [see figure 4.11a]. Thus, a preference for higher chirp rates driven by directional selection is unlikely to explain the occasional phonotaxis of Chirper females towards Double Chirper calls.

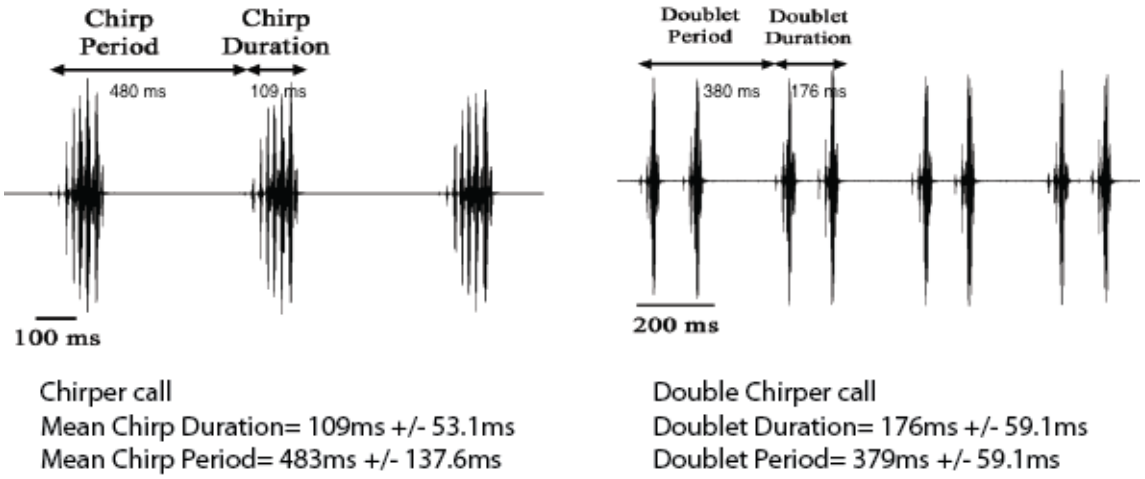


Figure 4.14: Representation of Chirper call and Double Chirper call along with their mean call parameters (adapted from Nityananda and Balakrishnan (2006))

Alternatively, some Chirper females might show less sensitivity to call structure in general and hence orient to both Chirper and Double Chirper calls. Most Chirper females strongly discriminate against the temporal structure of the Double Chirper call. When Double Chirper call is played at an optimum chirp rate of 480 ms, Chirper females do not show any significant increase in response to Double Chirper calls. This shows that most Chirper females seem to pay attention to the temporal structure of the call and are distinguishing the two call types based on differences in temporal structure other than the chirp rate. The most likely explanation for some Chirper females orienting towards Double Chirper call is, thus, a weaker preference for temporal parameters of Chirper call. They are perhaps evaluating Double Chirper doublet structure as a contiguous call rather than a doublet based on the total call duration. This might be because in species in which males call at higher chirp rate, females generally depend on gross structural cues (such as gaps or overall chirp duration) for species recognition (Bush et al., 2009; Deily and Schul, 2004; Schul, 1998). Chirper females may also ignore the doublet call duration of Double Chirper as the distribution of doublet duration of Double Chirper (117 ms to 236 ms) overlaps considerably (about 45 ms) with the distribution of chirp duration of Chirper calls (56 ms to 162 ms) [see figure 4.14, table 4.3]. Since Double Chirper chirp rate is higher than in Chirper and the doublet duration falls within the range of chirp duration of Chirper, Chirper females might be assessing Double Chirper call as a continuum of the Chirper call, albeit with suboptimal and unattractive parameters.

Item	Mean	Standard Deviation	Mean Temperature
Doubleton DC chirp period	379	$\pm$ 59.1	26.9 ($\pm$ 0.8)
Doubleton DC chirp duration	176	$\pm$ 59.1	26.9 ($\pm$ 0.8)
Chirper chirp period	483	$\pm$ 138	25.6 ($\pm$ 0.5)
Chirper chirp duration	109	$\pm$ 53.1	25.6 ($\pm$ 0.5)

Table 4.3: Call parameters of *Mecopoda* Chirper and *Mecopoda* Double Chirper that are overlapping (Nityananda and Balakrishnan, 2006)

4.5.3 Could behavioural isolation in *Mecopoda* be maintained by assortative mating?

The *Mecopoda* species provided an opportunity to study behavioural isolation among the five sympatric songtypes distributed in a small geographic area of southern India (Nityananda and Balakrishnan, 2006). The various sympatric distributions of various combinations of different *Mecopoda* songtypes known so far are shown in table 4.2. These data are preliminary and do not result from systematic sampling to examine the distribution of *Mecopoda* songtypes in southern India. In the absence of such a study, it is not possible to conclude that Chirper-Double Chirper and Chirper-Two Part combinations do not exist. Chirper appears to be distributed mostly east of the Western Ghats while Double Chirper and Two Part occur in the Western Ghats and there is a possibility of finding sympatric populations of Chirper, Double Chirper and Two Part at the eastern boundary of the Western Ghats in southern India. If such sympatric populations of Double Chirper and Chirper were present in the past, there could have been frequent encounter between the individuals of these two songtypes.

We can consider a situation in which Chirper and Double Chirper females belonged in the past to one continuous population of *Mecopoda*, say Chirper, such that a majority of females had preference functions that matched 'Chirper' call features. The process which leads to assortative mating by one sex based on the effect of traits present on the opposite sex is defined as mate choice (Edward, 2014). An acoustic preference function (Lande, 1981), is a measure of mate choice (Edward, 2014). In the case of *Mecopoda*, it would be a measure of the relationship between acoustic cues of males and phonotactic effort of females.

Preference functions however would vary between individual females and result in some degree of tolerance for either extreme values of traits, such as chirp rate,

or deviant temporal structures such as interrupted chirps. In this scenario, pre-existing variation in preference functions could drive assortative mating when a new signal form (for example, Double Chirper) arises within the population. Such assortative mating could have led to the development of a mate choice paradigm, which in turn may have led to behavioural isolation between *Mecopoda* song-types.

Chapter 5

Acoustically locating parasitoid fly found in the acoustically divergent *Mecopoda* populations

5.1 Abstract

While we are sure that population divergence in *Mecopoda* is being maintained as a result of reproductive barriers mediated by acoustic signals, chemical profiles and genital characters, probably through female preference, it is difficult to determine the exact cause of the evolution of such divergence in the first place. Here I describe the discovery of a Tachinid parasitoid affecting multiple *Mecopoda* songtypes, and speculate about its potential role in acoustic divergence within the group. In the evolutionary host-parasite race, hosts evolve mechanisms to avoid parasite attack and parasites evolve counter mechanism to detect host efficiently. Upon locating the host, parasitoid planidia enters a host body, develops into larvae inside the host, and eventually emerges to pupate, thereby killing the host. Adult of some Tachinid flies have evolved an orthopteroid hearing mechanism that can track down the sexual signal of a host much like that of its potential mate. The Tachinid fly affecting the *Mecopoda* songtypes does not appear to be any different. It has a well-developed hearing tympanum equipping it to be phonotactic towards Double Chirper, Two Part and the Helicopter songtypes of *Mecopoda*.

5.2 Introduction

Insect parasitoids, whose larval form depends on a host species for nutrition and development eventually causing death of the host, are quite abundant in nature (Godfray and Shimada, 1999; Santos and Quicke, 2011). They mostly belong to the Order Hymenoptera (78%) and Family Tachinidae under Order Diptera (20%) (Feener and Brown, 1997; Godfray and Shimada, 1999). These parasitoids have highly diverse life histories (Jervis and Ferns, 2011) and attack a range of hosts that are mostly herbivorous. In general, each such host may be parasitised by 2-8 parasitoid species and hosts for each parasitoid are likely to be closely related species (Santos and Quicke, 2011). The parasitoids can be grouped into ectoparasites or endoparasites depending on whether the larva enters the host body. They can be grouped into idiobionts or koinobionts depending on the host's ability to live upon being parasitised (Santos and Quicke, 2011). Typically, unlike idiobionts, most koinobionts are endoparasites that affect young hosts, have longer developmental stages and short adult life. They normally are pro-ovigenic and have a smaller host range compared to ectoparasitic idiobionts (Santos and Quicke, 2011).

While most initial research on parasitoids was conducted to understand the economic importance of the parasitoids in pest control, more recent scientific studies have started dealing with their behavioural ecology (Feener and Brown, 1997; Jervis and Ferns, 2011; Wajnberg et al., 2008). Apart from this, studies of the evolutionary implications of host-parasite interactions are also gaining importance (Feener and Brown, 1997; Godfray and Shimada, 1999). One particularly interesting aspect with respect to the current study is the exploitation of the host communication mechanisms by parasitoid flies to locate them (Xiaoyi and Zhongqi, 2008), more specifically acoustic exploitation of hosts by Dipteran parasitoids (Lakes-Harlan and Lehmann, 2014). Intraspecific auditory communication (advertising presence, location and potentially fitness information (Gerhardt and Huber, 2002)) is predominantly found in orthopteran and hemipteran species and dipteran parasitoids have evolved to take advantage of it (Lakes-Harlan and Lehmann, 2014).

One of the first studies to identify this phenomenon (Cade, 1975), found out that parasitoid females of *Euphasiopteryx ochracea* (a Tachinid fly) showed phonotaxis to playback of *Gryllus integer* (a field cricket) song. *Euphasiopteryx ochracea* also got attracted significantly more to the male choruses than to single callers (Cade,

1981) with preference for leading playback in a two speaker laboratory experiment when stimuli were not synchronous (Lee et al., 2009). The parasitoid affects the parasitised male host's calling ability that gradually declines with time until its death (Cade, 1984). Thus, calling and chorusing behaviour in orthopterans are thought to have evolved in a way to avoid exploitative parasites or predators (Cade and Wyatt, 1984; Greenfield, 1983; Henry, 1994). Currently, the two most studied dipteran parasitoid groups are Tachinids belonging to tribe Orminii and Sarcophagids belonging to tribe Emblemasomatini parasitising on crickets/bushcrickets (Orthoptera) and cicadas (Hemiptera) respectively with the help of independently evolved hearing mechanisms (Hedwig and Robert, 2014; Lakes-Harlan and Lehmann, 2014). The Sarcophagid genus *Emblemasoma* has been found to infect cicada males calling during the day (Farris et al., 2008). On the other hand, *Therobia leonidae* (a Tachinid parasitoid) flies locate males of the Tettigoniid host *Poecilimon* by eavesdropping on the host call's dominant frequency at night (Lakes-Harlan and Heller, 1992). Two species of *Ormia* parasitoids have also been successfully reared in the laboratory on their natural hosts, *Gryllus* sp. and *Scaptericus* sp. (Wineriter and Walker, 1990). *Ormia ochracea* females have been found to posses orthopteroid hearing organs, which are most sensitive between 4 to 6 kHz, corresponding to the frequency range of the calls of its hosts, *Gryllus rubens* (Robert et al., 1992). Other similar dipteran host-parasitoid systems include bushcricket *Neoconocephalus robustus* and Tachinid *E. brevicornis*, *Neoconocephalus triops* and Tachinid *Ormia leneifrons*, Cicada *Okanaga rimosa* and Sarcophagid *Colcondamyia auditrix* (Cade, 1984) and many more reviewed in (Feener and Brown, 1997; Zuk and Kolluru, 1998).

The Tachinids (Family Tachinidae) are generally considered a relatively recent group showing adaptive radiation (Cerretti et al., 2014; Crosskey, 1976) and consist of one of the highest number of species under the Order Diptera (Stireman et al., 2006). This highly diversified group of flies has a wide global distribution (Stireman et al., 2006) and all Tachinids that have been studied so far are parasitoids on a variety of insect Orders including Lepidoptera, Orthoptera, Coleoptera, Hymenoptera, Heteroptera and many other insect Orders (Cerretti et al., 2014; Feener and Brown, 1997; Stireman et al., 2006). This success of Tachinids as parasitoids is thought to be due to its larvae's special ability to avoid host immune response (Feener and Brown, 1997).Taxonomically, the family Tachinidae appears to be a monophyletic group that falls under Superfamily Oestroidea under Subsection Calyptratae (Stireman et al., 2006). Identification of parasitoids

beyond the Family level is quite challenging (Godfray and Shimada, 1999; Stireman et al., 2006). Although the taxonomic relationship between Tachinids and hierarchies placed above it are well established, relationships within the group are not clear (Smith et al., 2007; Stireman et al., 2006). There is no stable classificatory scheme beyond Tachinidae group and practical identification is fraught with ambiguity and difficulty (Cerretti et al., 2014; Crosskey, 1976; Smith et al., 2007; Stireman et al., 2006). There is also a dearth of studies regarding basic natural history for many parasitoid species in spite of their ecological importance (Santos and Quicke, 2011).

The bushcricket *Mecopoda* consists of five songtypes which are morphologically cryptic (Nityananda and Balakrishnan, 2006). The cause for such diversification in calling songs is as yet unknown. These songtypes show subtle differences in genital morphology and CHC profiles and females of one of the songtypes, Chirper, show a strong preference for their own call type (as shown in previous chapters). There has been no evidence of Tachinid parasitoids affecting *Mecopoda* males or any calling Orthopteran species from India until now (Crosskey, 1976). India has 1033 Orthopteran species/ subspecies within 398 genera and 21 families of Order Orthoptera mostly including acoustically communicating crickets and bushcrickets (Gryllidae and Tettigoniidae) as well as many Tachinids (>140 species) (Chandra and Gupta, 2013; O'Hara et al., 2009) and it is expected that there will be at least some exploitative interactions. There appear to be only a few records of Tachinid parasitoids parasitising on Lepidopterans from India and those having importance in pest control (Sathe, 2012; Shendage and Sathe, 2012). In this chapter, I describe a hitherto unknown host-parasite relationship between a Tachinid fly species and bushcrickets of the genus *Mecopoda*, including different songtypes. In case of Mecopoda, under the selection pressure of parasitisation once single population may have diverged into different songtypes to avoid detection by the Tachinid parasitoids. *Mecopoda* thus provides a good model system to study the possible role of parasitoids in song divergence.

The objectives of the study are as follows:

1. To describe the Tachinid parasitoids affecting *Mecopoda* populations.
2. To study the prevalence of parasitoid infection among *Mecopoda* songtypes.

5.3 Materials and methods

Field sampling was carried out in Kervashe, Hurabi and Heringe locality around Kadari village (N13° 13', E75° 05'), Dakshin Kannada district, Karnataka, India. Adult *Mecopoda* males were collected opportunistically from the three sites between January and March each year for a period of two years (2013-2014). The sampling took place at least twice for a given location each year between 7:30 pm to 9:30 pm at night. *Mecopoda* males were identified by listening to their calls and recording them when possible. *Mecopoda* males that were captured were housed individually in plastic boxes (15 cm x 7 cm x 5 cm) and fed ad libitum on oat flakes (Quaker Oats, Morten Seeds & Grains Pty. Ltd.), fish food (Taiyo Grow, Taiyo Petproducts [P] Ltd.) and water. These males were observed over subsequent days for parasitoid infection. A *Mecopoda* male was considered infected if it was found dead inside the plastic box along with presence of ovoid Tachinid pupa.

5.4 Results

5.4.1 Taxonomy of Tachinidae

There is a general agreement over classification of the Family Tachinidae (Stireman et al., 2006) into either four (Wood, 1987) or five subfamilies (Crosskey, 1976). Between these two alternative classificatory schemes, Subfamily Tachininae appears consistently as a taxonomic group in both but forms a diverse group in many respects and is probably not monophyletic (Cerretti et al., 2014). The classifications of groups within Order Tachinidae seem to be incomplete and await further refinement until more information about their reproductive behaviour (oviposition methods, host location and selection) and ecology (host utilization and life history) pours in.

Diagnostic features of Family Tachinidae (Crosskey, 1976):

1. Adults have subscutellum and meral bristles.
2. First Instar larvae do not have mandibles or they are vestigial.
3. Anterior cephalophryngeal skeleton appears as hook or axe-like beak.

Diagnostic feature of Subfamily Tachininae (with many exceptions):

1. Presence of naked prosternum.
2. No direct contact between host and the fly. Placement of numerous eggs on areas accessed by host species increases chance of infection in host.

The taxonomic status of the Tachinid fly that infect *Mecopoda* songtypes is summarized below (identified by Dr. Dhriti Banerjee, Zoological Survey of India, Kolkata):

Class Insecta
Subclass Pterygota
Infraclass Neoptera
Order Diptera
Suborder Brachycera
Section Schizophora
Subsection Calyptratae
Superfamily Oestroidea
Family Tachinidae
Subfamily Tachininae
Tribe Tachinini
Genus Mikia
Species *Mikia apicalis*

5.4.2 Morphology of Tachinid fly found on *Mecopoda*

Figure 5.1: Acoustically locating Tachinid parasitoid that was observed to be parasitising *Mecopoda*. The compound eyes, halteres and position of the hearing organ of the Tachinid fly are marked out

Tachinid flies are diverse ranging from 2 mm to more than 2 cm in size (Stireman et al., 2006). The Tachinid fly that was found to be parasitising on the *Mecopoda* songtypes appeared to be a typical muscoid Dipteran fly externally of 1 cm length [see figure 5.1]. It had a pair of compound eyes that were red in colour. The prothoracic auditory spiracles (AS) are relatively enlarged and it has a typical furry peritreme (represented by P in the figure 5.2: integument that surrounds the spiracles) and clearly visible hairs on the anterior side. It is a hearing Tachinid as it contains a hearing organ (a modified inflated prosternum) between its head and thorax on the ventral side, with a morphology and position similar to that of the well-studied ear of *Therobia leonidei* and *Ormia ochracea* (Hedwig and Robert, 2014; Lakes-Harlan and Heller, 1992). The prosternal tympanal membrane (PTM) is separated by a ridge from the coxa (Co) that is also large. The Tachinid fly also had distinct halteres. The larva that comes out of an infected animal is 2 cm long and forms an ovoid pupa.

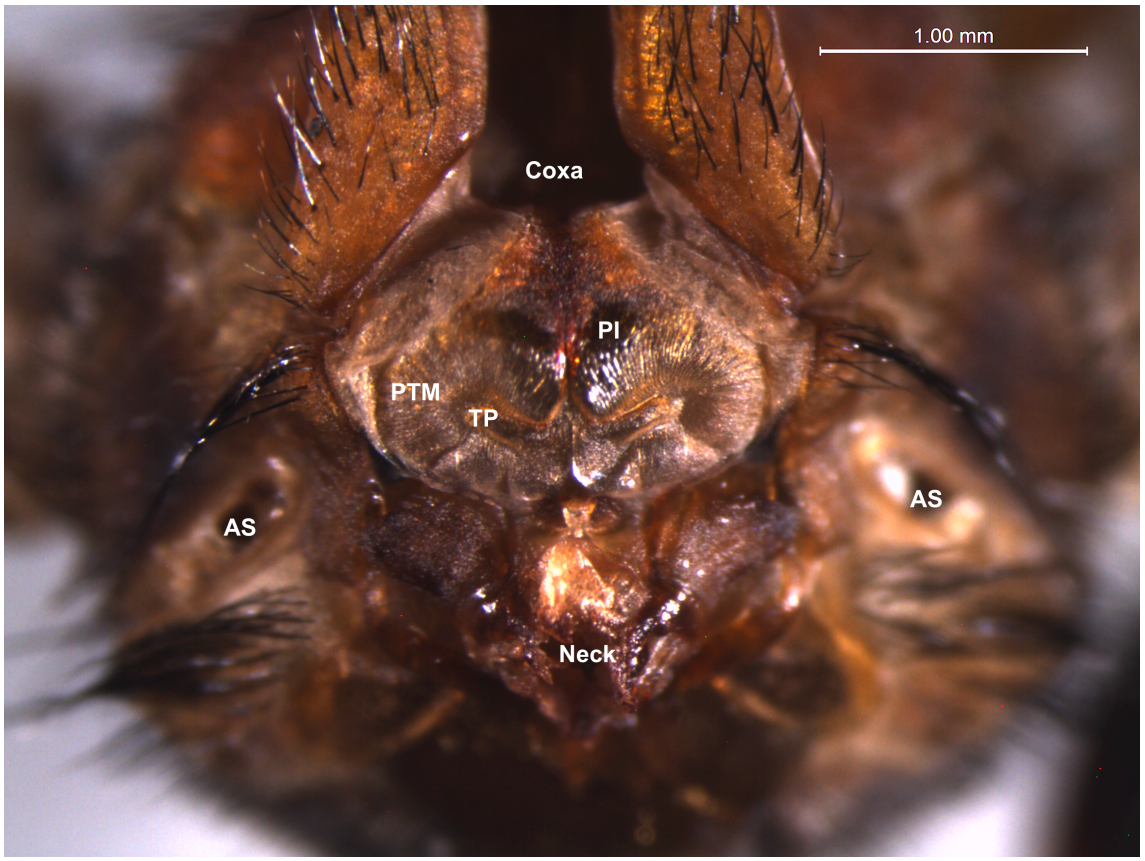


Figure 5.2: Magnified image of the orthopteroid hearing organ that helps the acoustically locating parasitoid Tachinid fly localise its host *Mecopoda*. The hearing organ is visible after the head of the Tachinid fly has been severed from the neck. AS is auditory spiracle, PTM is Prosternal Tympanal Membrane, TP is Tympanal Pit and PI is Prosternal Inflation Hedwig and Robert (2014)

Figure 5.3: Auditory spiracle of the acoustically locating Tachinid fly bound by peritreme (P)

5.4.3 Life cycle of Tachinid fly

Usually in Tachinids, the body piercing ovipositor found commonly on Hymenopteran parasitoids is not present (Feener and Brown, 1997). Rather planidia are deposited near or on the host by larviparous parasitoid females (Santos and Quicke, 2011; Stireman et al., 2006). The newly hatched larvae are expected to enter the host and remain as koinobionts where they grow without killing the host till they need to pupate (Stireman et al., 2006). These larvae after successful incubation within the *Mecopoda* abdomen have been observed to come out as white coloured larvae from an orifice the larva makes at the side of the abdomen. This process eventually kills the *Mecopoda* adult male as in other cases of Tachinid parasitism (Stireman et al., 2006). It was also observed that the white coloured larva transformed into a pupa within a few minutes of emergence from the body of the cricket, presumably due to exposure to air. The soft outer surface of the larva forms a hard cover after pupation. This cover appeared blackish red at first but changed to dark black after some time and with age.

From 2014 collection of infected *Mecopoda* males that included 11 infected individuals, mean larval emergence time was found to be 7 days and ranged from minimum of 5 days to maximum of 10 days which is usually the case with Tachinid flies (Stireman et al., 2006). Larval emergence led to slow death of *Mecopoda* males, which often lived for some hours after larval emergence but died within the same day. Usually one Tachinid larva emerged from a single infected *Mecopoda* individual (9 individuals led to 9 pupae in 2014) but sometimes two Tachinid larvae may emerge from a single individual (2 individuals had 2 pupae each in 2014). *Teleogryllus oceanicus* and *Gryllus* sp. on the contrary have ability to harbour more than 2 (up to 8 in extreme cases in *T. oceanicus*) *Ormia ochracea* larvae but they mostly harbour 1 to 2 larvae (Adamo et al., 1995; Kolluru and Zuk, 2001). Total number of pupae in 2014 collection was thus 13. Out of these 13 pupae, eight emerged as adult flies after a mean of 11 days and ranged from minimum of 9 days to maximum of 13 days. These adult Tachinid males and females would then mate with each other and gravid females would lay next progeny.

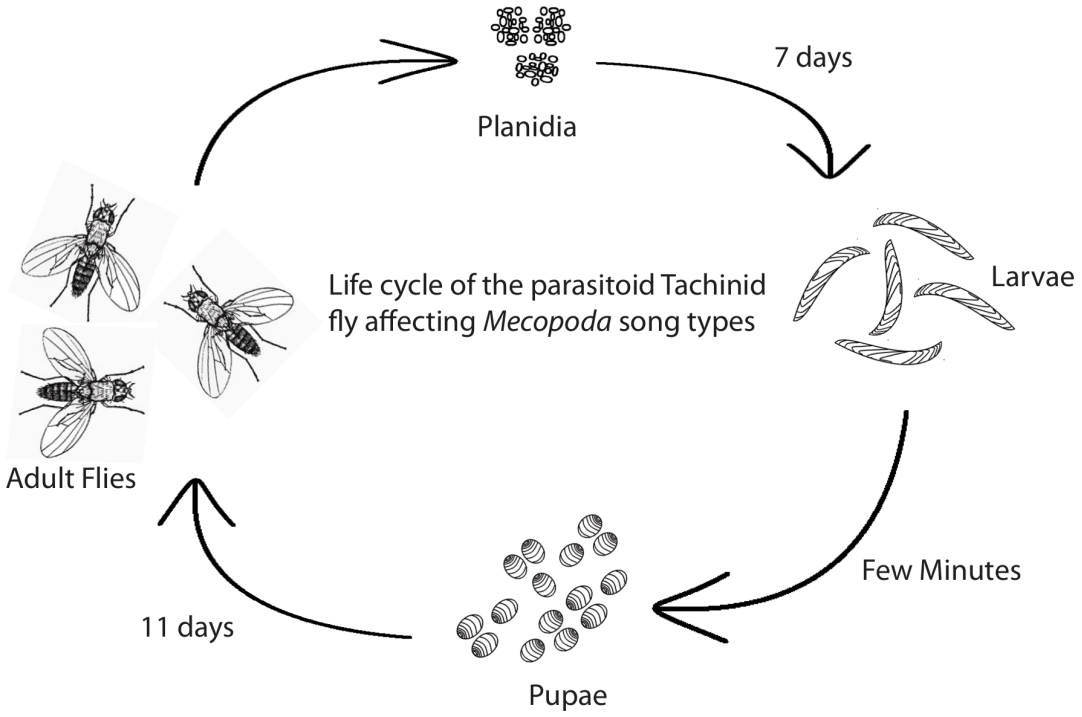


Figure 5.4: Life cycle of the Tachinid parasitoid that acoustically locates and parasitises *Mecopoda*. Larviparous Tachinid females infect the *Mecopoda* males. Typically Tachinid planidia are laid on the host or nearby it. The planidia then burrow inside the host and after a period of incubation emerges from the host as larvae. The larvae then pupates and the pupae subsequently transform into the adult flies

5.4.4 Field study of Tachinid fly: Tachinid infection of different songtypes

From the field study of two years (2013 & 2014), it was found that all three songtypes found in this area between January and March (Double Chirper, Two Part and Helicopter) have chances of infection from Tachinid flies. The details of the samplings and recorded infection are shown in the tables 5.1 and 5.2.

Although unequal sample size makes it difficult to formally compare the results from two years, it is still evident that the proportion of infected *Mecopoda* among the three songtypes and among the three sampling locations differs considerably between the two sampling periods as shown in the tables 5.3 and 5.4. The 95 % confidence interval estimate of the proportion of infected *Mecopoda* among songtypes and among sampling locations during the sampling years 2013 and 2014 are shown in figures 5.5 & 5.6.

Sampling Location	Songtype	Sample Size	Infected	Not Infected
Kervashe	Double Chirper	15	7	8
	Helicopter	13	5	8
	Two Part	1	1	0
Heringe	Double Chirper	3	3	0
	Helicopter	15	11	4
	Two Part	0	0	0
Total		47	27	20

Table 5.1: Number of infected *Mecopoda* songtypes found among the *Mecopoda* samples from different sampling locations in the sampling year 2013

Sampling Location	Songtype	Sample Size	Infected	Not Infected
Kervashe	Double Chirper	56	0	56
	Helicopter	4	0	4
	Two Part	13	1	12
Heringe	Double Chirper	20	0	20
	Helicopter	17	0	17
	Two Part	4	0	4
Hurabi	Double Chirper	39	5	34
	Helicopter	0	0	0
	Two Part	20	5	15
Total		173	11	162

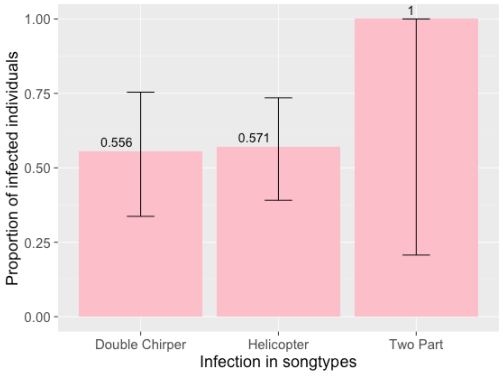
Table 5.2: Number of infected *Mecopoda* songtypes found among the *Mecopoda* samples from different sampling locations in the sampling year 2014

Songtype	Sampling Year (2013)	Sampling Year (2014)
Double Chirper	10/18 = 56%	5/ 115 = 4%
Helicopter	16/28 = 57%	0/ 21 = 0%
Two Part	1/1 = 100%	6/ 37 = 16%
Total	27/47 = 57%	11/173 = 6%

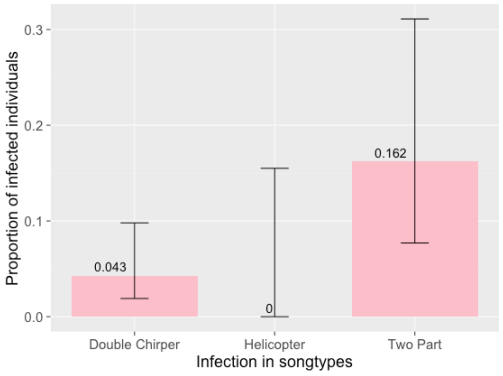
Table 5.3: Details of the infection percentages of the sampled *Mecopoda* songtypes in the sampling years, 2013 and 2014

Sampling Location	Sampling Year (2013)	Sampling Year (2014)
Kervashe	13/29 = 45%	1/ 73 = 1%
Hurabi	-	10/ 59 = 17%
Heringe	14/18 = 78%	0/41 = 0%

Table 5.4: Details of the locationwise infection percentages of sampled *Mecopoda* in the sampling years, 2013 and 2014

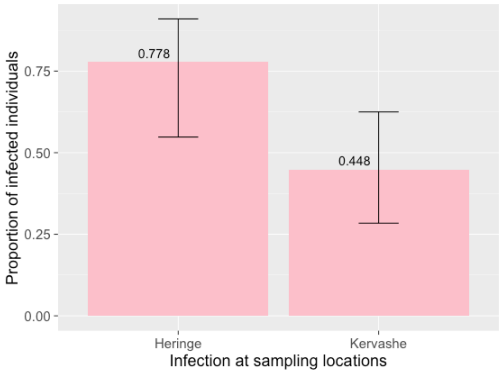


(a) Proportion of parasitised *Mecopoda* songtypes, Double Chirper, Helicopter and Two Part, observed in the year 2013

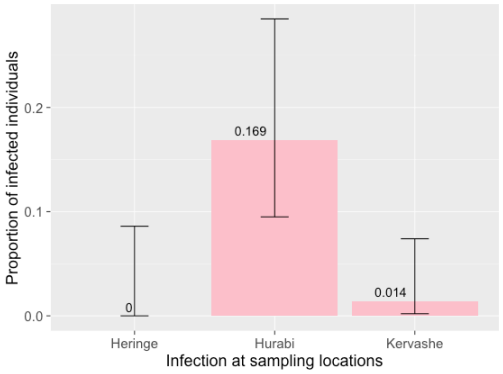


(b) Proportion of parasitised *Mecopoda* songtypes, Double Chirper, Helicopter and Two Part, observed in the year 2014

Figure 5.5: Proportion of Tachinid parasitoid fly infected *Mecopoda* songtypes found in the sampling years, 2013 and 2014, with 95% confidence intervals.



(a) Proportion of parasitised *Mecopoda* at Heringe and Kervashe observed in the year, 2013



(b) Proportion of parasitised *Mecopoda* at Heringe, Hurabi and Kervashe observed in the year, 2014

Figure 5.6: Proportion of Tachinid parasitoid fly infected *Mecopoda* found at different sampling locations in the sampling years, 2013 and 2014, with 95% confidence intervals.

In Pearson's χ-square test with simulated p-value (based on 2000 replicates), parasitoid infection of Double Chirper and Helicopter were found to be significantly greater (p=0.0005 for both songtypes) in the year 2013 than the year 2014. The population of Two Part callers could not be meaningfully compared among years due to insufficient sample size of my collection in the year 2013. Similarly, parasitoid infection in Kervashe and Heringe location was found to be significantly greater (p=0.0005 for both locations) in the year 2013 than in the year 2014. The *Mecopoda* population at Hurabi could not be compared between two years since it was not sampled in the year 2013.

5.5 Discussion

Tachinid flies (Order Diptera, Family Tachinidae) which are parasitoids of many groups of insects (Cerretti et al., 2014; Stireman et al., 2006) are not extensively studied and little is known about their evolution, ecology and behaviour (Stireman et al., 2006). The diversification of host use might have evolved after acquisition of parasitoid life history since all known Tachinids are parasitoids (Feener and Brown, 1997). Apart from being of economic importance in biological control of pests, they are thus steadily becoming tools for basic host-parasite research.

The breeding season of *Mecopoda* starts from October in the areas where collections for infected *Mecopoda* individuals were done (personal observation). However, most of the infection appears to be occurring in February and March. This suggests that the Tachinid parasitoid life cycle lags that of *Mecopoda* cycle in Kervashe, Hurabi and Heringe locality by four months. This trend was also seen in *Ormia ochracea* (Paur and Gray, 2011). It will be interesting to find out the population abundance of the Tachinid fly infecting *Mecopoda* after *Mecopoda* season is over. If they were to survive in the interim period when *Mecopoda* is not available, there must be an alternate host or their pupae must go into diapause.

This study is the first record of parasitoids affecting *Mecopoda* males or any calling Orthopteran species from India. This confirmation follows a very recent anecdotal discovery of Malaysian *Mecopoda* infected by larvae of parasitoid fly belonging to family Tachinidae (Hartbauer et al., 2011). All the three songtypes under study could be infected by the Tachinid parasitoid [see tables 5.1, 5.2, 5.3, 5.4]. This might be because the Tachinid parasitoids pay attention to the dominant frequency of the *Mecopoda* call, which are identical irrespective of the songtypes as also seen in *Therobia leonidei* (Lakes-Harlan and Heller, 1992). However, it may be expected that Tachinid fly found to affect *Mecopoda*, has locality-wise songtype preference. In the wild, Tachinid species *Ormia ochracea* has shown locality specific host preference (Gray et al., 2007). The preference specificity of host by Tachinids has been demonstrated in few instances where Tachinids have been less successful when grown in laboratory using closely related alternate hosts rather than their specific host (Thomson et al., 2012; Wineriter and Walker, 1990).

In another study of two closely related bushcricket species *Poecilimon* that have calls differing in the number of syllables in chirps, it was found that parasitoid *Therobia* parasitised the *Poecilimon* species with polysyllables three to four times

more than the species with monosyllable (Lehmann and Heller, 1998). This study along with study by (Wagner, 1996) indicates that Tachinid parasitoids find it easier to locate hosts with longer calls. There is no indication that this may be true for the parasitised *Mecopoda* songtypes (chirp period ranging from 0.38 s to 35s) from the current study but more directed study is needed to elucidate host preference in the parasitoid fly at finer scale. Although distribution and abundance of *Mecopoda* has not been systematically studied yet, preliminary opportunistic sampling in known *Mecopoda* habitats indicates that there is possibility of a high proportion of the *Mecopoda* population being infected by Tachinid parasitoids. For example, 57% of *Mecopoda* sampled during 2013 were infected by the Tachinid fly. This kind of high rate (more than 50 %) of infection is also seen in *Therobia leonidae-Poecilimon* parasitoid-host system (Heller and von Helversen, 1993). At high infection rates, we can expect a high selection pressure on *Mecopoda* from the Tachinid parasitoids on intraspecific sound signals (Hedwig and Robert, 2014). In the case of *Mecopoda*, infection rates seem to differ signficantly between consecutive years. Although more detailed study on this aspect is required, my data shows that the infection rates at Kervashe and Heringe were significantly higher in 2013 than in 2014. The reason for this remains unknown but site specific and season specific selection pressure may lead to a more unpredictable trajectory of local song divergence within *Mecopoda* distribution, a condition indicated by different *Gryllus* species of USA acting as primary host to *Ormia ochracea* parasite in different locations(Gray et al., 2007). It appears as though Tachinid infection in *Mecopoda* was more prevalent in songtypes Double Chirper and Helicopter in 2013 than in 2014. This trend is interesting since it leaves open the potential for parasitoids to target different songtypes in different seasons. The potential influence of a process such as this needs to be examined but it may be that the less infected songtypes in one season may be proportionally more common in the next season making them naturally more susceptible to parasitoid infection.

Parasites have long been acknowledged to be a potentially important factor for both sympatric and allopatric divergence of host species leading to host speciation (Buckling and Rainey, 2002). It has also been argued that investment in immunity to parasites in males may trade off with investment in secondary sexual characteristics (Simmons, 2013). This would mean that parasite resistance divergence among males would affect the diversification of secondary sexual characters in the population that might lead to sexual selection. Assortative mating would restrict gene flow and eventually barriers may form separating the different populations of

the same species. However, there is limited direct evidence of parasitism leading to host diversification (Buckling and Rainey, 2002).

Mecopoda songtypes may turn out to be the promising system that could throw light on the role of parasites in speciation. It is interesting that *Mecopoda* calls that are sexual signals for *Mecopoda* females are being used by Tachinid parasitoids to parasitise them. To receive this signal, both *Mecopoda* females and parasitoid females may have undergone a convergent evolution of their hearing organs [see figure 5.2] as seen in Tachinid species *Ormia ochracea* and *Therobia leonidae* (Hedwig and Robert, 2014; Lakes-Harlan and Heller, 1992; Robert et al., 1992; Zuk and Kolluru, 1998). Those *Mecopoda* song parameters that attracts *Mecopoda* females more may also elicit enhanced response from the parasitoid flies as seen in *Ormia ochracea* (Wagner, 1996). There would be pressure from both natural selection and sexual selection on the calling song and there is likely to be an evolutionary interaction between two different selection pressures on calling song structure. This evolutionary interaction could lead to host species making calls that are less attractive for parasitoids and more attractive for potential mates, which could result in an evolutionary arms race to achieve novel calls in host species to ward off parasites and novel mechanisms in parasites to tune in to and locate the host species call (Zuk and Kolluru, 1998). This might, in turn, lead to divergence of calls in host species as we see in *Mecopoda*. Thus, discovery of parasitoids affecting *Mecopoda* population throws open a new question of whether their divergence is also a product of the effect of infection by the parasitoids. It will be also interesting to test if Tachinid parasitoids of *Mecopoda* are themselves diverged populations over their distribution like another Tachinid species, *Ormia ochracea* that has shown strong attraction to the song of the local hosts (Gray et al., 2007).

Chapter 6

Conclusion

6.1 Reproductive isolation in *Mecopoda*

Acoustically divergent groups of *Mecopoda* form the study system for this thesis. It is relatively rare to find a study system that is so well suited to addressing so many pending issues in speciation research. Previous to my work, these divergent groups had been described as differing only in the temporal parameters of the male calls, and in their natural distributions, they are found in various sympatric combinations with one another and have overlapping reproductive seasons. These populations were considered morphologically indistinguishable based on a traditional multivariate morphometric study of 75 quantitative and 61 qualitative characters. These features of *Mecopoda* populations led us to assume that they could be a cryptic species complex (Nityananda and Balakrishnan, 2006).

Mecopoda males use acoustic signals to attract potential mates from a distance. The divergence and maintenance of such sexual signals in the population indicates the probable presence of a reciprocal female preference, such that females of a particular songtype are expected to respond preferentially to males of their 'own' songtype. How this divergence in the population was initiated is unknown but we can investigate the possible mechanisms of reproductive isolation in these divergent population.

In common with other orthopteran species, it is likely that *Mecopoda* might be affected by exploiters (predators and parasites) in the wild. If acoustically orienting exploiters require a specific signal in order to locate potential hosts/prey this

could provide a with possible explanation for songtype divergence in *Mecopoda*. Tachinid flies were found to infect Double Chirper, Two Part and Helicopter males and seemed to have evolved a hearing mechanism as seen in other studied Tachinid systems (Lakes-Harlan and Heller, 1992; Robert et al., 1992). Given this evidence, we can conclude that gravid Tachinid females can track down a singing *Mecopoda* male with the help of its hearing organ and parasitise it. This provides direct evidence of selection pressure on *Mecopoda* sexual signal and could have been a contributing factor for initiation of acoustic signal divergence.

It is seldom found that population divergence in one trait involved in reproduction does not lead to divergence in other sexual traits. Nevertheless, for *Mecopoda* external morphology has diverged only very slightly, and this divergence appears to be concentrated in external genitalic structures, suggesting that it has been driven by sexual selection, rather than natural selection. The degree of morphological divergence expected out of ecological isolation was not evident. This would indicate a minimal role of ecology in the maintenance of acoustic divergence in *Mecopoda* population.

My behavioural experiments suggest that there is a complete behavioural isolation between the Chirper songtype and the three trilling songtypes (Two Part, Helicopter and Train) whereas there is an incomplete isolation between Chirper and Double Chirper songtypes. Although incomplete, the strength of this behavioural isolation between Chirper and Double Chirper was found to be strong through mating experiments and it appears that a successful mating event between Chirper females and Double Chirper males in the wild is improbable. There is good evidence that there is stabilizing selection on the chirp rates of Chirper calls and it is unlikely that the chirp rate acts as the explanation for the occasional phonotactic response by Chirper females towards Double Chirper calls. Besides, Chirper females appear to have strong specificity for Chirper call structure, which might prevent Chirper females from approaching Double Chirper males. Although the exact call trait(s) responsible for such aberrant phonotactic behaviour of Chirper females need to be ascertained by further experiments, it appears that the divergent acoustic signals of Chirper and Double Chirper may be acting as the basis of mate choice in Chirper females. This study indicates that mate choice both via phonotaxis and subsequently once the two are in contact with one another in sympatric populations of *Mecopoda* may be sufficiently strong to maintain behavioural isolation among songtypes in *Mecopoda*.

The other line of evidence that there may be behavioural isolation is derived from profiling the chemical components of *Mecopoda* cuticle. In this study, we have found differences in the chemical profiles of *Mecopoda* songtypes which allowed us to statistically differentiate the songtypes into five groups with high confidence. Cuticular chemicals are used for species level identification during direct encounters between potential mating pairs and if the chemical profiles of *Mecopoda* songtypes do not match, actual copulation may be stalled. This is because the differences in chemical components found on *Mecopoda* males of a particular songtype could lead to reduced attraction of female individuals from acoustically divergent groups. Since CHCs can act as sexual signals at close range, the divergence in cuticular profiles indicates premating reproductive isolation in *Mecopoda* as also indicated by acoustic sexual signal divergence.

Genital morphology is usually used for species level identification in insects. Although general morphology was found to be broadly similar in *Mecopoda* songtypes, robust shape analysis tool such as geometric morphometrics gave us an opportunity to look at some genital characters in detail. Surprisingly, the two morphological characters found prominently on *Mecopoda* males, cerci and subgenital plate that were studied were able to differentiate the songtypes into five distinct clusters in a statistical analysis. We demonstrate, however, that the differences in shape are subtle and this does not evidently change the cryptic nature of the *Mecopoda* songtypes. Cerci and the subgenital plate have, in fact, sensory roles and take part before and during copulation. The subtle differences in shape of these possible secondary sexual characters can act as tactile isolating mechanisms where female of a particular songtype may be able to feel the differences in male genital morphology of a different songtype and subsequently avoid mating with the male. This indicates that divergence in genital morphology has the potential to lead to isolation in *Mecopoda*. Although subtlety in differences in cuticular profiles and the genital morphology of *Mecopoda* do not match the major divergence of acoustic signals, they produce ample evidence of population divergence. It is interesting to note that the divergence appears to follow a similar trajectory in both the cases but a quantitative study is needed to establish it. This population divergence in *Mecopoda* may lead to reproductive isolation of the divergent populations due to assortative mating causing restriction of gene flow among the divergent populations. This may ultimately lead to their speciation.

6.2 Drawbacks of *Mecopoda* as model system for studying speciation

One of the most critical preconditions for this study was to establish a pure bred population of each *Mecopoda* songtype. This was very important given the fact that populations of *Mecopoda* songtypes seldom occur in allopatry. With females not calling, there is no way to identify songtypes of females in the wild (Nityananda and Balakrishnan, 2006) and they are at least necessary for the very important behavioural assays. Even males are difficult to identify if they do not call. An attempt to rear *Mecopoda* in the laboratory, before I commenced this study failed. This is unexpected because *Mecopoda* (albeit the Malaysian species) has been successfully reared in at least one other lab (Römer et al., 2002).

During field collecting, I observed that Chirper and Train songtypes occur predominantly west of the Western Ghats, a narrow hill range along the western coast of southern India, in spatial and temporal sympatry whereas the other three songtypes occur on and east of the Western Ghats. Even as *Mecopoda* Chirper distribution overlapped with that of Train, I was able to establish a *Mecopoda* Chirper culture from an allopatric population of *Mecopoda* found in the Indian Institute of Science campus. No other songtypes were found in such allopatry during the course of study. For other songtypes, I attempted to lab rear a population from wild caught *Mecopoda* females but failed repeatedly because of high mortality during the nymphal stage. It appeared as if *Mecopoda* (Double Chirper, Helicopter and Two Part) from the forested region of the Western Ghats were sensitive to some aspect of culture conditions. In general, *Mecopoda* seem to be less prolific breeders than field crickets where it is possible to rapidly establish large populations. Non-availability of pure bred populations of the different songtypes other than Chirper is a significant drawback of this system.

Another practical drawback was the non-availability of the different songtypes outside of their relatively brief breeding seasons. Typically, the active reproductive period lasts around three months annually. Thus, most of the research work concentrated around brief periods, making it difficult to work simultaneously on different experiments. Additionally, it was not feasible to use wild *Mecopoda* females in behaviour experiments as they mostly lacked motivation for phonotaxis. In a pilot experiment, the percentage of wild *Mecopoda* females showing phonotaxis to stimuli was found to be around 10 %. It was also very difficult to spot female

nymphs in the wild especially in the forest habitats. The wild caught females of unknown status were also more agitated in the lab condition even after giving them ample time to acclimatize. Moreover, we could never be certain about the ages of the wild caught females, which could potentially affect their motivation to mate.

Mecopoda are found in a variety of landscapes: undisturbed forests, degraded forests, plantations and near human habitats in urban gardens. They are conspicuous by their loud, broadband calls, which can be heard from a long distance at night. They have tendency to be active during the new moon phase and appear to hide under vegetation during the day, *Mecopoda* are omnivorous and appear to like many different plant materials. They are sturdy animals and adult females have been found to have the ability to survive for six months in laboratory conditions. Survivability in field would however be dependent on many other factors not encountered in the laboratory. They tend to hide or freeze on detecting possible danger. They appear to be slow fliers but in adverse condition, they tend to jump with help of their strong hind legs to escape and have been found to autotomize their hind legs to escape capture. Apart from these observational information, nothing much is known about their ecology nor has there been any dedicated study in India in these regards.

6.3 Future aspects regarding studies on *Mecopoda* speciation

Studies on the *Mecopoda* system would benefit from information on its ecology. We lack detailed information on the distribution and abundance of *Mecopoda* in India. The occurrence of *Mecopoda elongata* has been reported widely from different places in India but they are just presence absence reports. Moreover, such reports do not have information on their calls or songtypes as well as seasonality. As such, there is dearth of knowledge about much of their natural history apart from personal observations and anecdotes.

Similarly, not much is known about the population structure of *Mecopoda* and its songtypes. A preliminary unpublished study has suggested a low genetic divergence based on a single marker among the songtypes that indicates a relatively fast differentiation and recent divergence in song patterns in Indian *Mecopoda*

population. This study nevertheless can be expanded to include more mitochondrial markers that are available now and other nuclear markers such as microsatellites. An all encompassing phylogeographic study would need a countrywide field sampling followed by analysis of population genetic structures. In 2013 (Zhi Jun et al., 2013), a complete mitochondrial genome sequence was made available for the two *Mecopoda* species, *Mecopoda elongata* (GenBank Accession No: JQ917910.1) and *Mecopoda niponensis* (GenBank Accession No: JQ917909.1). Apart from this, we also have accessible data on 18s ribosomal RNA sequence, cytochrome oxidase subunit I & II and subunits of RNA polymerase as mitochondrial markers. Microsatellite markers need to be developed also and it might lead to an immense step forward towards understanding the process of speciation in *Mecopoda*. In a haplotype network analysis, old haplotypes will generally be shared by all groups while newer haplotypes will mostly be limited to the derived groups. If we find this trend in *Mecopoda* population genetic structure by taking all markers into consideration, we will get additional support for the song type divergence. Besides we may construct phylogenetic trees and derive relations between the ancestral songtype and the derived groups. We should use *Mecopoda niponensis* as an outgroup in this study. This will make a strong case for species status of the *Mecopoda* songtypes. Loss of heterozygosity in sub populations of a species can indicate that the genetic drift may have been responsible in their divergence. The possibility exists that Chirper songtypes in south India could have diverged due to genetic drift in sympatry but testing this hypothesis will require detailed information on the distribution of the different songtypes with further behavioural and phylogenetic studies.

Another aspect that is understudied is the various causes leading to songtype divergence in the first place. Bat predation and Tachinid infection points to possible causes of such divergence but a dedicated study on this aspect could help us find the vital clues about the initiation of divergence.

This study on divergent *Mecopoda* populations points to a significant contribution of mate choice in what may be incipient speciation in *Mecopoda*. One of the most important step that has to be followed after this current study is to compare the divergence of different sexual traits. It is imperative to measure the effect of female preference for these different sexual traits through behavioural assays to test if sexual selection is the main driver for population diversification, and which other cues are most important in creating isolation.

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