Sexual conflict in the penduline tits (Remizidae): implications for sperm competition and speciation

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Sexual conflict in the penduline tits (*Remizidae*): implications for sperm competition and speciation

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Contents

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II. Declaration of Contributions

I hereby declare that this thesis is my own work, written and compiled by Alexander Ball. However the contributions of co-authors with whom this work is being prepared for submission are described below. I also include the names of the relevant co-contributors in the heading page of each chapter. Tamás Székely and Steve Dorus have supervised me throughout my research and as such are acknowledged at the start of all chapters.

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III. Summary

This thesis explores the far-reaching impacts of sexual conflict over care on a suite of traits in the penduline tit family (*Remizidae*), further confirming the intricate relationships between parental care, mating systems and sexual selection. The results reveal the first genetic phylogeny of this family and suggest that uniparental care evolved once in this group. The transition to uniparental care is associated with rapid evolution of male plumage ornaments most likely driven by increased sexual selection. The results also suggest a relationship between male care and the likelihood of paternity on an evolutionary time-scale, as the biparental species exhibit much lower levels of promiscuity than the uniparental European penduline tit. Increased promiscuity was also found to impact sperm morphology in the penduline tits and allies with greater sperm length uniformity in more promiscuous species. This sperm trait was also discovered to co-vary with a sexually selected plumage trait in the European penduline tit suggesting potential interactions between female mate choice and male fertility. An investigation into genetic diversity within the *Sylvioidea* super-family finds large variation but does not suggest any link between promiscuity and genetic diversity as predicted if promiscuity maintains a higher effective population size in these passerines. The work highlights the interlinked relationships between parental care, mating systems and sexually selected traits, which are increasingly studied in concert. The consequences of sexual conflict over care appear to be far-reaching in the penduline tits, however the degree to which they feedback upon each other and the effect that it has on speciation remains to be seen. The penduline tits further prove their ability to provide valuable insight into the evolution of sexual conflict.

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VI. Introduction

The historic focus on the harmonious relationships thought necessary for successful propagation have been swept aside in the last century by the realisation that sex is awash with conflicts of interest (Trivers, 1972; Parker, 1979; Halliday & Arnold, 1984; Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005; Gavrilets & Rice, eds, 2014). In the last few decades the study of these conflicts has laid the foundations for sexual conflict theory. All aspects of reproduction are now coming under scrutiny and revealing an on-going tug-of-war between traits such as mate choice, mating frequency and parental investment (David F Westneat & Sargent, 1996; Chapman, Arnqvist, *et al.*, 2003; Parker, 2006).

Sexual conflict theory

Sexual conflict arises when the sexes diverge in their evolutionary optima, thus male and female fitness is not maximised via the same processes. Sexual conflict can act on a shared trait that has a different optimum value in both males and females (intra-locus conflict) or it can arise if a trait exhibited by one sex is detrimental to the fitness of the other (inter-locus conflict). Intra-locus conflict over a shared trait is predicted to favour differential expression of the genes controlling the trait in each sex, likely leading to the evolution of sexual dimorphism (Bonduriansky & Chenoweth, 2009). However inter-locus conflict has gained more attention and refers to the expression of an advantageous trait in one sex, which is detrimental to the fitness of the other. An important example of this form of conflict has been discovered in the chemicals transferred in seminal fluid, which can manipulate the behaviour and physiology of females (Johnstone & Keller, 2000; Chapman, Bangham, *et al.*, 2003; Wigby & Chapman, 2005). In this case selection would favour a counter adaptation that could limit the influence of these chemicals (Wigby & Chapman, 2004). Inter-locus conflict is theorized to lead to co-evolutionary arms-races in which each sex is constantly trying to counter the negative effects of the other (Dawkins & Krebs, 1979; Parker, 1979; Lessells, 2012; Székely, 2014).

Sexual conflict is thought to emerge from divergent interests stemming from anisogamy, the differential investment in female compared to male gametes. This idea of the differential selection pressures on the rare sex compared to the more common sex is fundamental to sexual conflict theory. In populations with lifelong monogamy a male's and female's reproductive success will be completely dependent on each other and the variance in reproductive success will be identical for each sex. However if this variance is greater in males than females, selection pressures on each will diverge. Thus recent research is focusing on the differences between the number of mature adult males compared to females, the adult sex ratio (ASR), in a population compared to the actual numbers of males and females that provide offspring to the next generation, the operational sex ratio (OSR) (Liker *et al.*, 2013, 2014). Any deviations from equality in these ratios would lead to differences in the reproductive pressures acting on males and females, setting up the potential for conflict.

Thus sexual conflict stems from the differing selection pressures that are predicted to operate on the more frequent compared to the rarer sex (Emlen & Oring, 1977). These differing selection pressures can create sexual conflict over a variety of traits which in turn cascade into multiple aspects of reproduction (Kokko & Jennions, 2008; Szekely *et al.*, 2014). However it is extremely difficult to determine either the ASR or the OSR, especially in highly mobile species such as birds, and so most research has relied upon manipulations of the ASR in lab organisms (Clark, 1988; Berglund, 1994; Alonso-Pimentel & Papaj, 1996; Burley & Calkins, 1999; Jirotkul, 1999; Klemme *et al.*, 2007). However this is not the only obstacle in sexual conflict research with further complexities created by the myriad of variables that could affect both the ASR and OSR, including both the mating system and parental care behaviours which in turn feedback on each other (Kvarnemo & Ahnesjo, 1996; Kokko & Jennions, 2008). For example polygamy could cause uncertain parentage for one sex but not the other leading to lower parental investment by one sex, returning one sex to the mating pool sooner than the other. Alternatively this heavy bias in the ASR could favour

individuals of the more common sex providing greater parental care rather than returning to a mating pool in which they have little chance of success. Thus the complex feedbacks operating between these multiple traits are far from clear and have made it very difficult to pin down universal predictors of mating systems, parental care and sex roles (Kokko *et al.*, 2006; Kokko & Jennions, 2008). Thus an in-depth knowledge of individual study systems seems vital in order to disentangle the traits most likely to be important in individual contexts.

Sexual conflict and sperm competition

Multiple mating by females is an evolutionary conundrum. If one male can provide more sperm than the number of eggs a female possesses, why would any female gain higher fitness by mating with multiple males? In essence, by mating multiply, a female reduces the potential fitness of her mate with no obvious gains to her own. The revelation of the pervasive nature of female promiscuity has therefore gained enormous interest. There are many competing hypotheses to account for this behaviour with a large proportion attempting to identify the fitness benefits that females could gain from such behaviour. In some cases multiple mating by females is thought to stem from sexual conflict over mating rate, as males are selected to coerce females at the detriment to a female's fitness. This hypothesis posits sperm competition as a by-product of female behaviour that attempts to alleviate male aggression (Svard & Wiklund, 1986; Halliday & Arnold, 1987; Ebensperger, 1998).

However the positive solicitation of additional mates by females in many species suggests that it is not only driven by male coercion (Davies *et al.*, 1996). Research into the direct (such as increased parental investment or greater fertility) and indirect benefits (such as increased offspring heterozygosity) of multiple mating by females have been investigated (reviewed in (Arnqvist & Nilsson, 2000; Jennions & Petrie, 2000)). As sperm competition has a direct impact on a male's potential fitness, multiple mating by females produces large conflicts of interest between the sexes. This has been instrumental in spearheading work on sexual conflict in *Drosophila* sp. In order to limit sperm

competition, males have evolved to transfer chemicals in their seminal fluid that reduce a females propensity to remate (Chapman, Bangham, *et al.*, 2003), but they also have negative consequences for a female's lifetime fitness. However in birds, where large parental investment is often required, work has focused on the influence of multiple mating to male parental effort. Research has indicated that conflict over care could be influenced by the infidelity of parents (Terry Burke *et al.*, 1989; García-Navas *et al.*, 2013).

As the pervasive nature of promiscuity in birds became clear the significance of post-copulatory processes to reproductive success moved to the fore. Work has revealed a substantial impact of promiscuity on male investment in gonadal tissue and also on sperm morphology (Moller, 1991; Briskie *et al.*, 1997; Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lüpold, Calhim, *et al.*, 2009) . What is not so clear is the relationship between pre and post-copulatory traits, are there trade-offs between them? Do they covary? Are they indicative of genetic or direct benefits? (Andersson & Simmons, 2006; Lüpold *et al.*, 2014). As our understanding of these individual aspects deepens our ability to place them in a unifying context with parental care, mating systems and sex roles moves closer.

Sexual conflict and speciation

It is the idea of run-away co-evolutionary "arms races" that has lead to the supposition that sexual conflict could play a large role in the divergence of species (Parker & Partridge, 1998; Arnqvist *et al.*, 2000; Gavrilets, 2000, 2014). These arms-races are likely to lead to rapid evolution of traits intimately entwined with reproductive success. Traits involved in mate-choice and fertilisation are those which define a species as they are critical for the union of male and female gametes. Sexual conflict therefore has the ability to rapidly affect traits at the cusp of reproduction, providing the potential for rapid impacts on pre and post-mating isolation, which subsequently define the separation of species (Gavrilets & Waxman, 2002; Martin & Hosken, 2003).

Although theory has predicted a link between sexual conflict and speciation, empirical evidence in support of this is limited (Martin & Hosken, 2003; Ritchie, 2007; Butlin, 2011). One promising avenue of research that could remedy this is to narrow the focus on closely related species with large differences in their levels of sexual conflict. The comparative approach is a fundamental tool in evolutionary biology that allows inferences to be drawn from the natural experiments that have played out through time (Harvey & Pagel, 1991; Martins, 1996; Freckleton & Pagel, 2010). Presently studies on disparate groups are revealing parallel effects of sexual selection on speciation rates (Kraaijeveld *et al.*, 2011). Comparative studies in fish and insects have also found a relationship between sexual conflict and speciation, using both post-mating and pre-mating measures of sexual conflict (Arnqvist *et al.*, 2000; Hosken *et al.*, 2009; Wagner *et al.*, 2012). However in birds, a group in which there is extensive knowledge of breeding systems and mating behaviours similar studies have revealed contrasting results (Morrow *et al.*, 2003; Krüger, 2008).

Sexual selection theory predicts the rapid evolution of selected traits and the genes that control them, thus the ability to sequence the relevant genes across species has provided evidence for the rapid evolution of these regions compared to the majority of the genome (Dorus *et al.*, 2004; Haerty *et al.*, 2007; Nadeau *et al.*, 2007). However work has also begun to explore the additional genetic consequences which sexual selection can have upon the evolution of the genome and in turn species divergence. Theory predicts that highly polygamous species, in which a reduced number of individuals of one sex monopolise the reproductive success, will have much lower effective population sizes (Ne) than a comparably sized monogamous population (Wright, 1931; Chesser, 1991). Recently a study in shorebirds has linked polygyny to decreased levels of genetic diversity on the Z chromosome, the sex chromosome which is homogametic in males (ZZ) compared to females (ZW) (Corl & Ellegren, 2012). However there is controversy over the effect that mating systems will have on whole genome genetic diversity, which is also impacted by the effective population size (Ne), recent theory proposes that the maintenance of genetic diversity in promiscuous species hinges on a range of life-history traits,

including longevity, level of promiscuity and variance in female breeding success (Lotterhos, 2011). If promiscuity is able to maintain genetic diversity it provides an advantage to promiscuous species in the face of environmental change, perhaps leading to greater capacity for rapid divergence.

Sexual conflict in birds

Birds have provided some of the most important insights into sexual selection research due to the detailed behavioural data available and the conspicuous visual ornaments which have interested biologist's for centuries (Darwin, 1871; Andersson, 1994). As the conflicts of interest between the sexes were revealed, this wealth of knowledge on birds provided a very large resource for studies on sexual conflict.

A large focus of sexual conflict research in birds has focused on conflict over parental care (Slagsvold & Lifjeld, 1989; Székely *et al.*, 2007; LaBarbera *et al.*, 2012; van Dijk *et al.*, 2012; Parker *et al.*, 2014). It is theorised that in polygamous systems each parent should attempt to reduce its parental investment whilst maximising the effort provided by its partner (Trivers, 1972; Queller, 1997). This would conserve resources for future reproduction whilst maintaining high fitness from the current offspring. Obviously this strategy would only be beneficial in polygamous systems where lifetime reproductive success is not dependent entirely on your partner. This prediction has found support in a range of species where investment by one sex can be manipulated by the other (Davies *et al.*, 1992; Valera *et al.*, 1997; Komdeur *et al.*, 2002). However the implications of conflict over care are far-reaching and are likely to affect a range of other traits. Proposals for a more inclusive approach to the study of sexual conflict are being made, in order to untangle the complex interplay between, mating systems, parental care and sexual selection (Kokko *et al.*, 2006). Two important factors theorised to link these three complex areas are biases in operational sex ratios and the differing abilities of males and females to allocate care to their genetic offspring (Kokko & Jennions, 2008).

As research has revealed the large scale of female infidelity, work on post-copulatory traits has moved to the fore (Birkhead & Pizzari, 2002). However combining study of post and pre-copulatory processes has only recently gained traction. This thesis attempts to explore the relationships between sexual conflict over care and pre and post-copulatory sexual selection, using the penduline tit as a model system.

Penduline tits as ecological model systems

The penduline tits (*Remizidae*) are a small family (~13 species) of passerine birds closely related to the true tits (*Paridae*). They all share a small body size (6-10g) and a diet consisting predominantly of insects and spiders. They have a large global distribution, being found throughout Eurasia and Africa, with an additional species occurring in North America (Figure 1.1). This large distribution is unsurprisingly coupled with a wide diversity of habitats, and species are found from arid deserts to tropical rainforests. However it is their breeding behaviour that is also extremely unusual having captured the interest of biologists in the last few decades.

The discovery of the unusual mating system of the European penduline tit (*Remiz pendulinus*) catapulted the penduline tits into the path of evolutionary ecologists (Persson & Ohrstrom, 1989). They were the first avian species defined as ambi-sexually polygamous; in essence they exhibit a mating system of rapid sequential polygamy by both sexes within a single breeding season (van Dijk, Szentirmai, Komdeur, *et al.*, 2007). Its implications for parental care are extensive with large proportions (~30%) of nests abandoned by both parents, as instead they attempt to find a new mate. Further investigation has attributed this supposedly maladaptive behaviour to sexual conflict, as each parent increases its fitness by shifting care to their partner while they leave to start a new breeding attempt (Szentirmai *et al.*, 2007; Pogány *et al.*, 2008, 2012; van Dijk *et al.*, 2012). A behavioural arms race between the two sexes has been documented with females hiding their investment by covering their eggs and attacking the male when he attempts to enter the nest (Valera *et al.*, 1997). In

this way it is thought they attempt to delay the males abandonment until a clutch of eggs is produced which is large enough to be in his interest to incubate.

Both sexes therefore attempt to reduce their investment in offspring care at the expense of their partner's fitness (Szentirmai *et al.*, 2007). The aim of this thesis is to reveal the interplay between sexual conflict over care and the evolution of other aspects of the species biology likely to affect speciation, including mate choice, sperm competition and genetic diversity. Questions are posed from an evolutionary perspective and attempts are made to test predictions using the diversity found within the penduline tit family and its close relatives.

Thesis structure

In **Chapter 1** the taxonomic relationships of the penduline tit family are revealed by inferring the first molecular phylogeny of this group. The penduline tits have provided contentious debate among taxonomists with classifications varying throughout the last century. Five gene regions were sequenced and multiple phylogenetic methods are used to infer the most likely phylogeny for use in the analyses in later chapters.

Chapter 2 investigates the relationship between parental care and genetic parentage. Evolutionary theory predicts that in socially monogamous species loss of paternity will lead to sexual conflict resulting in reduced paternal compared to maternal care. Here the parental care systems of two biparental species of penduline tit are described and genetic markers are used to assess extra pair paternity levels in each. The results are contrasted with the previous studies in the uniparental European penduline tit.

Chapter 3 focuses on the evolution of sexual dimorphism within the penduline tit family. Sexual dimorphism provides an indication of the selection pressures that differ between the sexes. An attempt is made to discover the extent and drivers of sexual dimorphism in this family and use it to test whether sexual

selection is greater in males or females in species undergoing high conflict over care.

Chapter 4 explores post-copulatory sexual selection pressures. Penduline tits and other passerine allies are used to investigate the impact that infidelity has on sperm morphology. The prediction that under high levels of sperm competition, males will evolve longer sperm is tested.

Chapter 5 investigates the trade-offs between post-copulatory (sperm morphology) and pre-copulatory (plumage) traits in the European penduline tit and to test whether pre-copulatory traits can be used by females to assess post-copulatory competition. Two populations are also compared to investigate the within-species variation in these sexually selected traits.

Chapter 6 tackles the wider genomic implications of sexual conflict by investigating the relationship between female infidelity (via its effect on Ne) and genetic diversity in the penduline tits and allies.

The main findings are summarised and interesting avenues for future work are discussed.

Chapter 1

Molecular phylogeny of the penduline tits (*Remizidae*)

Alexander D. Ball, René E. van Dijk, Martin Irestedt, Ákos Pogány, Sander Bot, Duŝan Brinkhuisen, Jan Komdeur, Per Ericson, Steve Dorus & Tamás Székely

Abstract

The Penduline tits (*Remizidae*) are a family of small passerine birds currently emerging as a model system in studies of sexual conflict. One species, the European penduline tit (*Remiz pendulinus*) is a highly polygamous passerine species, with high rates of nest desertion, however, other species such as the Cape penduline tit, appear to be biparental and monogamous; a more typical strategy in passerine birds. The ability to investigate the ecological and life-history traits influencing this diversity has been hampered by the lack of a robust phylogeny. Here we provide the most comprehensive phylogeny of penduline tits by sequencing 5 gene regions across all species currently classified within this group. We use 3 nuclear introns [glyceraldehyde-3-phosphodehydrogenase intron 11 (G3P-11), ornithine decarboxylase introns 6 and 7 (ODC-6-7), and brahma intron 15 (BRM-15)] and 2 mitochondrial genes [cytochrome b (Cyt-B) and nicotinamide adenine dinucleotide dehydrogenase subunit 3 (ND3)] to infer the evolutionary relationships via a number of methods. We find 1) that the current classification systems of penduline tits are not satisfactory given that they refer to a polyphyletic group 2) Three genera make up a monophyletic clade which we subsequently define as *Remizidae*; 3) Speciation patterns are significantly different in each of these three genera. We produce a robust phylogeny for this ecologically interesting passerine family, providing the most comprehensive opportunity to test competing evolutionary hypotheses.

Introduction

One of the most puzzling topics in evolutionary biology is the presence of sex and its diverse manifestations across sexually reproducing organisms. However, in order to understand its evolutionary success we also have to be aware of its costs. Sexual conflict arises because of the competing interests of the two partners required for successful reproduction. Conflict can occur over many different aspects of reproduction from mating frequency to parental investment and affects the evolution of a range of physical and behavioural traits (Chapman, Arnqvist, *et al.*, 2003). The mating system and parental care of species are intimately entwined with levels of sexual conflict (David F. Westneat & Sargent, 1996; Kokko & Jennions, 2008; Székely, 2014). Both of these aspects of reproduction are not randomly distributed across species; exhibiting strong phylogenetic signal (Webb *et al.*, 2010). This allows broad interpretations of the underlying mechanisms that maintain sexual conflict but makes it difficult to pinpoint the key attributes that lead to changes in its intensity. Therefore the study of the exceptions, closely related species that show large differences in sexual conflict, allow insights into the key traits that affect the complex milieu of sexual reproduction.

In birds a large knowledge of mating systems and parental care strategies have been documented by ornithologists over the last couple of centuries. This huge repository reveals that the majority (81%) of birds exhibit social monogamy with biparental care (Cockburn, 2006). However there are key exceptions to this rule which provide the opportunity to disentangle the drivers of sexual conflict. The penduline tits are one group which show unusually diverse mating and parental care behaviours, the European penduline tit was the first species in which substantial sequential polygamy by both sexes was documented, termed ambi-sexual polygamy by (Persson & Ohrstrom, 1989). This behaviour was coupled with high levels of clutch desertion by both sexes (>30% of nests abandoned by both parents) (Persson & Ohrstrom, 1989; Pogány *et al.*, 2008; van Dijk, Brinkhuizen, *et al.*, 2010). Subsequent studies has revealed that the most likely cause of this aberrant behaviour is sexual conflict over offspring

care, with both sexes increasing their reproductive success if they are able to transfer parental care of their current brood onto their partner (van Dijk, Szentirmai, Komdeur, *et al.*, 2007; van Dijk *et al.*, 2012). However it is not only this unusual behaviour but the fact that other species of penduline tit exhibit the typical passerine behaviour, of social monogamy coupled with biparental care, that make this group of interest to the understanding of the drivers of sexual conflict. The contrasting reproductive strategies of these close relatives could allow us to isolate the recent cause of these different evolutionary trajectories and thus illuminate potential drivers of sexual conflict.

The key to understanding the differences between species however is an accurate knowledge of their evolutionary history. Currently the relationships of the penduline tits are in flux; 9 conflicting classification systems have been proposed within the last century (Table 1.1). These have involved differences not only at the species and genus levels but also the family status of the penduline tits has been hotly debated (Madge, 2008). This state of flux has been driven by various anatomical and behavioural studies which have failed to reach a consensus, only recently has genetic evidence been put forward, and this has also provided varying conclusions.

As their name suggests the penduline tits share many anatomical and behavioural traits with the true tits (*Paridae*). This led to taxonomists initially grouping the penduline tits within the family *Paridae* as the sub-family *Remizinae* (Mayr & Amadon, 1951), however Vaurie (1957) advocated separating the *Paridae* into three families, the true tits (*Paridae*), long-tailed tits (*Aegithalidae*) and the penduline tits (*Remizidae*)(Vaurie, 1957). *Aegithalidae* is now unanimously treated as a family in its own right since molecular studies confirmed its tenuous relationship to *Paridae* (Ericson & Johansson, 2003). However, the treatment of the penduline tits still differs between authors, some advocating full family status and others content with defining them as a sub-family within *Paridae* (see Table 1.1). What is clear is that the two are closely related, and the limited genetic evidence suggests that they are sister clades (Gill *et al.*, 2005; Johansson *et al.*, 2008; Dai *et al.*, 2010). This recent molecular

work also suggests that the split between these two clades is extremely basal, occurring soon after their split from other diverse passerine families. For this reason a distinction between the two is recognised in this work and throughout will refer to the True tits and the Penduline tits by their familial names, *Paridae* and *Remizidae* respectively. This is also in accordance with Harrap & Quinn (1996), the classification system used for the nomenclature throughout this chapter.

Another area of contention has been the inclusion of certain genera within the *Remizidae*, currently five are recognized to varying degrees; *Remiz*, *Anthoscopus*, *Auriparus*, *Cephalopyrus* and *Pholidornis*. *Remiz* and *Anthoscopus* make up the backbone of the family, comprising a Eurasian and African clade respectively (Figure 1.1). The other three are distinct monotypic genera and have been included in (or rejected from) *Remizidae* by various taxonomists (summarised in Table 1.1). These are the Verdin (*Auriparus flaviceps*), the Fire-capped tit (*Cephalopyrus flammiceps)* and the Tit-hylia (*Pholidornis rushiae*). There has been much debate over whether these are closely related to the other penduline tits (Taylor, 1971; Madge, 2008). The Tit-hylia as its name suggests was once placed in the family *Hyliidae* with the Green hylia (*Hylia prasina*), and has been placed in no less than 5 other families throughout history, including *Dicaeidae, Nectariniidae, Estrilidae, Meliphagidae* and *Sylvidae* before being tentatively placed in the *Remizidae* (Vernon & Dean, 1975; Madge, 2008). The fire-capped tit has also been placed in another family, the *Regulidae*, before placement within the penduline tits (Bates, 1952). Finally the Verdin was recently placed within the gnatcatchers (*Polioptilidae*) until skeletal evidence in favour of the penduline tits moved it back (Webster, 2000), however the disparity of its geographic position still provides some contention (see Figure 1.1).

Further controversy exists among the species level relationships, most notably within the Eurasian genus *Remiz*. As can be seen in Table 1.1, authors often vary in the number of species they include within *Remiz*. For a current review of *Remiz* identification and classification see (Bot *et al.*, 2011). Species within the *Anthoscopus* genus also show differing treatment throughout the last century,

with the African penduline tit (*Anthoscopus caroli*) providing particular problems. It is highly variable across its range leading to some taxonomists splitting it into 2 species and suggesting that even four might exist (Sibley & Monroe, 1990; Harrap & Quinn, 1996); implying that the subspecies *ansorgei*, *sylviella* and *rankinei* could all be elevated to species status. The last two of these already have common names distinguishing them from the African penduline tit, the Buff-bellied and Zambezi penduline tit respectively. Other taxonomists have gone further and elevated the Buff-bellied penduline tit (*Anthoscopus sylviella*) to species status (Sibley & Monroe, 1990).

The discrepancies in the systematics of the penduline tits are understandable, many of the species have not been studied in any great detail, and genetic data has been extremely sparse. The fundamental conclusion by most authors is that more work is needed before any of these classification systems can be fully validated. The aim of this study is to produce a comprehensive genetic phylogeny for the *Remizidae* family, providing us with a unique opportunity to clarify the taxonomic situation and provide the phylogenetic basis for detailed evolutionary studies in this complex group.

Table 1.1. A summary of the contradicting classification systems for the penduline tits (*Remizidae*), put forward during the last 60 years.

(Portenko, 1955)	(Vaurie, 1957)	(Dolgushin *et al.*, 1972)	(Howard & Moore, 1980)	(Stepanyan, 1990)	(Sibley & Monroe, 1990)	(Harrap & Quinn, 1996)	(Fry *et al.*, 2000)	(Eck & Martens, 2006)	(Gill & Wright, 2006)	(Madge, 2008)
Unknown	Family	Unknown	Family	Unknown	Sub-family of *Certhiidae*	Sub-family of *Paridae*	Family	Family	Family	Family
R. pendulinus	*R. pendulinus*	*R. pendulinus*	*Remiz pendulinus*	*R. pendulinus*	*R. pendulinus*	R. pendulinus	*R. pendulinus*	*R. pendulinus*	R. pendulinus	R. pendulinus
in *R. pendulinus*	in *R. pendulinus*	*R. macronyx*	in *R. pendulinus*	*R. macronyx*	in *R. pendulinus*	R. macronyx	*R. macronyx*	in *R. pendulinus*	R. macronyx	R. macronyx
in *R. pendulinus*	in *R. pendulinus*	*R. coronatus*	in *R. pendulinus*	in *R. pendulinus*	*R. coronatus*	R. coronatus	*R. coronatus*	in *R. consobrinus*	R. coronatus	R. coronatus
in *R. pendulinus*	in *R. pendulinus*	-	in *R. pendulinus*	*R. consobrinus*	*R. consobrinus*	R. consobrinus	*R. consobrinus*	*R. consobrinus*	R. consobrinus	R. consobrinus
-	-	-	*Anthoscopus punctifrons*	-	*A. punctifrons*	A. punctifrons	*A. punctifrons*	-	A. punctifrons	A. punctifrons
-	-	-	*Anthoscopus musculus*	-	*A. musculus*	A. musculus	*A. musculus*	-	A. musculus	A. musculus
-	-	-	*Anthoscopus caroli*	-	*A. caroli*	*A. caroli*	*A. caroli*	-	A. caroli	A. caroli
-	-	-	*Anthoscopus sylviella*	-	*A. sylviella*	in *A. caroli*	in *A. caroli*	-	in *A. caroli*	in *A. caroli*
-	-	-	*Anthoscopus flavifrons*	-	*A. flavifrons*	*A. flavifrons*	*A. flavifrons*	-	*A. flavifrons*	*A. flavifrons*
-	-	-	*Anthoscopus parvulus*	-	*A. parvulus*	*A. parvulus*	*A. parvulus*	-	*A. parvulus*	*A. parvulus*
-	-	-	*Anthoscopus minutus*	-	*A. minutus*	*A. minutus*	*A. minutus*	-	*A. minutus*	*A. minutus*
-	-	-	*Cephalopyrus flammiceps*	-	*C. flammiceps*	*C. flammiceps*	excluded	*C. flammiceps*	*C. flammiceps*	*C. flammiceps*
-	-	-	excluded	-	*Pholidornis rushiae*	*P. rushiae*	*P. rushiae*	-	excluded	*P. rushiae*
-	-	-	*Auriparus flaviceps*	-	excluded	*Au. flaviceps*	excluded	-	*Au. flaviceps*	*Au. flaviceps*

- = Species not included in the given study

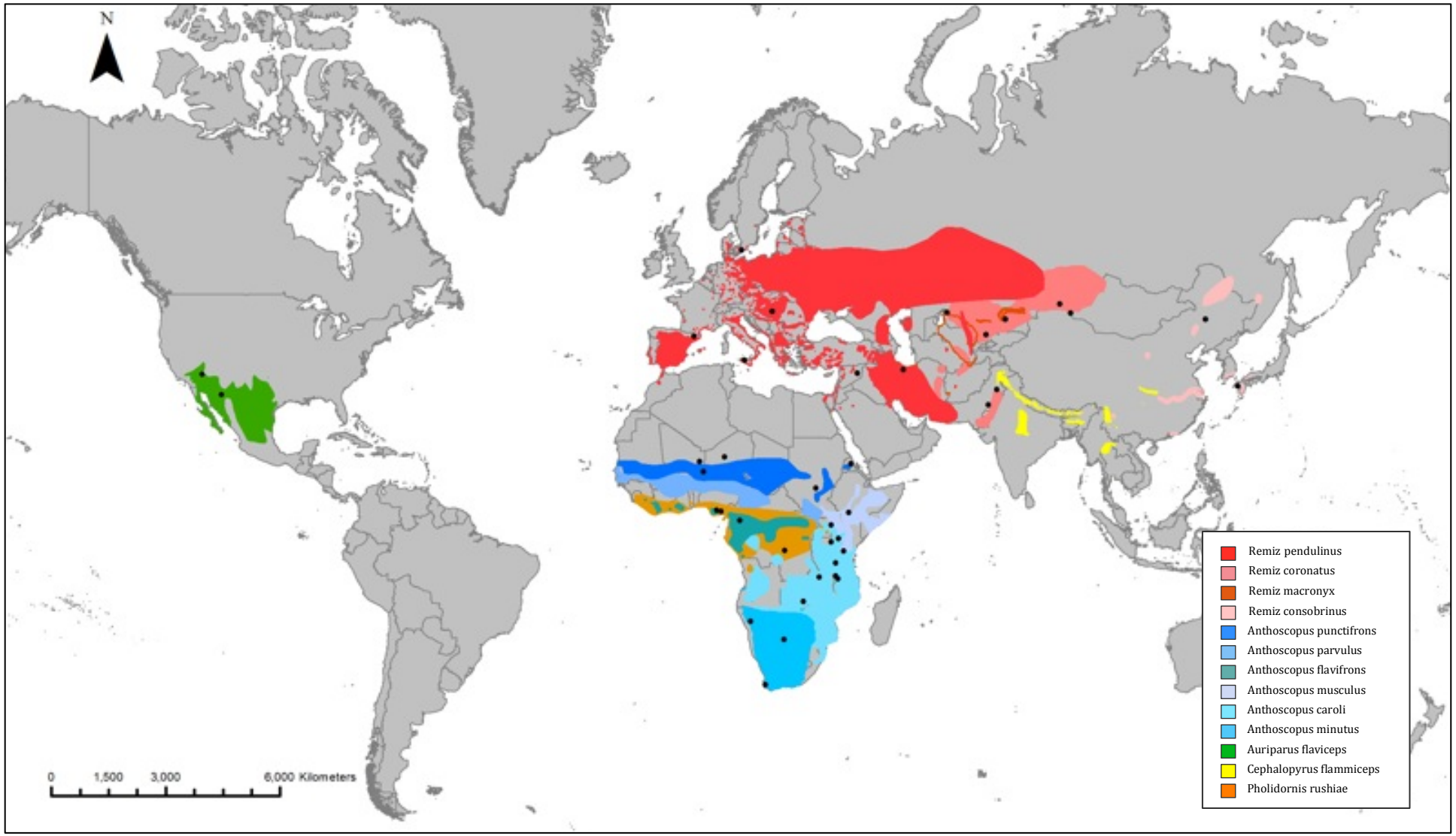

Figure 1.1. Geographic distribution of the 13 *Remizidae* species according to (Harrap & Quinn 1996). Ranges obtained from Birdlife international and the 5 genera are colour coded (*Remiz*=reds, *Anthoscopus*=blues, *Auriparus*=green, *Pholidornis*=orange and *Cephalopyrus*= yellow). Known sampling locations for this study are indicated by a black dot.

Methods

Penduline tit specimens used in this study are from a combination of museum collections (British Natural History Museum, Swedish Museum of Natural History, Bell Museum of Natural History and the Netherlands National Museum of Natural History) and fieldwork conducted throughout Eurasia and in South Africa during the years 2003-2008. All 13 species within the *Remizidae* family have been sampled (species according to (Harrap & Quinn, 1996)). Of the 45 subspecies described, 21 have been obtained for DNA sequencing. The details of the 71 individuals used for sequencing and phylogeny construction are given in Table 1.2.

DNA extraction and sequencing

DNA was extracted and sequenced using two protocols, one for fresh blood samples and the other for dried toe-pad samples collected from museum specimens. For all samples we attempted to amplify and sequence five gene regions; two mitochondrial genes, cytochrome b (Cyt-B) and nicotinamide adenine dinucleotide dehydrogenase subunit 3 (ND3), and three nuclear regions, glyceraldehyde-3-phosphodehydrogenase intron 11 (GAPDH-11), ornithine decarboxylase introns 6 and 7 (ODC-6-7) and brahma intron 15 (BRM-15). They have all been used to produce avian phylogenies in other species (Sheldon *et al.*, 2005; Irestedt *et al.*, 2006; Küpper *et al.*, 2009; Ribeiro *et al.*, 2011), due to their rapid mutation rates and to gain representatives from maternally inherited mitochondrial genes (CytB and ND3), unlinked autosomal genes (GAPDH-11 and ODC-6-7) and a sex linked gene on the Z chromosome (BRM-15).

Oligo primers designed for previous passerine sequencing efforts were initially tested for amplification success in a sub-set of individuals, however new *Remizidae* specific primers were required for the amplification and sequencing of some regions. We designed the required primers using primer-designing programs accessible via the internet, including primer3 (Rozen & Skaletsky, 2000). These allowed us to check primer melting

temperatures (Tm) and avoid primers that might have palindrome or hairpin loop structures. Pairs of primers that could form primer-dimers were identified using the program Amplify and subsequently avoided (Engels, 1993). Primer melting temperatures (Tm) were kept as similar as possible (between 60-65°C). All PCR amplification was performed using hot-start touchdown PCR, with initial denaturation of 95°C for 5 minutes. The annealing temperatures used were 1-2°C lower than the melting temperature (Tm) of the primer with the lowest Tm. An example of the most commonly used PCR protocol is, 95°C for 5 mins, then 2 cycles of 95°C for 30sec, 58°C for 30 secs, 72°C for 60 secs then followed by a four cycle phase and a 36 cycle phase where all temperatures were the same except that the annealing temperature was dropped to 56°C and 54°C respectively. The 5 gene regions (Cyt-B, ND3, GAPDH-11, ODC-6-7 and BRM-15) were successfully sequenced in the majority of species (see Table 1.2 for exceptions).

Table 1.2. Specimen details of all individuals used for the phylogenetic inference. Genus, species and subspecies columns are classified according to (Harrap & Quinn, 1996), with our revised classification in the final column.

Individual I.D.	Museum/Ring #	English name	Genus	Species	Subspecies	Location	Country	Coordinates (WGS84)	Museum /Fresh	CytB Accession #	ND3 Accession #	BRM Accession #	GAPDH Accession #	ODC Accession #	Our Phylogenetic classification
Au.fla01	VERD-10-CA	Verdin	*Auriparus*	*flaviceps*	*acaciarum*	California	USA	33N 116W	Fresh	complete	complete	complete	complete	complete	*Auriparus flaviceps*
Au.fla02	VERD-34-SO	Verdin	*Auriparus*	*flaviceps*	*ornatus*	Sonora	Mexico	28N 109W	Fresh	complete	complete	complete	complete	complete	*Auriparus flaviceps*
Au.fla03	from NCBI	Verdin	*Auriparus*	*flaviceps*	*acaciarum*	California	USA	-	NCBI	AF347969	-	-	-	-	*Auriparus flaviceps*
Au.fla04	Conacyt233	Verdin	*Auriparus*	*flaviceps*	*flaviceps*	Tiburón island	Mexico	28N 112W	Fresh	-	-	-	complete	-	*Auriparus flaviceps*
R.con.con01	108	Chinese penduline tit	*Remiz*	*consobrinus*	*consobrinus*	Xianghai	China	45N 122E	Fresh	complete	complete	complete	complete	complete	*Remiz consobrinus*
R.con.con02	109	Chinese penduline tit	*Remiz*	*consobrinus*	*consobrinus*	Xianghai	China	45N 122E	Fresh	complete	complete	complete	complete	complete	*Remiz consobrinus*
R.con.con03	NHM1898.9.20.457	Chinese penduline tit	*Remiz*	*consobrinus*	*consobrinus*	Nagasaki	Japan	32N 129E	Museum	complete	complete	complete	complete	complete	*Remiz consobrinus*
R.cor.cor01	KA46263	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Topar	Kazakhstan	45N 75E	Fresh	complete	complete	complete	complete	complete	*Remiz coronatus*
R.cor.cor02	KA46264	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Topar	Kazakhstan	45N 75E	Fresh	complete	complete	complete	complete	complete	*Remiz coronatus*
R.cor.cor03	KA46276	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Jabagly	Kazakhstan	42N 70E	Fresh	complete	complete	complete	complete	complete	*Remiz coronatus*
R.cor.cor04	NHM1949.25.3514	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Ghullapur	Pakistan	32N 73E	Museum	complete	complete	complete	complete	complete	*Remiz coronatus*
R.cor.cor05	NHM1949.Whi.1.5012	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Bahawalpur	Pakistan	29N 71E	Museum	complete	complete	complete	complete	~200bp	*Remiz coronatus*
R.cor.cor06	KA46279	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Jabagly	Kazakhstan	42N 70E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.cor.cor07	KA46271	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Jabagly	Kazakhstan	42N 70E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.cor.cor08	KA46268	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Jabagly	Kazakhstan	42N 70E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.cor.cor09	KA46265	White-crowned penduline tit	*Remiz*	*coronatus*	*coronatus*	Topar	Kazakhstan	45N 75E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.cor.sto01	A24-7901	White-crowned penduline tit	*Remiz*	*coronatus*	*stoliczkae*	Xinjiang	China	47N 87E	Fresh	complete	complete	complete	complete	complete	*Remiz coronatus*
R.cor.sto02	A24-7990	White-crowned penduline tit	*Remiz*	*coronatus*	*stoliczkae*	Xinjiang	China	46N 90E	Fresh	complete	complete	complete	complete	complete	*Remiz coronatus*
R.cor.sto03	A24-7999	White-crowned penduline tit	*Remiz*	*coronatus*	*stoliczkae*	Xinjiang	China	46N 90E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.cor.sto04	A24-7970	White-crowned penduline tit	*Remiz*	*coronatus*	*stoliczkae*	Xinjiang	China	46N 90E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.cor.sto05	A24-7938	White-crowned penduline tit	*Remiz*	*coronatus*	*stoliczkae*	Xinjiang	China	47N 87E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.cor.sto06	A24-7939	White-crowned penduline tit	*Remiz*	*coronatus*	*stoliczkae*	Xinjiang	China	46N 90E	Fresh	complete	-	-	-	-	*Remiz coronatus*
R.pen.pen01	NHM1941.5.30.649	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Castjon	Spain	42N 01E	Museum	complete	complete	complete	~350bp	~400bp	*Remiz pendulinus*
R.pen.pen02	NRM20036910	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Falsterbo	Sweden	55N 12E	Fresh	complete	complete	complete	complete	complete	*Remiz pendulinus*
R.pen.pen03	T228277	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Feherto	Hungary	46N 20E	Fresh	complete	complete	complete	complete	complete	*Remiz pendulinus*
R.pen.pen04	T228186	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Feherto	Hungary	46N 20E	Fresh	complete	complete	complete	complete	complete	*Remiz pendulinus*
R.pen.pen05	T228376	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Feherto	Hungary	46N 20E	Fresh	complete	complete	complete	~100bp	complete	*Remiz pendulinus*
R.pen.pen06	AV30408	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Sicily	Italy	37N 13E	Fresh	complete	complete	complete	complete	complete	*Remiz pendulinus*
R.pen.pen07	AV30410	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Sicily	Italy	37N 13E	Fresh	complete	complete	complete	complete	complete	*Remiz pendulinus*
R.pen.pen08	AV30409	European penduline tit	*Remiz*	*pendulinus*	*pendulinus*	Sicily	Italy	37N 13E	Fresh	complete	-	-	-	-	*Remiz pendulinus*
R.pen.men01	NHM1924.3.20.501	European penduline tit	*Remiz*	*pendulinus*	*menzbieri*	Deir ez-Zor	Syria	35N 40E	Museum	complete	complete	-	~300bp	-	*Remiz pendulinus*
R.pen.cas01	RMNH.AVES.130840	European penduline tit	*Remiz*	*pendulinus*	*caspius*	unknown	unknown	unknown	Museum	complete	complete	-	-	~200bp	*Remiz pendulinus*
R.pen.cas02	NRM570106	European penduline tit	*Remiz*	*pendulinus*	*caspius*	S.Ö. Ural	Russia	unknown	Museum	complete	complete	-	complete	-	*Remiz pendulinus*
R.pen.cas03	RMNH.AVES.130839	European penduline tit	*Remiz*	*pendulinus*	*caspius*	unknown	unknown	unknown	Museum	complete	complete	complete	complete	~200bp	*Remiz pendulinus*
R.pen.jax01	NRM570105	European penduline tit	*Remiz*	*pendulinus*	*jaxarticus*	Kyzylorda	Kazakhstan	44N 65E	Museum	-	complete	-	-	-	*Remiz pendulinus*
R.mac.ssa01	KA46260	Black-headed penduline tit	*Remiz*	*macronyx*	*ssaposhnikowi*	Topar	Kazakhstan	45N 75E	Fresh	complete	complete	complete	complete	complete	*Remiz pendulinus*
R.mac.ssa02	KA46261	Black-headed penduline tit	*Remiz*	*macronyx*	*ssaposhnikowi*	Topar	Kazakhstan	45N 75E	Fresh	complete	complete	complete	complete	complete	*Remiz pendulinus*
R.mac.mac01	NHM1901.5.4.478	Black-headed penduline tit	*Remiz*	*macronyx*	*macronyx*	Bugan	Kazakhstan	46N 61E	Museum	complete	complete	-	complete	~400bp	*Remiz pendulinus*
R.mac.mac02	NHM1907.12.21.440	Black-headed penduline tit	*Remiz*	*macronyx*	*macronyx*	Caspian sea	Iran	36N 51E	Museum	complete	complete	~150bp	complete	~400bp	*Remiz pendulinus*
R.mac.nig01	NHM1901.5.4.477	Black-headed penduline tit	*Remiz*	*macronyx*	*nigricans*	unknown	Iran	unknown	Museum	complete	complete	-	-	-	*Remiz pendulinus*
A.par.par01	NHM1946.43.3	Yellow penduline tit	*Anthoscopus*	*parvulus*	*parvulus*	Talodi	Sudan	10N 30E	Museum	complete	-	-	complete	complete	*Anthoscopus parvulus*
A.par.sen01	NHM1932.8.6.344	Yellow penduline tit	*Anthoscopus*	*parvulus*	*senegalensis*	Fiko	Mali	14N 03E	Museum	complete	complete	complete	complete	complete	*Anthoscopus parvulus*
A.par.sen02	NHM1940.12.4.28	Yellow penduline tit	*Anthoscopus*	*parvulus*	*senegalensis*	Argungu	Nigeria	12N 04E	Museum	complete	-	-	-	-	*Anthoscopus parvulus*
A.min.gig01	W83644	Cape penduline tit	*Anthoscopus*	*minutus*	*gigi*	Koeberg	South Africa	33S 18E	Fresh	complete	complete	complete	complete	complete	*Anthoscopus minutus*
A.min.gig02	W83645	Cape penduline tit	*Anthoscopus*	*minutus*	*gigi*	Koeberg	South Africa	33S 18E	Fresh	complete	complete	complete	complete	complete	*Anthoscopus minutus*
A.min.dam01	NHM1950.50.653	Cape penduline tit	*Anthoscopus*	*minutus*	*damarensis*	Damaraland	Namibia	20S 15E	Museum	complete	complete	complete	complete	complete	*Anthoscopus minutus*

Individual I.D.	Museum/Ring #	English name	Genus	Species	Subspecies	Location	Country	Coordinates (WGS84)	Museum /Fresh	CytB Accession #	ND3 Accession #	BRM Accession #	GAPDH Accession #	ODC Accession #	Our Phylogenetic classification
A.min.dam02	NHM1957.36.113	Cape penduline tit	*Anthoscopus*	*minutus*	*damarensis*	Mokatsi pan	Botswana	24S 22E	Museum	complete	complete	complete	complete	complete	*Anthoscopus minutus*
A.min.dam03	RMNH.AVES.130844	Cape penduline tit	*Anthoscopus*	*minutus*	*damarensis*	Potchefstroom	South Africa	26S 27E	Museum	complete	-	-	-	-	*Anthoscopus minutus*
A.min.min01	NHM1923.8.7.3016	Cape penduline tit	*Anthoscopus*	*minutus*	*minutus*	Bloemfontein	South Africa	29S 26E	Museum	complete	-	-	-	-	*Anthoscopus minutus*
A.fla.fla01	NHM1926.8.8.326	Forest penduline tit	*Anthoscopus*	*flavifrons*	*flavifrons*	Bitye	Cameroon	03N 12E	Museum	complete	complete	complete	complete	complete	*Anthoscopus flavifrons*
A.fla.fla02	NHM1926.8.8.328	Forest penduline tit	*Anthoscopus*	*flavifrons*	*flavifrons*	Bitye	Cameroon	03N 12E	Museum	complete	complete	complete	complete	complete	*Anthoscopus flavifrons*
A.fla.fla03	NHM1950.9.44	Forest penduline tit	*Anthoscopus*	*flavifrons*	*flavifrons*	Owerri	Nigeria	05N 07E	Museum	complete	complete	complete	complete	complete	*Anthoscopus flavifrons*
A.fla.fla04	NHM1966.16.3415	Forest penduline tit	*Anthoscopus*	*flavifrons*	*flavifrons*	Itu	Nigeria	05N 07E	Museum	complete	complete	complete	complete	complete	*Anthoscopus flavifrons*
A.pun.pun01	NHM1932.8.6.347	Sennar penduline tit	*Anthoscopus*	*punctifrons*	*punctifrons*	Timbuktu	Mali	16N 03E	Museum	complete	complete	complete	~300bp	complete	*Anthoscopus punctifrons*
A.pun.pun02	NHM1932.8.6.350	Sennar penduline tit	*Anthoscopus*	*punctifrons*	*punctifrons*	Tillia Wells	Niger	17N 08E	Museum	complete	complete	complete	complete	complete	*Anthoscopus punctifrons*
A.pun.pun03	NHM1953.35.26	Sennar penduline tit	*Anthoscopus*	*punctifrons*	*punctifrons*	Afabet	Eritrea	16N 38E	Museum	complete	complete	complete	complete	-	*Anthoscopus punctifrons*
A.pun.pun04	NHM1953.35.27	Sennar penduline tit	*Anthoscopus*	*punctifrons*	*punctifrons*	Afabet	Eritrea	16N 38E	Museum	complete	complete	complete	complete	complete	*Anthoscopus punctifrons*
A.mus.mus01	NHM1946.5.2702	Mouse-coloured penduline tit	*Anthoscopus*	*musculus*	*musculus*	Yabello	Ethiopia	04N 38E	Museum	complete	complete	complete	complete	complete	*Anthoscopus musculus*
A.mus.mus02	NHM1933.2.6.20	Mouse-coloured penduline tit	*Anthoscopus*	*musculus*	*musculus*	Lotomi	Uganda	02N 34E	Museum	complete	complete	complete	complete	complete	*Anthoscopus musculus*
A.mus.mus03	NRM552437	Mouse-coloured penduline tit	*Anthoscopus*	*musculus*	*musculus*	Guasso Nyiro	Kenya	01S 35E	Museum	complete	complete	complete	complete	complete	*Anthoscopus musculus*
A.car.tar01	NHM1946.83.3	African penduline tit	*Anthoscopus*	*caroli*	*taruensis*	Naberera	Tanzania	04S 36E	Museum	complete	complete	complete	complete	complete	*Anthoscopus musculus*
A.car.syl01	NHM1932.5.10.1084	Buff-bellied penduline tit	*Anthoscopus*	*caroli*	*sylviella*	Iringa uplands	Tanzania	07S 35E	Museum	complete	~200bp	complete	complete	complete	*Anthoscopus sylviella*
A.car.syl02	NRM570104	Buff-bellied penduline tit	*Anthoscopus*	*caroli*	*sylviella*	Serengeti	Tanzania	02S 34E	Museum	complete	complete	complete	complete	complete	*Anthoscopus sylviella*
A.car.ans01	NHM1936.4.13.211	African penduline tit	*Anthoscopus*	*caroli*	*ansorgei*	Kasai district	DRCongo	04S 23E	Museum	complete	complete	complete	complete	complete	*Anthoscopus ansorgei*
A.car.rob01	NHM1949.72.14	African penduline tit	*Anthoscopus*	*caroli*	*robertsi*	Mazabuka	Zambia	15S 27E	Museum	complete	complete	complete	complete	complete	*Anthoscopus caroli*
A.car.rho01	NHM1955.41.15	African penduline tit	*Anthoscopus*	*caroli*	*rhodesiae*	Kasama	Zambia	10S 31E	Museum	complete	complete	complete	complete	complete	*Anthoscopus caroli*
A.car.rho02	RMNH.AVES.36701	African penduline tit	*Anthoscopus*	*caroli*	*rhodesiae*	Songea district	Tanzania	10S 35E	Museum	complete	complete	complete	complete	complete	*Anthoscopus caroli*
A.car.rho03	RMNH.AVES.44420	African penduline tit	*Anthoscopus*	*caroli*	*rhodesiae*	Namalungo	Tanzania	10S 35E	Museum	complete	complete	complete	complete	complete	*Anthoscopus caroli*
A.car.rho04	RMNH.AVES.37677	African penduline tit	*Anthoscopus*	*caroli*	*rhodesiae*	Kidugallo	Tanzania	06S 38E	Museum	-	complete	-	complete	complete	*Anthoscopus caroli*
C.fla01	NRM570107	Fire-capped tit	*Cephalopyrus*	*flammiceps*	*flammiceps*	Kishtwar	India	33N 75E	Museum	-	-	-	complete	complete	*Cephalopyrus flammicep*
P.rus01	NRM570103	Tit-hylia	*Pholidornis*	*rushiae*	*rushiae*	Bibundi	Cameroon	04N 08E	Museum	-	-	-	complete	complete	*Pholidornis rushiae*
Paridae	from NCBI	-	-	-	-	-	-	-	NCBI	AY495412	NC_014341	AJ890555	EU272098	EU680749	*Paridae*
Corvidae	from NCBI	-	-	-	-	-	-	-	NCBI	AY527270	GQ494146	EU554525	EF052755	EU680746	*Corvidae*
Tyrannidae	from NCBI	-	-	-	-	-	-	-	NCBI	EF458572	AY489522	-	DQ470527	EU231849	*Tyrannidae*

(i) *Blood DNA extraction and sequencing*

DNA extraction from blood was the preferred method, but requires the sampling of live birds in the field. Where this was not possible museum samples were used as detailed in the section below. Approximately 10ul of blood was collected from the brachial vein of adult birds and stored in 1ml of Queen's lysis buffer at 5°C. DNA was extracted using a standard proteinase K digestion followed by DNA extraction using a GeneMole® automated nucleic acid extraction instrument (Mole Genetics). The manufacturers instructions were followed using the MoleStrips™ DNA Tissue kit.

For all five gene regions, previously published primer sequences were first tested for PCR amplification in a subset of DNA samples using the previously published PCR conditions. To test for amplification success, 2ul of the PCR products were stained with ethidium bromide and electrophoresis was performed on a 1.2% agarose gel with a 100bp ladder. Amplification was deemed successful if the visualized gels exhibited a clean band of the expected molecular weight; indicating highly specific primer binding for the correct gene product. After success in a subset of samples all other DNA samples were then PCR amplified, tested for success and the PCR products prepared for sequencing. However if amplification failed or non-specific binding was observed for a primer pair, PCR conditions were first adjusted and if failure still occurred then *Remizidae* specific primers were designed using either closely related species or any *Remizidae* individuals where the primers were successful. This was the case for gene regions CytB, and GAPDH-11. All successful primer pairs are listed in Table 1.3.

PCR products were purified by adding 5ul of ExoSap and incubating at 37°C for 30mins followed by a deactivation step at 80°C for 15min. BigDye Terminator v1.1 Cycle sequencing (20ul reactions) was performed using 2ul of purified PCR product following the manufacturer's protocol. Each PCR product was used for two sequencing reactions; one with the forward

primer and the second with the reverse primer from the PCR amplification step. This allowed us to perform bi-directional sequencing for every sample. Cycle sequencing products were sequenced on an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The sequences were assembled in SeqMan II (DNASTAR) and the primer sequences were removed from either end. Positions where the nucleotide could not be determined with certainty were coded with the relevant IUPAC code.

(ii*) Museum DNA extraction and sequencing*

DNA was extracted and sequenced from museum specimens closely following the protocol described by (Irestedt *et al.*, 2006). Toe-pad samples measuring approximately $2mm^3$ were cut from the base of the middle toe using a sterile scalpel blade. They were stored in an Eppendorf tube in dry, dark conditions until DNA extraction. A DNeasy Tissue Kit (QIAGEN®) was used to extract DNA from the entire toe-pad sample. The manufacturer's instructions were followed except that the quantity of buffers and enzymes during the lysis stage were doubled due to the tough structure of toe-pads. The museum samples had been collected between the years 1858 and 1957 and thus the DNA would have undergone variable amounts of degradation, therefore contamination was kept to a minimum by conducting all extractions in a room designated to the sole use of old DNA material. All equipment was sterilized on a UV bench and the proximity of samples from closely related individuals was restricted so that any contamination between samples could be more easily detected.

Due to the presence of degradation, DNA from museum samples cannot be amplified and sequenced in large sections, as it has been sheared over time into small fragments. Irestedt et al. found that success of PCR amplification dramatically increases when primers are designed to amplify PCR products <200 base pairs (bp) in length (unpublished data). Therefore to obtain an entire gene region, amplification and sequencing is performed on 100-200bp sections. Using the complete sequences obtained from the fresh DNA

material we designed *Remizidae* specific internal primers in conserved regions. These were designed as described previously and were chosen to amplify overlapping regions of ~200bp. Thus the entire gene region was obtained by amplifying and sequencing contiguous 200bp overlapping sections. These sections were assembled using SeqMan (DNASTAR package) to create one continuous gene sequence. All other protocols were the same as for the DNA extracted from fresh material, except the BigDYE Terminator v3.1 cycle sequencing kit was used in replacement of v1.1. The internal primers were used for both the PCR amplification and the sequencing reactions and details are supplied in Table 1.3.

Table 1.3. Primer sequences for all 5 gene regions (Cyt-B, ND3, GAPDH-11, ODC-6-7 and BRM-15) used in this study. For fresh specimens the forward primer of the first fragment and the reverse primer of the final fragment were used to amplify and sequence the gene region. For museum specimens each gene region was amplified and sequenced in fragments, which were subsequently aligned to form the complete gene region. The primers for each of these fragments are shown in the table below, note that slightly different primers were used on the *Anthoscopus* and *Remiz/Auriparus* individuals to amplify and sequence the CytB gene.

Gene Region	Length approx. (bp)	Gene fragment	Primer ID	Primer sequence
ND3	395 (2 fragments)	Fragment 1 (Forward)	ND3-H11151	GATTTGTTGAGCCGAAATCAAC
		Fragment 1 (Reverse)	ND3-RemizR1	GAAGAGGAGAAATAGGATTGCTAC
		Fragment 2 (Forward)	ND3-RemizF1	TCCCCTTCTCAATCCGATTCTTC
		Fragment 2 (Reverse)	ND3-L10755	GACTTCCAATCTTTAAAATCTGG
ODC introns 6-7	580 (3 fragments)	Fragment 1 (Forward)	ODC-RemizF1	GCGTGCAAAAGAACTTGC
		Fragment 1 (Reverse)	ODintR3B	CAAATACACAGCGAGCATCAGA
		Fragment 2 (Forward)	ODCbopF2	CAGACCCAGAGACCTTTGTTCA
		Fragment 2 (Reverse)	ODCbopF3R	TGCCAAGCTGGTCAAAGTAAGCTAC
		Fragment 3 (Forward)	ODCbopF3	GTAGCTTACTTTGACCAGCTTGGCA
		Fragment 3 (Reverse)	ODCintR4	CATATTGAAGCCAAGTTCAGCCTA
BRM intron 15	355 (2 fragments)	Fragment 1 (Forward)	BRM15-RemizF1	ATTTGCCATGACTGGAGAAAGG
		Fragment 1 (Reverse)	BRM15-RemizR1	GCAAAAAGTACACCTTAAACATTAAA
		Fragment 2 (Forward)	BRM15-RemizF2	GACCCCTTTTTTATAATACCACA
		Fragment 2 (Reverse)	BRM15R	TACTTTATGGAGACGACGGA
GAPDH intron 11	310 (2 fragments)	Fragment 1 (Forward)	G3P14c	AGTCCACAACACGGTTGCTGTATCCA
		Fragment 1 (Reverse)	G3P-RemizR1	TGGTGATCCAGGTGGATACACA
		Fragment 2 (Forward)	G3P-RemizF1	ACAACTGAATTCCCACCTACTCT
		Fragment 2 (Reverse)	G3PintL1	GAACGACCATTTTGTCAAGCTGGTT
CytB-*Remiz* genus	820 (4 fragments)	Fragment 1 (Forward)	CytBbopF1	TCACACAAATTATCACAGGCCT
		Fragment 1 (Reverse)	CytB-RemizR1	TCCTACGAAGGC**R**GTTGCTATGA
		Fragment 2 (Forward)	CytB-RemizF2	TGCT**Y**CTGGCCCTCATAGCAAC
		Fragment 2 (Reverse)	CytB-RemizR2	GGGGGTTGTTGGATCCTGTTTC
		Fragment 3 (Forward)	CytB-RemizF3	CCTCCATGAAACAGG**M**TCCAACAA
		Fragment 3 (Reverse)	CytB-RemizR3	GGAGGATGGCGTATGCAAA**I**AGGAA
		Fragment 4 (Forward)	CytB-RemizF4	ACTCCCCCCCACATTAAACCCGA
		Fragment 4 (Reverse)	CytB-RemizR4	AATGG**R**TGTTCGACTGGTTGGCT
CytB-*Anthoscopus* genus	820 (4 fragments)	Fragment 1 (Forward)	CytB-caroliF1	CACATCTGCCGAAACGTCCA
		Fragment 1 (Reverse)	CytB-RemizR1	TCCTACGAAGGC**R**GTTGCTATGA
		Fragment 2 (Forward)	CytB-caroliF2	CAAAGAAACCTGAAACATCGGAGT
		Fragment 2 (Reverse)	CytB-RemizR2	GGGGGTTGTTGGATCCTGTTTC
		Fragment 3 (Forward)	CytB-RemizF3	CCTCCATGAAACAGG**M**TCCAACAA
		Fragment 3 (Reverse)	CytB-RemizR3	GGAGGATGGCGTATGCAAA**I**AGGAA
		Fragment 4 (Forward)	CytB-RemizF4	ACTCCCCCCCACATTAAACCCGA
		Fragment 4 (Reverse)	CytB-RemizR4	AATGG**R**TGTTCGACTGGTTGGCT

Phylogeny construction

Phylogenies were estimated using a variety of approaches in order to address the controversies surrounding the many phylogenetic methods implemented in the current literature. Firstly gene trees for the 5 sequenced gene regions were created via two methods, a maximum likelihood (PhyML) and a Bayesian inference approach (MrBayes). These same five gene regions were then used to estimate a species level phylogeny simultaneously using all sequence information. This was also performed using two approaches, a concatenation method implemented in MrBayes and a coalescent approach recently proposed using the BEAST software (termed *BEAST).

Each set of gene sequences were aligned using the software MEGA 5.0 and the ClustalW alignment algorithm (Tamura *et al.*, 2011), using the default parameters. The alignments were occasionally adjusted by eye following implementation of ClustalW. The sequence alignments were converted into required formats for input into the phylogenetic software using the ClustalX 2.1 program (Larkin *et al.*, 2007).

For both maximum likelihood and Bayesian inference methods an accurate DNA substitution model was estimated. These were obtained for each gene using the program MrModelTest 2.3 (Posada & Crandall, 1998) in conjunction with PAUP*4.0 (Swofford, 2003), where the most appropriate model selected by the Akaike Information Criterion (AIC) was used in further analyses. The results suggested the most suitable substitution model for the mitochondrial sequences (Cyt-B and ND3) was a General Time Reversible (GTR) model with invariable sites and gamma distributed rates. The GTR model with gamma distributed rates was selected for the ODC sequences, however for the two other nuclear regions (GAPDH-6-7 and BRM-15) the most suitable fit was provided by the Hasegawa, Kishino and Yano (HKY) model with gamma distributed rates.

Out-group sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). We required a member of the *Paridae* in order to determine its relationship with *Remizidae*. Representatives were also selected from both *Corvidae* and *Tyrannidae* as they represent two basal clades of the Passeriformes, to which *Remizidae* belongs. Where all five gene sequences could not be obtained for the same species a closely related species with the available gene sequence was supplemented, thus creating a multi-species composite sequence (NCBI accession numbers in Table 1.2). Phylogenetic trees were visualized in either R, using the APE 2.7-2 package (Paradis et al. 2004) or in the program FigTree v1.3.1 (can be found at http://tree.bio.ed.ac.uk/software/figtree/).

Gene trees

(i) *Maximum likelihood*

The aligned sequences were analysed by the online program PhyML 3.0 (Guindon *et al.*, 2005). The parameters for the substitution model were obtained from the MrModelTest 2.3 output as described above. The program PhyML estimates phylogenies based on maximum-likelihood principles. We used the default parameters except that the Sub-tree Pruning and Regrafting (SPR) algorithm was selected, the number of random starting trees was set to 5, and topology and branch lengths were optimized. The internal branch supports were estimated using a non-parametric bootstrap analysis consisting of 1000 replicates.

(ii) *Bayesian inference*

We used the program MrBayes 3.2.1 to produce gene-trees for all 5 genes (Huelsenbeck & Ronquist, 2001). All runs were implemented on the Oslo bioportal (http://www.mn.uio.no/ibv/bioportal/index.html). MrBayes is a widely used program for phylogeny reconstruction using posterior probabilities approximated via Markov Chain Monte Carlo (MCMC) simulation techniques. Sequence alignments were input in nexus format and the relevant substitution model obtained from the MrModelTest 2.3 was denoted for each respective gene tree. The default parameters were used

except that the number of generations of the MCMC run was set at 20,000,000, leading to all trees exhibiting an average standard deviation of split frequencies < 0.01. The first 25% of generations were discarded as burnin. The number of parallel runs was set at three, 1 cold and 2 heated chains (Nchains=3) to optimize time and convergence success.

Multi-gene trees

(i) *Concatenated*

These were performed in MrBayes 3.2.1 using the partition function to allow each gene to run under its respective evolutionary model as estimated above, whilst constraining them all to the same tree in this super matrix based approach. For each individual the aligned sequences for each included gene region were concatenated into one continuous sequence. For the 5 gene analysis this created an alignment of 53 sequences each totalling 2638bp in length (BRM-15 = 345bp, GAPDH = 396bp, ND3 = 395bp, ODC = 661bp and Cyt-B = 841bp). The MCMC algorithm was run for 20,000,000 generations and the first 25% of generations were discarded as burn-in.

(ii) *Coalescent*

This is a recent method proposed by Heled and Drummond 2010, which is a coalescent based approach using a Bayesian framework implemented in the software BEAST (Heled & Drummond, 2010). Known as *BEAST it works by embedding all 5 gene trees within one species tree as the branches coalesce back through time. It is claimed to provide a robust alternative to the highly used concatenation method, which can produce highly supported but incorrect species trees in certain situations (Kubatko & Degnan, 2007). The evolutionary models were the same as used in the analyses above for each gene region. The analysis was run for 200,000,000 generations. The two mitochondrial genes (Cyt-B and ND3) were analysed together as they are genetically linked and evolve under the same evolutionary model. In *BEAST a species classification system has to be included prior to the analysis, thus we allocated each individual to a subspecies. We classified these according to Harrap and Quinn, based on morphology, plumage and sampling location.

We also checked that these classifications were consistent with the results of the MrBayes concatenated run. Results were checked in tracer v 1.5 and then summarised using the program TreeAnnotator v 1.6.2. The burnin was set to 25% and the maximum clade credibility tree was inferred with the node heights set as the means for each clade. The final trees were viewed in Fig tree v 1.4.0.

Results

Consistency between methods

The maximum likelihood and Bayesian methods produced highly consistent trees from each gene although the Bayesian approach showed higher supported nodes as expected from this robust approach. The main difference between the gene trees was the resolution provided by the mitochondrial genes compared to the nuclear gene regions. The 3 nuclear genes resolved the deeper genus level relationships but the mitochondrial genes were much more informative at the species level relationships within each clade. This is consistent with the faster mutation rates of mitochondrial genes.

The two methods used to produce the species level phylogeny were also largely consistent. The only discrepancy between the two methods was the placement of the Cape penduline tit and the Forest penduline tit, *Anthoscopus flavifrons*. In the concatenated approach using MrBayes the Cape penduline tit is positioned more posteriorly; with the Forest penduline tit branching from the other *Anthoscopus* species slightly later (Figure 1.3.). However in the coalescent approach in *BEAST they are shown as sister species diverging at the same time from the other *Anthoscopus* species soon after the Yellow penduline tit, *Anthoscopus parvulus* (Figure 1.4.). The latter's branching structure does show lower support in this phylogeny (a posterior probability of 82).

Phylogenetic patterns in Remizidae

We were able to amplify and sequence at least 2 complete gene regions in 57 individuals, these included representatives of all species classified in the *Remizidae* according to (Harrap & Quinn, 1996) (see Table 1.2). We created a phylogeny using the two autosomal nuclear gene regions (ODC and G3P) with representatives of all species. This phylogeny shows that all species group into their currently classified genera, creating 5 distinct lineages.

However the phylogeny shows that the *Remizidae* classification put forward by (Harrap & Quinn, 1996) is polyphyletic (Figure 1.2). Two of the monotypic genera are found to be highly divergent from the other three genera, with both splits highly supported. *Cephalopyrus flammiceps* is shown to be a member of the *Paridae* and *Pholidornus rushiae* is even more distantly related. The remaining 3 genera (*Remiz, Anthoscopus and Auriparus*) form a monophyletic sister clade to the *Paridae*. All branches separating the 5 genera are highly supported (see Figure 1.2) although the species level relationships lack resolution.

Of note is the diversity of speciation patterns between the 3 genera comprising the monotypic penduline tits. *Auriparus* is monotypic with only one representative in North America, however the other two contain multiple species. The *Remiz* contain three genetically pauperate species. The African clade, *Anthoscopus,* is formed of a diverse assemblage of species with large separation both between and within species, with the majority containing genetically distinct subspecies.

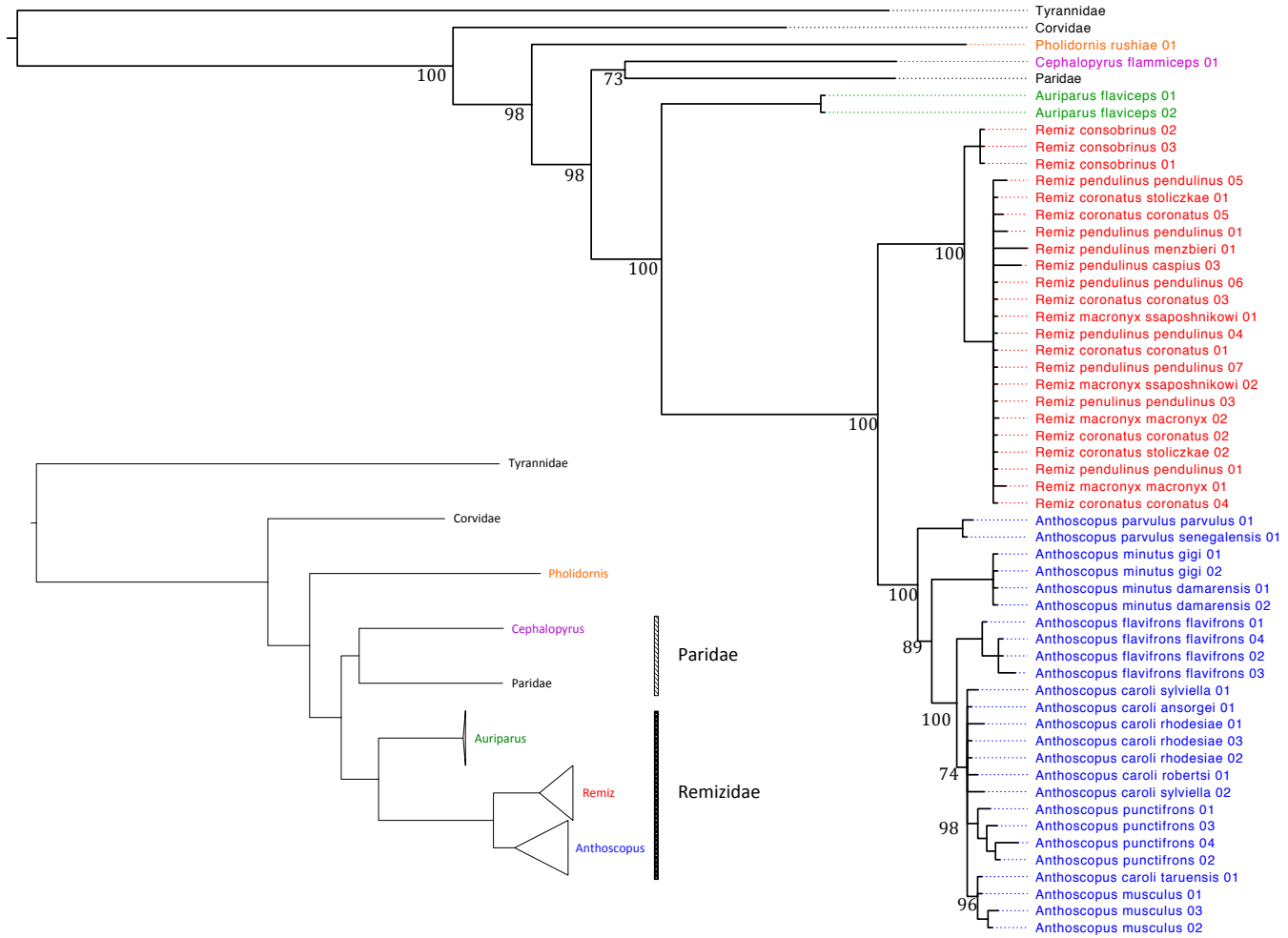

Figure 1.2. Molecular phylogeny of the *Remizidae* inferred using two nuclear gene regions (GAPDH intron 11 and ODC introns 6 & 7). All five genera according to (Harrap & Quinn, 1996) are included showing that this current classification is paraphyletic. Only 3 of the genera form a monophyletic clade (*Remiz, Anthoscopus and Auriparus*) as a sister group to the *Paridae*. *Cephalopyrus* is inferred as a member of the *Paridae* and *Pholidornis* is even more distantly related to the other *Remizidae*. This phylogeny was produced via the MrBayes concatenation method and all node supports with posterior probabilities >50 are shown.

Species level relationships

All 5 gene regions were successfully amplified and sequenced in 39 individuals, these included representatives of all species within the three monophyletic *Remizidae* genera (*Remiz, Anthoscopus* and *Auriparus*; see above). An additional 11 individuals with at least 3 gene regions sequenced, were also included in the multi-gene species level phylogenies to increase the representation of subspecies. The two methods produced highly consistent phylogenies with the majority of branches highly supported (see Figures 1.3 & 1.4). The results show the three genera as a monophyletic sister clade to the *Paridae*. *Anthoscopus* and *Remiz* form the backbone of the *Remizidae* as two monophyletic sister groups with the monotypic *Auriparus* more distantly related; diverging soon after the split from the *Paridae*.

The *Remiz* genus is formed of three distinct clades, the Chinese penduline tits, the White-crowned penduline tits and the large number of individuals from the European and Black-headed penduline tits grouping together (Figure 1.3). However the *Anthoscopus* is much more variable, formed of 5 distinct groups plus the *Anthoscopus caroli* clade. This *A. caroli* clade appears to be formed of 3 groups, the *sylviella, ansorgei* and *Rhodesia/robertsi* individuals. The separations between them suggest species level distinctions, however more intensive sampling would clarify the relationships within this clade. Based on our phylogenies the *Remiz* genus contains 3 species and the *Anthoscopus* exhibits 8 species, along with the monotypic *Auriparus:* our results therefore suggest the *Remizidae* is comprised of 12 distinct species.

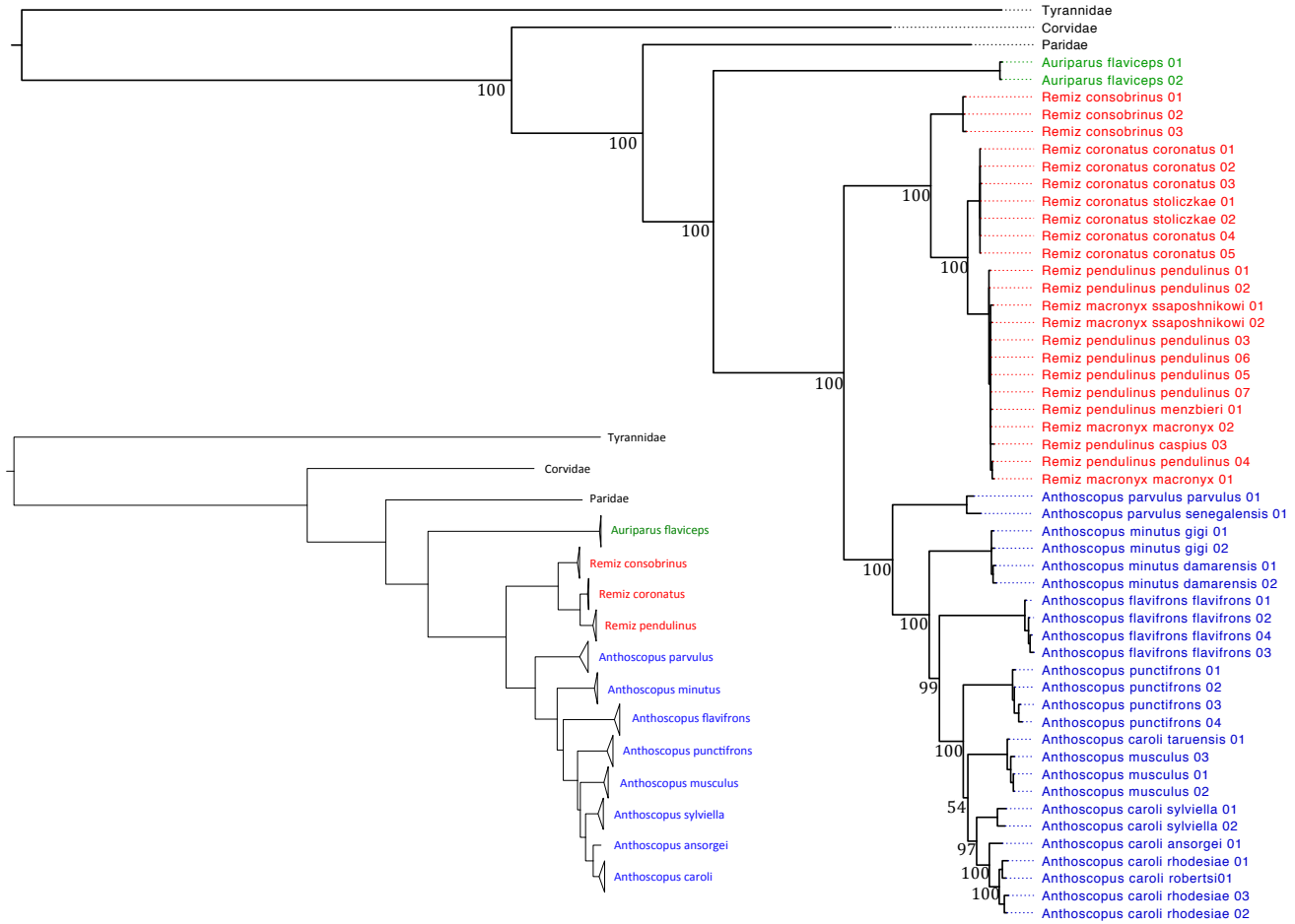

Figure 1.3. Phylogeny of the monophyletic *Remizidae* inferred using 5 gene regions (Cytb, ND3, GAPDH-11, ODC-6-7 and BRM-15) by the MrBayes concatenation method. The 3 genera are colour coded, *Auriparus* (Green), *Remiz* (Red) and *Anthoscopus* (Blue). All node supports with a posterior probability >50 are shown. A summary of the species level relationships suggested by the phylogeny is shown on the left.

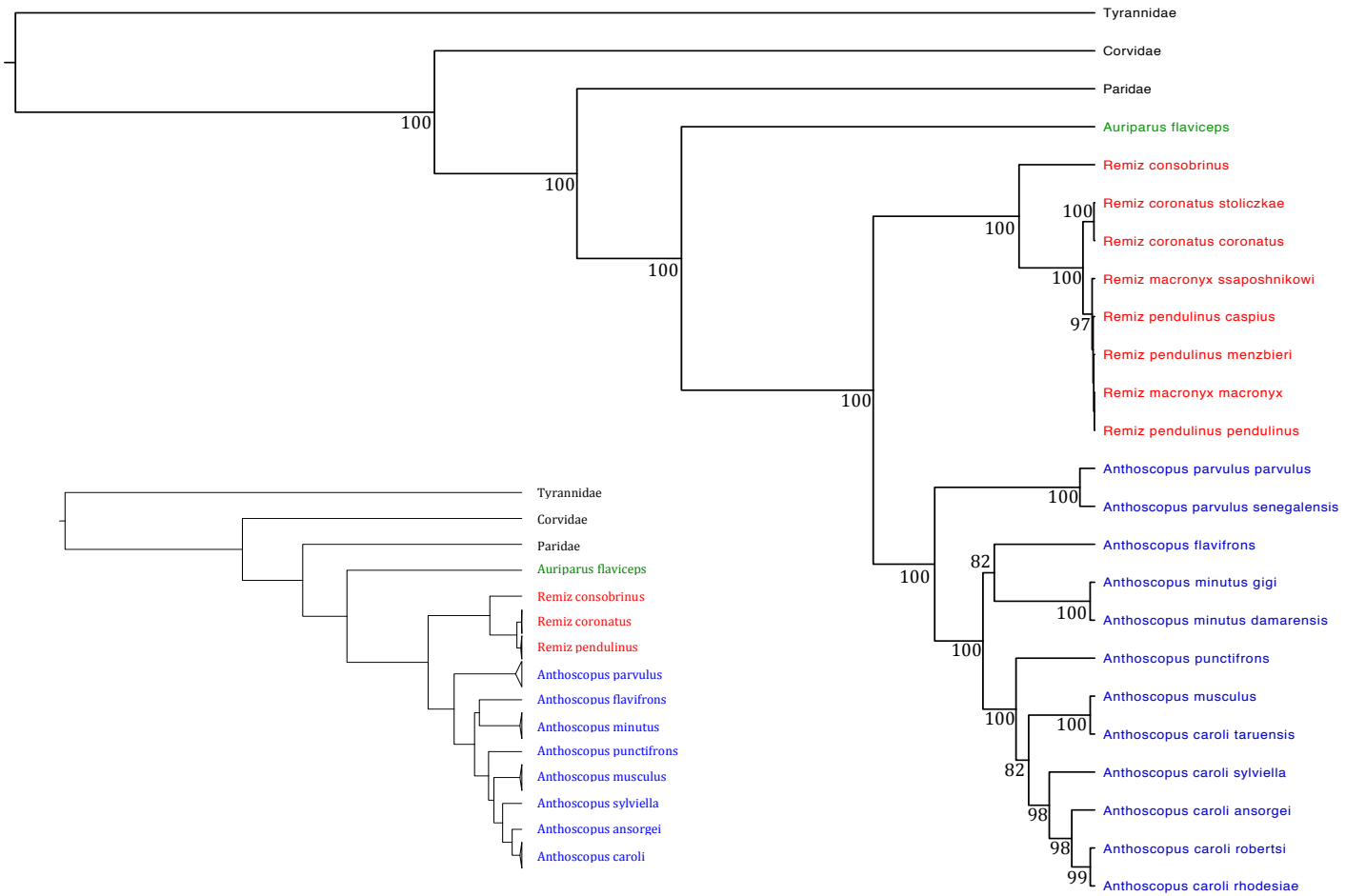

Figure 1.4. Phylogeny of the monophyletic *Remizidae* inferred using 5 gene regions (Cyt-B, ND3, GAPDH-11, ODC-6-7 and BRM-15) using the *BEAST coalescence approach. The subspecies are colour coded according to genera, *Remiz* (red), *Anthoscopus* (blue) and *Auriparus* (green). Node supports with posterior probabilities >50 are shown. A species summary is show in the bottom left.

Discussion

The results provide a robust and detailed description of the relationships within the *Remizidae* family. This not only provides insights into the phylogeography of the penduline tits but also allow accurate comparative analyses of the diverse traits found within this group. The multiple phylogenetic methods have provided highly consistent results further supporting the accuracy of our findings. Our use of 5 gene regions, both mitochondrial and nuclear also aid in improving the accuracy of our phylogeny, with the individual gene trees confirming the need for both types of markers during phylogeny construction.

This molecular phylogeny is the most comprehensive of the Penduline tit family to date, and provides strong evidence for the inclusion of three monophyletic genera within the group, *Auriparus*, *Remiz* and *Anthoscopus,* with the rejection of both *Pholidornis* and *Cephalopyrus*. As far as we are aware this is not in accordance with any classification system put forward previously (see Table 1.1). Our phylogeny supports the two main genera, *Anthoscopus* and *Remiz* as monophyletic sister groups within the family, with the monotypic genus, *Auriparus*, being a more distant relative. We found no support for the inclusion of *Pholidornis rushiae* or *Cephalopyrus flammiceps* within this group, instead finding support, as did (Tietze & Borthakur, 2012), that *C. flammiceps* is a member of the *Paridae*.

The polyphyly of the current classification system is not surprising given the large discrepancies among taxonomists on the relationships within the *Remizidae*, both *C. flammiceps* and *P. rushiae* have been placed in other families by various taxonomists. A recent phylogeny of the Tits suggested *Cephalopyrus flammiceps* was a basal monotypic group of the *Paridae*, which our results also support (Tietze & Borthakur, 2012). The common name of *Pholidornis rushiae*, tit-hylia, also highlights its uncertain taxonomic position, as it exhibits traits common to both the tits (*Paridae* and *Remizidae*) and the green hylia (*Hylia prasina*). Our results are consistent with its exclusion from both *Remizidae* and *Paridae*, as suggested by a molecular phylogeny which instead supported a sister relationship with the green hylia (Sefc *et al.*, 2003).

We found that the monotypic genus Auriparus is the most basal genus within the *Remizidae*, diverging soon after their split with the *Paridae*, supporting the relationship shown by previous molecular phylogenies (Johansson *et al.*, 2008). This is highly consistent with their separate geographic position, being the only species of penduline tit to be found in the Americas. Its morphology and behaviour have also been found to mark it out as distinct from the other *Remizidae* (Taylor, 1970, 1971).

The results are enlightening with reference to the often contentious species level relationships of *Remiz* (Vaurie, 1957). Our phylogenies support the existence of three distinct species within the *Remiz* genus, *Remiz consobrinus, Remiz coronatus* and *Remiz pendulinus*. The genetic separation of *consobrinus* from the other two is expected given its distinct geographic separation in East Asia (Figure 1.1). The range overlap plus the previous work highlighting the differences in song and behaviour between *Remiz pendulinus* and *Remiz coronatus* is also consistent with the closer but distinct relationship presented by our results (Bot *et al.*, 2011). However the finding that the *Remiz macronyx* individuals grouped with *Remiz pendulinus* using all 5 gene regions, causes us to suggest at present that *R. macronyx* be treated as a subspecies of *Remiz pendulinus,* pending further investigation. This follows the classification system of (Sibley & Monroe, 1990). Surprisingly little differentiation was found between the subspecies within each of the *Remiz* species suggesting either very recent range expansion or a large degree of gene-flow within each species. The rapid range expansion of *Remiz pendulinus* across Europe has been documented throughout the last century, and could perhaps go some way to explaining the low variability within this group (Valera *et al.*, 1993).

The *Anthoscopus* species have previously been classified as a super-species complex (excluding *A. flavifrons*) as each species is found in a distinct environment across sub-Saharan Africa but they exhibit little morphological variation (Gill & Wright, 2006). However we find that this morphological similarity actually belies substantial underlying genetic differences. This genus

shows much higher genetic variation than the *Remiz* genus with even subspecies exhibiting substantial divergence. Our phylogeny supports the existence of all six species described by (Harrap & Quinn, 1996). In addition it suggests that two subspecies of *Anthoscopus caroli* could be elevated to species status.

Both *Anthoscopus caroli sylviella* and *Anthoscopus caroli ansorgei* show pronounced differentiation from the other *caroli* subspecies, forming distinct highly supported clades diverging from the other *caroli* soon after the split with *A. musculus* (Figures 1.3 & 1.4). This provides evidence for the previous assertion that A. *caroli sylviella* should be elevated to species status based on its distinct highland geographic range with limited overlap and no evidence of hybridisation with other *caroli* subspecies (Howard & Moore, 1980; Sibley & Monroe, 1990). As we only have one individual of *A. caroli ansorgei* within our study we have to be careful in our interpretation of this additional result. But both subspecies appear to show substantial genetic differentiation from the other two *A. caroli* subspecies included in our study (*rhodesiae* and *robertsi*). The complex relationships of the *A. caroli* group have always provided contention and both *A. caroli sylviella* and *A. caroli rankenei* already have common English names, the Buff-bellied and Zambezi penduline tit respectively. Although we were unable to procure a sample of *A. caroli rankenei* our finding that the *A. caroli* clade exhibits larger amounts of genetic divergence than expected highlights the need for further investigation into the relationships within the *A. caroli* complex. At present our results appear to suggest that the *A. caroli* group has undergone cryptic speciation with morphologically similar but genetically distinct species emerging.

Another discrepancy with previous classifications is our grouping of *A. caroli taruensis* with the *A. musculus* individuals. *A. caroli taruensis* is the most northeastern subspecies of *A. caroli* and is situated on the boundary between *A. musculus* and *A. caroli*. There are potentially 3 reasons for our analyses placing it as a member of *A. musculus*, 1) *A. caroli taruensis* could in fact be a sub-species of *A. musculus*, 2) *A. caroli taruensis* could represent a hybrid zone between *A.*

caroli and *A. musculus*, or 3) this *A. caroli taruensis sample* could be an aberrant *A. musculus*. Further work would need to be undertaken to resolve this as we currently only have one individual of *A. caroli taruensis*.

The use of both mitochondrial and nuclear gene regions has allowed high resolution of both the ancient and recent divergence events due to the differing rates of evolution of these genomic regions. Our results are consistent with the rapid mutation rate of mitochondrial genes compared to nuclear autosomal regions. The cytochrome-b gene provides the most robust resolution of any of the single gene trees but the older nodes are more highly supported when also including nuclear gene regions in the analysis.

Both species level methods, the traditional MrBayes concatenation approach and the recently devised coalescence method, *BEAST, inferred consistent phylogenies. Previous studies have suggested that the traditional methods can lead to overestimated divergence times and inaccurate inference of the true relationships due to incomplete lineage sorting (Heled & Drummond, 2010). However the coalescent approach is able to control for stochasticity during allele sorting even when alleles are shared among species (Maddison & Knowles, 2006). However, the coalescent approach requires prior knowledge of the relationships between individuals. We used the subspecies classification system of (Harrap & Quinn, 1996) to group individuals and these were also consistent with the results from the MrBayes analysis. However if there were cryptic differences within these subspecies the coalescent approach would be unable to reveal these and could lead to spurious results. Thus this method is best used in combination with another such as MrBayes to check for potential cryptic divergence as implemented by (Kearns *et al.*, 2013).

The main purpose of this work has been to create a robust phylogeny of this ecologically interesting family. We have used multiple gene regions which have each inferred highly similar relationships and in combination provide high support of a monophyletic family incorporating 3 genera (A*uriparus, Remiz* and *Anthoscopus*). These genera are geographically separated, each occurring on a

different continent, suggesting allopatric speciation has dominated the evolutionary history within this group. We have thus created the first molecular phylogeny of all species currently classified within the *Remizidae*, elucidating the problems with the conflicting classification systems of the past. This work now allows detailed testing of evolutionary hypotheses within the penduline tits, a fascinating group in evolutionary ecology research.

Chapter 2

Mating systems and parental care in the penduline tits (*Remizidae*): parentage analysis in the Eurasian (*Remiz pendulinus*) White-crowned (*Remiz coronatus*) and Cape penduline tit (*Anthoscopus minutus*)

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Abstract

Evolutionary theory predicts a close association between mating and parental care systems. Parental care will only evolve if it can be accurately directed towards an individual's genetic offspring and any sex bias in the ability to do this is predicted to bias care roles. In organisms with internal fertilisation such a bias could manifest itself via multiple mating by a female during a single reproductive cycle. This has the ability to decrease a male's share of parentage without decreasing the females; lowering the benefits of male but not female care. Thus theory would predict that as promiscuity increases, the proportion of paternal care would decrease. To test whether paternal care is associated with the level of female promiscuity across the penduline tits we compare the levels of Extra Pair Paternity (EPP) in three closely related species. The European penduline tit (*Remiz pendulinus*) has been studied extensively and we draw data from the previous work on this uniparental species. Comparisons are made with our new results for the White-crowned (*Remiz coronatus*) and Cape penduline tit (*Anthoscopus minutus*), both of which exhibit biparental care systems with long-term care by both sexes. In line with the prediction, the biparental species (White-crowned and Cape penduline tit) exhibit much lower levels of EPP (0% and 7% respectively) than the uniparental species, the European penduline tit (24% EPP).

Introduction

Until the 1980's the mating systems of birds were thought to be dominated by monogamous relationships (Lack, 1968). The strong pair bonds formed during the breeding season and the dedicated care of offspring provided a paragon of devotion for human societies (Birkhead, 2000). But with the advent of molecular parentage analysis it soon became clear that birds were far from faithful (Burke & Bruford, 1987; T. Burke *et al.*, 1989; Birkhead, 2000). The extreme prevalence of extra pair young (EPY) discovered across bird species has established infidelity as a fundamental aspect in the mating system of most bird species (Griffith *et al.*, 2002), a complete contrast to the view held just decades before.

This revelation of infidelity has not only changed our view of avian mating behaviour but also the parental care roles of male and female birds, as mating systems and parental care are intimately entwined in evolutionary theory (Trivers, 1972; Westneat & Sherman, 1992; Székely *et al.*, 2000). In fact before the extent of avian promiscuity came to light it was predicted that socially monogamous males should still attempt to gain extra pair copulations (EPCs) to increase their fitness without increasing the demands of parental care (Trivers, 1972). The trade-offs involved between parental investment and mate attraction are key focuses of sexual conflict research as both males and females are thought to differ in the costs and benefits associated with parental care and EPCs (David F. Westneat & Sargent, 1996; Chapman, Arnqvist, *et al.*, 2003).

For parental care to increase an individual's fitness it needs to be directed towards the individual's genetic offspring. A female's ability to successfully determine her genetic progeny is thought to be higher than a males, as all fertile eggs she produces are related to her, however a male cannot be certain that all her eggs were fertilized by him except in a completely monogamous mating system. In mating systems dominated by infidelity, such as birds, males would be less certain of an offspring's parentage than females and so likely have reduced ability to direct parental care correctly. This is one argument put

forward to explain the higher prevalence of female compared to male parental care across animals (Queller, 1997).

Thus the level of polygamy within a mating system could have profound effects on parental care behaviour via its effect on paternal but not maternal ability to determine parentage. Evidence for this has been sought across bird species with some studies finding that male parental care effort declines with increasing levels of EPP (Moller & Birkhead, 1993; Arnold & Owens, 2002). The impact of EPP on parental care has also been studied on an ecological timescale with varying results, some males show the predicted decline in parental investment with increasing loss of paternity (Lifjeld *et al.*, 1998). However males of other species seem to lack the ability to direct parental care appropriately, with more than half of studies failing to observe the predicted positive relationship between paternity and parental investment (Alonzo, 2010). This could be attributed to the lack of a mechanism to determine paternity in these species. Studies have subsequently attempted to determine the cues that allow some species to ascertain paternity levels while others cannot. In some species males use cues to detect the presence of other males near their partner during her fertile period, and direct their parental care accordingly (García-Navas *et al.*, 2013). However in many species it seems likely that parental care behaviour is less flexible and instead changes on an evolutionary time-scale. This lack of flexibility fits with the high repeatability of parental care behaviours exhibited within individuals across breeding attempts and also with the heritability of parental care behaviour found in the few studies to date (Freeman-Gallant & Rothstein, 1999; MacColl & Hatchwell, 2003; Charmantier *et al.*, 2007; Nakagawa *et al.*, 2007). This lack of flexibility is also indicated by the consistency of parental care systems across populations of the same species. One species in which parental care behaviour varies between the sexes but is consistent across populations and repeatable within individuals is the European penduline tit, *Remiz pendulinus* (Pogány *et al.*, 2008; van Dijk, Brinkhuizen, *et al.*, 2010).

The European penduline tit fits the expected pattern of high extra pair paternity (EPP) (23% of offspring) and low levels of male parental care (van Dijk, Mészáros, *et al.*, 2010). The majority of successful clutches are cared for by the female alone (72-90%) with the male leaving to find a new mate (Pogány *et al.*, 2008). This pattern of parental care is consistent across populations throughout Europe (van Dijk, Brinkhuizen, *et al.*, 2010). However it has been found that male abandonment is not associated with his level of paternity within the clutch, suggesting that paternity plays little role in a male's decision to provide parental care (van Dijk, Mészáros, *et al.*, 2010). However, due to the consistent pattern of parental care provisioning in this species across populations it is perhaps more suitable to study the relationship between parental care and infidelity on an evolutionary timescale. Here we assess rates of EPP in two closely related species of the European penduline tit, which show markedly different parental care behaviour.

The White-crowned (*Remiz coronatus*) and Cape penduline tit (*Anthoscopus minutus*) are both thought to exhibit greater levels of male parental care than the European penduline tit (Cockburn, 2006; van Dijk, Pogány, *et al.*, 2010). We would therefore predict them to have lower levels of infidelity, with the level of paternal care related to the level of EPP in each species. Here we aim to compare the level of EPP across these three species by using molecular genetic techniques to calculate EPP in both the White-crowned and Cape penduline tit. The results of these are compared with the previous work conducted on the European species (van Dijk, Meszaros, et al., 2010). The White-crowned penduline tit is the sister species of the European penduline tit and the Cape penduline tit is a member of the sister genus (see chapter 1). This close relatedness allows us to control in a large part for differing evolutionary histories, which have been found to explain the majority of variation in parental care strategies (Arnold & Owens, 2002).

Methods

Behavioural observations and field sampling

Fieldwork was conducted on one population of each of two species of penduline tit. The White-crowned penduline tit (*Remiz coronatus*) was studied in 2008 at a site in the Tien Shan foothills near Jabagly, Kazakhstan (42°25'N, 70°29'E). The Cape penduline tit (*Anthoscopus minutus*) of Southern Africa was studied over a 6 year period (2002-2007) at Koeberg Nature Reserve, a coastal site in the Western cape, South Africa (33°38'S, 18°26'E) consisting largely of low lying *strandveld*.

Both populations were studied during their respective breeding season, White-crowned penduline tit (May-June) and the Cape penduline tit (August-November). Nests were located by searching for audio-visual cues of each species. All penduline tit species create fairly conspicuous nests usually dangling from a branch in a small shrub or tree (Figure 2.1) and are usually fairly vocal in the breeding season, calling quietly but often. Birds were caught at or near the nest using a small net placed at the entrance to their nest or by a mist net placed within ~10m of the nest. The White-crowned penduline tit respond readily to song play back, thus this was used often in combination with a dummy bird to lure birds close to the net, in the majority of cases the males were caught in this way.

All adult birds were banded with a numbered metal ring and a unique combination of three plastic colour rings (A. C. Hughes). A blood sample (~30ul) was taken from the brachial wing vein of all adults and nestlings, and then transferred to an eppendorf tube containing 1ml of Queen's lysis buffer. These were stored at 5°C until use. Morphometric measurements were taken of all birds including weight, right wing length and left tarsus length. Each side of the head and the back of all adult birds were also photographed before birds were released at the site of capture.

Observations were made every 2 days at each nest during which the adult birds that visited the nest were recorded along with their behaviour. This allowed the social parents at each nest to be identified and also the timing of abandonment of each or both of the parents at each nest could be confidently recorded. Fifteen minutes of observation has been found to correctly ascertain whether either or both parents have abandoned the nest according to previous studies on European penduline tit (van Dijk, Szentirmai, Komdeur, *et al.*, 2007; van Dijk, 2009).

The sampling effort was substantially different between the years of study for the Cape penduline tit population. This is the most likely reason for the strikingly different numbers of nests located and offspring blood sampled, ranging between 3 and 12 nests per year (sample sizes of both nests and individuals in each year are summarised in Appendix 2.1). Across all years the total number of sampled birds was 197 in the Cape penduline tit population (52 Adults, 11 juveniles and 143 nestlings). These figures include individuals that were ringed as nestlings and subsequently recruited into the breeding population in both metrics. This occurred to a higher degree among males (n=7) than females (n=2). Of the sampled adults 19% (10/52) were observed as adults in multiple years. This is a higher retention rate than that observed in the European penduline tit where only 7% of individuals are caught across successive years (van Dijk *et al.*, 2008). For the White-crowned penduline tit population parental care was observed at 18 nests and nestlings blood sampled at 6 nests. In total 63 birds were blood sampled (32 Adults and 31 nestlings).

Figure 2.1. Examples of penduline tit nest construction. A European penduline tit (*Remiz pendulinus*) and Cape penduline tit (*Anthoscopus minutus*) nest are pictured in the left and right photos respectively. They are fairly conspicuous and all species within *Remizidae* make nests of a similar appearance.

Microsatellite selection

The microsatellites used in the current study were obtained from three previously published sources. Nine were designed specifically for use in penduline tits and had been selected from an enriched microsatellite library created using DNA from 10 European penduline tit individuals (see (Mészáros *et al.*, 2008) for the detailed methods). An additional 29 microsatellites were tested having been created as conserved microsatellites for cross-species utility in birds (Dawson *et al.*, 2010, 2013). These microsatellites were discovered by comparing the sequenced genomes of the zebra finch and chicken, two highly divergent bird species. The primers were created for the microsatellites which showed most conservation in the flanking sequence, thus allowing the design of primer pairs which would complement both the chicken and the zebra finch sequence (and presumably the many species related to the two divergent lineages, galliformes and passeriforms). Both sets of these conserved microsatellites were designed using slightly differing methods, the 'TG'

microsatellites were initially located from zebra finch expressed sequence tag (EST) sequences and the 'CAM' microsatellites were located by searching both the zebra finch and chicken whole genome sequences. For other differences in the design methods please see the respective papers (Dawson *et al.*, 2010, 2013). Both sets of conserved microsatellites were indeed found to amplify polymorphic microsatellites in a range of bird species, including many passerines. The nineteen 'TG' microsatellites tested for use in this study had previously been found to be polymorphic in the great tit, *Parus major*, the most closely related species to the penduline tits of those that had been tested previously (Dawson *et al.*, 2010). The ten 'CAM' microsatellites were tested either because they were the most conserved between the zebra finch and chicken and thus likely to amplify (CAM06, CAM13, CAM17, CAM18, CAM20 & CAM24) or they had previously been found to be polymorphic in the great tit (CAM03, CAM10, CAM15 & CAM16) (Dawson *et al.*, 2013).

A European penduline tit paternity study had been conducted previous to this current work and had used the 9 microsatellites designed specifically from European penduline tit sequences (named Remiz# primers) for the paternity analysis. For the White crowned penduline tit and Cape penduline tit paternity analyses only 7 of the Remiz primers were tested as only these 7 had previously been found to exhibit polymorphism when tested in 10 Cape penduline tits from the Koeberg population (Mészáros *et al.*, 2008).

All 36 selected microsatellites were initially tested for amplification and polymorphism in 4 individuals from each population of White-crowned and Cape penduline tit. All markers that amplified and were polymorphic in the respective species were then genotyped in every individual from which DNA had been obtained. A sexing marker designed for use across bird species was also tested. This locus exhibits a different size allele in both the Z and W sex chromosomes of many bird species allowing the sex of each individual to be genetically assigned (Dawson, 2007). All individuals were genotyped at this locus (Z-002A) to confirm that their sex assigned based on morphology and

behaviour was correct. All nestlings were unsexed in the field and thus were sexed using this genetic marker in both populations.

Of the 36 microsatellites tested in 4 adult birds from the Cape penduline tit population 97% (35/36) amplified successfully and of these 63% (22/35) were polymorphic (possessed ≥2 alleles). The results of the tests with the same microsatellites in the White-crowned penduline tit population were similarly successful with 94% (34/36) amplifying and of those 62% (21/34) exhibiting polymorphism (possessed ≥2 alleles) (see Table 2.1 for detailed results). The sexing marker (Z-002A) also successfully sexed the 8 test individuals.

Table 2.1. Details of the 36 microsatellites tested for amplification and polymorphism in 4 individuals of both the White-crowned (WCPT) and Cape penduline tit (CPT).

Microsatellite details				Genotyping Results			
Locus	Primer sequences*	Tm (°C)*	Reference	Species	# individuals	Allele size range	# alleles
Remiz-01	F: [6-FAM]TGCCTTCTATCAAGCATGAGC	59.02	(Mészáros *et al.*, 2008)	CPT	4	169-189	2
	R: TGTGCATGTAAGATTTCCATCTATC	58.09		WCPT	4	174-182	3
Remiz-07	F: [HEX]GGTAAGCTGGTGCACAAAATG	59.08	(Mészáros *et al.*, 2008)	CPT	4	206-210	2
	R: GGTCTATGAAAGATGATAGATGATGG	58		WCPT	4	159-175	4
Remiz-09	F: [6-FAM]AATTACTGAAGAACAACACATCTGG	57.34	(Mészáros *et al.*, 2008)	CPT	4	126-169	6
	R: GGACAGCTGGAGAGCAACTC	58.56		WCPT	4	116-132	5
Remiz-10	F: [6-FAM]ATCACTCCCCAGTGATAGCC	57.43	(Mészáros *et al.*, 2008)	CPT	4	178-182	3
	R: CCTTCAGCACTGAGAATAGGG	57.47		WCPT	4	195-211	4
Remiz-14	F: [HEX]CTTCTGCTTGCCTTTTGAAAC	57.71	(Mészáros *et al.*, 2008)	CPT	4	134-185	3
	R: AACGATTTGAAATATGACTGC	53.05		WCPT	4	208-224	5
Remiz-17	F: [6-FAM]CCTATCTGTCCATAGCCTTCTCTAC	57.62	(Mészáros *et al.*, 2008)	CPT	4	104	1
	R: GGATGAGAAAGTTCATGTTTTATGG	58.81		WCPT	4	147-160	4
Remiz-18	F: [HEX]CATTAATGATTGGATATGGCAAG	57.04	(Mészáros *et al.*, 2008)	CPT	4	112-368	5
	R: GTCCCTCTGCCTGTCGTTC	59.2		WCPT	4	87-104	4
CAM03	F: [HEX]ATTAGCATAGCTCAGCATTGCC	60.74	(Dawson *et al.*, 2013)	CPT	4	134	1
	R: CGAGCATTCAA**M**CCTGTCATC	60.65 (A)		WCPT	4	134	1
CAM06	F: [HEX]GTGATGGTCCAGGTCTTGC	59.04	(Dawson *et al.*, 2013)	CPT	4	286	1
	R: CAAGAGGAACAGATGAGGGTC	58.73		WCPT	4	282	1
CAM10	F: [6-FAM]TATCC**M**GAGAATGGGCATC	55.94 (A)	(Dawson *et al.*, 2013)	CPT	4	183-191	4
	R: **K**GCTCTCATTGTCATGCTG	57.83 (G)		WCPT	4	179-183	2
CAM13	F: [HEX]TCAAATACAGCAGCAGGCAG	60.16	(Dawson *et al.*, 2013)	CPT	4	214-218	3
	R: TTCATTACCAAACAGCATCCAG	60		WCPT	4	215-221	4
CAM15	F: [6-FAM]**S**GACGACTCCTTTATTTCCC	57.58 (G)	(Dawson *et al.*, 2013)	CPT	4	263-303	6
	R: TTCTGACTTCC**Y**CAGGTAACAC	56.05 (T)		WCPT	4	277-283	4
CAM16	[F] [HEX]AGCCTTGAT**M**TTGGGAAGAGC	59.7 (A)	(Dawson *et al.*, 2013)	CPT	4	292	1
	[R] ATCCATACTC**Y**GTGCAACCTG	57.68 (T)		WCPT	4	284	1
CAM17	F: [6-FAM]CGGGTTGTAATCAAGAAGATGC	60.85	(Dawson *et al.*, 2013)	CPT	4	207-210	3
	R: CTGCGGAGCAATTAACGC	60.51		WCPT	4	203-204	2

Microsatellite details				Genotyping Results			
Locus	Primer sequences*	Tm (°C)*	Reference	Species	# individuals	Allele size range	# alleles
CAM18	F: [HEX]TTAAGAAGTTTACACCCAGCG	57.16	(Dawson *et al.*, 2013)	CPT	4	336-338	2
	R: GCTAAATAACAGAGCCAGGAAG	57.38		WCPT	4	326	1
CAM20	F: [HEX]TAACAGGCAGGAATGCAGG	59.81	(Dawson *et al.*, 2013)	CPT	4	203-209	4
	R: TCAGCCAGTGTTGGAGGTC	59.81		WCPT	4	203-210	5
CAM24	F: [HEX]CCCACTTCAGTCTTCAGAGC	57.58	(Dawson *et al.*, 2013)	CPT	4	102	1
	R: TGGAGTATTTGGGATTGGAG	57.46		WCPT	4	97-102	2
TG01-000	F: [6-FAM]TTGCTACCA**R**AATGGAATGT	55.67 (A)	(Dawson *et al.*, 2010)	CPT	4	190-191	2
	R: TCCTAACCATGAGAAGCAGA	55.99		WCPT	4	212-213	2
TG01-040	F: [6-FAM]TGGCAATGGTGAGAAGTTTG	59.69	(Dawson *et al.*, 2010)	CPT	4	291-293	2
	R: AGAATTTGTACAGAGGTAATGCACTG	60.01		WCPT	4	289	1
TG01-114	F: [HEX]TTGAAACATTGTGAAGCAG	53.07	(Dawson *et al.*, 2010)	CPT	4	182	1
	R: CAGATAGTGTCATAACAATACTTTTC	53.56		WCPT	4	180	1
TG01-124	F: [6-FAM]AGTACTACTTGCCTGCAGAGTTTAT	57.15	(Dawson *et al.*, 2010)	CPT	3	404-406	2
	R: TGTGTATGGCAGCATTTACAA	57.74		WCPT	3	396-400	2
TG01-147	F: [HEX]TGAGCCACTACAGAGTGGAAA	58.51	(Dawson *et al.*, 2010)	CPT	4	272	1
	R: GCCACTCAATGAAGAAAATATTACAG	58.51		WCPT	4	272	1
TG02-088	F: [6-FAM]TGTGTGTTGACAGTATTCTCTTGC	59.36	(Dawson *et al.*, 2010)	CPT	4	FAIL	FAIL
	R: TTTAAACCTAATAAACGTCACACAGTC	59.09		WCPT	4	FAIL	FAIL
TG03-098	F: [HEX]TTTGCCTTAATTCTTACCTCATTTG	59.92	(Dawson *et al.*, 2010)	CPT	4	233-234	2
	R: TTGCAACCTCTGTGGAAGC	59.98		WCPT	4	229-230	2
TG04-004	F: [HEX]CTGGAGCAGTATTTATATTGATCTTCC	59.83	(Dawson *et al.*, 2010)	CPT	3	168	1
	R: GAAGATGTGTTTCACAGCATAACTG	60.11		WCPT	4	170	1
TG04-012	F: [HEX]TGAATTTAGATCCTCTGTTCTAGTGTC	58.55	(Dawson *et al.*, 2010)	CPT	4	137-141	3
	R: TTACATGTTTACGGTATTTCTCTGG	58.63		WCPT	4	139-143	3
TG04-041	F: [HEX]CTGAATTGTTGACCTTTGCTTAC	58	(Dawson *et al.*, 2010)	CPT	4	170-190	4
	R: GTCCTTTTAGAAAGCAGCACAG	58.34		WCPT	4	FAIL	FAIL
TG04-061	F: [HEX]GACAATGGCTATGAAATAAATTAGGC	60.42	(Dawson *et al.*, 2010)	CPT	3	191-195	4
	R: AGAAGGGCATTGAAGCACAC	60.26		WCPT	4	189-193	2
TG05_046	F: [6-FAM]AAAACATGGCTTACAAACTGG	56.86	(Dawson *et al.*, 2010)	CPT	4	326-332	3
	R: GCTCAGATAAGGGAGAAAACAG	57.26		WCPT	4	329	1
TG05-053	F: [6-FAM]GCATCATCTGGTTGAACTCTC	57.3	(Dawson *et al.*, 2010)	CPT	4	204-207	4
	R: ACCCTGTTTACAGTGAGGTGTT	57.63		WCPT	4	231-239	4

Microsatellite details				Genotyping Results			
Locus	Primer sequences*	Tm (°C)*	Reference	Species	# individuals	Allele size range	# alleles
TG06-009	F: [6-FAM]AAGCCTTGCTTACATTTTATGGTG	60.72	(Dawson *et al.*, 2010)	CPT	4	119	1
	R: GGGGTGGTAACTGAAATAAAGTATAGG	60.56		WCPT	4	119	1
TG07-022	F: [HEX]CAGAAGACTGTGTTCCTTTTGTTC	59.36	(Dawson *et al.*, 2010)	CPT	4	416	1
	R: TTCTAATGTAGTCAGCTTTGGACAC	58.94		WCPT	4	415	1
TG11-011	F: [6-FAM]ACAAACTAAGTACATCTATATCTGAAG	52.02	(Dawson *et al.*, 2010)	CPT	4	215	1
	R: TAAATACAGGCAACATTGG	52.07		WCPT	3	212-214	2
TG12_015	F: [HEX]ACAACAGTGGCTTTACTGTGTGA	59.76	(Dawson *et al.*, 2010)	CPT	4	279-281	2
	R: TACAGCAGCTGCAGCAAAGT	59.96		WCPT	4	277	1
TG13-009	F: [HEX]TGTGGTGGGATAGTGGACTG	59.39	(Dawson *et al.*, 2010)	CPT	4	196	1
	R: CTGTAAAATGTGCAAGTAACAGAGC	59.46		WCPT	4	196	1
TG13-017	F: [6-FAM]GCTTTGCATCTTGCCTTAAA	58.19	(Dawson *et al.*, 2010)	CPT	4	281	1
	R: GGTAACTACAACATTCCAACTCCT	57.74		WCPT	3	310-312	2

* Degenerate basepairs in primer sequences are underlined and highlighted in bold font, the primer melting temperatures (Tm) of these primers are calculated based on one of the degenerate base pair combinations, signified in brackets.

DNA extraction

All DNA was extracted from blood samples using the ammonium acetate precipitation method. Birds have nucleated red blood cells and so DNA can be readily obtained from even a minimal sample. A small amount (30-50ul) of the globular contents from the blood samples collected in the field was transferred by pipette to an eppendorf containing 250ul Digsol buffer and 15ul of Proteinase K. These were then placed in a rotating oven at 36°C overnight. Once digested 300ul of 4M ammonium acetate was added to each eppendorf. These were vortexed several times over a period of 15 minutes and then centrifuged for 20 minutes at 13,000rpm. The supernatant was then aspirated into an empty 1.5ml flip-top tube and the eppendorf containing the pelleted protein was discarded. The DNA was then precipitated by adding 1ml of 100% ethanol to the supernatant and vortexed briefly. The tubes were then centrifuged for 15 minutes at 13,000 rpm and the ethanol was poured off leaving a DNA pellet at the bottom of the tube. This was then rinsed by adding 500ul of 70% ethanol to the pellet and vortexing briefly. Once again this was centrifuged at 13,000 rpm for 5 minutes and the ethanol poured off in a smooth movement. The tube was then placed upside down on tissue for 30 minutes to allow the remaining ethanol to evaporate. When dry the DNA pellet was resuspended in 50ul of TE by briefly vortexing and leaving in a water bath at 65°C for 30 minutes. The DNA extractions were then stored at -20°C until quantification.

The DNA samples were quantified using a FLUOstar Optima Spectrophotometer (BMG labtech). A 2ul volume of each sample was loaded into a BMG black plate along with 7 calf thymus quantification standards (0, 3.24, 6.49, 12.98, 25.95, 51.9 & 103.8ng/ul). A volume of 200ul of Hoesct dye, which fluoresces when bound to DNA, was then added and the readings taken in the fluorometer. Aliquots of all samples were diluted to 10ng/ul concentration with T.E. and before use in the microsatellite genotyping.

Microsatellite genotyping

All Polymerase Chain reactions (PCRs) were carried out as 2ul reactions in either a TETRAD DNA Engine (MJ Research, Biorad) or a Touchdown thermal cycler (Hybaid). A DNA sample (1ng) was dried onto the bottom of each well in a 96 well plate. Then 1ul of primer mix was added to each well, the primer mix consisted of the forward and reverse primers diluted in ddH_2O so that each was at a concentration of 0.2ng/ul. If it was a singleplex reaction then just one primer pair was in the mix but multiplex reactions were also run with each primer at 0.2ng/ul. All initial tests of each microsatellite were conducted as singleplex reactions but multiplexes containing up to 7 primer pairs were subsequently designed for use across all individuals. These were designed by combining markers labelled with different dyes (6-FAM and 5HEX) or by combining those that did not overlap in allele size. After adding the primer mix, 1ul of Qmix was added to each well. The plate was then briefly centrifuged and 20ul of mineral oil was added to each well before running the PCR. All primers used in this study were designed to run at the same annealing temperature and so every PCR was run using the exact same protocol, this started with a Hotstart at 95°C for 15 mins to activate the Qmix and then 45 cycles of the following temperatures and times were used, a denaturation step of 94°C for 30seconds, followed by an annealing temperature of 56°C for 90 seconds, finishing with an elongation step of 72°C for 90 seconds. After the 45 cycles the whole reaction ends with a 72°C elongation step for 10 minutes. The PCR product in each well is then prepared for input onto an ABI 3730 DNA Analyzer (Applied Biosystems) for genotyping.

All PCR products are diluted 1/160 times in T.E. Then 0.5ul of this dilution is placed in the well of a greiner skirted PCR plate and 9.5ul of a Rox/Formamide mix (4ul Rox in 1ml Formamide) is added to the well. The plate is briefly centrifuged and denatured for 5 minutes at 95°C before being placed in an ice bath for 4 minutes. The plate is then run on the 3730 with a 2 second injection time. All genotypes were visualised and scored using Genemapper 3.7 (Applied Biosystems).

Table 2.2. Results of the genotyping in all individuals of the White-crowned penduline tit (WCPT) and Cape penduline tit (CPT) populations.

Locus	Species	# individuals genotyped	Allele size range	# alleles	Ho	He	Null allele frequency
Remiz-01	CPT	148	107-269	20	0.291	0.754	0.4421
	WCPT	67	169-190	6	0.627	0.725	0.0639
Remiz-07	CPT	168	206-214	3	0.232	0.227	-0.0186
	WCPT	67	159-179	6	0.806	0.787	-0.0164
Remiz-09	CPT	166	113-168	11	0.373	0.74	0.3338
	WCPT	67	108-137	9	0.448	0.787	0.2688
Remiz-10	CPT	168	177-206	5	0.458	0.482	0.0303
	WCPT	67	187-211	7	0.776	0.774	-0.0054
Remiz-14	CPT	-	-	-	-	-	-
	WCPT	67	206-223	9	0.761	0.8	0.0257
Remiz-17	CPT	-	-	-	-	-	-
	WCPT	67	142-164	6	0.776	0.714	-0.0423
Remiz-18	CPT	167	100-394	24	0.892	0.89	-0.0047
	WCPT	67	83-114	9	0.851	0.798	-0.0344
CAM10	CPT	172	183-194	7	0.68	0.722	0.0359
	WCPT	67	179-187	4	0.433	0.421	-0.041
CAM13	CPT	172	213-218	5	0.506	0.502	-0.0046
	WCPT	67	215-221	7	0.761	0.772	-0.0007
CAM15	CPT	168	283-385	11	0.845	0.853	0.004
	WCPT	-	-	-	-	-	-
CAM17	CPT	173	203-211	5	0.37	0.386	0.0454
	WCPT	67	203-204	2	0.463	0.493	0.0275
CAM18	CPT	167	334-340	4	0.497	0.538	0.0405
	WCPT	-	-	-	-	-	-
CAM20	CPT	-	-	-	-	-	-
	WCPT	66	198-211	11	0.864	0.834	-0.0263
CAM24	CPT	-	-	-	-	-	-
	WCPT	67	97-102	3	0.224	0.348	0.208
TG01-040	CPT	171	291-293	2	0.351	0.327	-0.0374
	WCPT	-	-	-	-	-	-

Locus	Species	# individuals genotyped	Allele size range	# alleles	Ho	He	Null allele frequency
TG01-124	CPT	149	404-406	2	0.161	0.182	0.0585
	WCPT	64	396-400	2	0.281	0.307	0.04
TG03-098	CPT	169	233-234	2	0.432	0.493	0.0645
	WCPT	67	229-230	2	0.582	0.503	-0.0763
TG04-012	CPT	171	136-141	3	0.398	0.417	0.0125
	WCPT	67	137-145	5	0.642	0.667	0.0104
TG04-041	CPT	173	170-190	10	0.705	0.705	0.0092
	WCPT	-	-	-	-	-	-
TG04-061	CPT	173	184-195	7	0.671	0.765	0.0657
	WCPT	67	188-196	4	0.269	0.245	-0.0645
TG05-046	CPT	171	326-332	3	0.579	0.529	-0.0468
	WCPT	-	-	-	-	-	-
TG05-053	CPT	173	201-295	9	0.792	0.775	-0.013
	WCPT	67	231-240	9	0.881	0.829	-0.0348
TG11-011	CPT	-	-	-	-	-	-
	WCPT	67	210-216	4	0.642	0.632	-0.0128
TG12-015	CPT	170	279-281	2	0.094	0.101	0.0317
	WCPT	-	-	-	-	-	-
TG13-017	CPT	-	-	-	-	-	-
	WCPT	66	308-320	4	0.424	0.431	0.0042

Ho & He represent observed and expected heterozygosity respectively, calculated in CERVUS v 3.0.

Parentage analysis

After genotyping each marker was assessed to ascertain its suitability for paternity analysis in the two species. All markers that were difficult to score e.g. had an unclear multi-peak profile or unaccountable peaks were discarded. Null allele frequencies were estimated in the program CERVUS v3.0 using all genotyped individuals within each population, and any markers with frequencies ≥0.2 were excluded from further analyses.

Paternity was allocated using the program COLONY v 2.0.5, which assesses the likelihood of sibships within the population as well as allocating paternity and maternity via a maximum likelihood method. COLONY requires information on allele scoring error to calculate likelihood scores. Errors due to null alleles can to an extent be calculated based on allele frequencies within the population. These were therefore calculated within CERVUS using the entire dataset. However an overall mis-typing error rate was calculated by running duplicate samples in parallel for ~7% of each population, these were duplicated from the start of DNA extraction in the White-crowned penduline tit and from the point of capture in the Cape penduline tit and then alleles compared after the final datasets were compiled.

Colony uses a maximum likelihood approach to ascertain both maternity and paternity for each offspring as well as full and half sibship relationships of each nestling. Since we had data from multiple years for the Cape penduline tit population we analysed each year separately as offspring in one year could be parents in subsequent years. For each analysis the candidate parents were all birds that had been caught as adults in that year or in any of the previous years. The likelihood of the parent being sampled was kept at 0.5 for all runs. The full likelihood model with a long-run was selected for all analyses. Both males and females were assumed to be polygamous and a single iteration was performed for each year. No a priori information about known social relationships was provided. Birds were accepted as assigned correctly if they had a likelihood score ≥80 and there were no other birds with a likelihood score ≥50.

Results

Microsatellite genotyping

In the Cape penduline tit population 167 individuals were successfully genotyped across the 6 breeding seasons (yearly sample sizes are given in Appendix 2.1). A roughly equal number of adult males and females were genotyped (16 males, 18 females). Of the genotyped adults sexed in the field 93% (27/29) were sexed successfully. The nestlings were found to be of a roughly equal sex ratio (62 males: 58 females, $\chi^2 = 0.1333$, df = 1, p = 0.715) using the genetic sexing marker. This pattern was also found in the juveniles caught (5 males: 8 females, Pearson's $\chi^2 = 0.039$, df = 1, p = 0.8435).

In the White-crowned penduline tit population, 63 (32 adults and 31 nestlings) individuals were successfully genotyped. In the field 100% (32/32) of adults were sexed successfully and the offspring sex ratio was not found to be significantly different from parity (18 Males: 13 females, Pearson's $\chi^2 = 0.1461$, df = 1 p=0.7023).

Patterns of parental care

In the Cape penduline tit parental care was performed by both male and female at the observed nests for the duration of care (n=21/21, 100%) and nest attendance was highly synchronous between the pair (see (van Dijk, Pogány, *et al.*, 2010)). Of the 18 nests observed in the White-crowned penduline tit population, incubation was carried out by both male and female, and biparental care continued through nestling provisioning at 14/18 nests (78%), at the other 4 nests uniparental care was provided after incubation by either the male (2/18 nests, 11%) or the female (2/18 nests, 11%) (Figure 2.2).

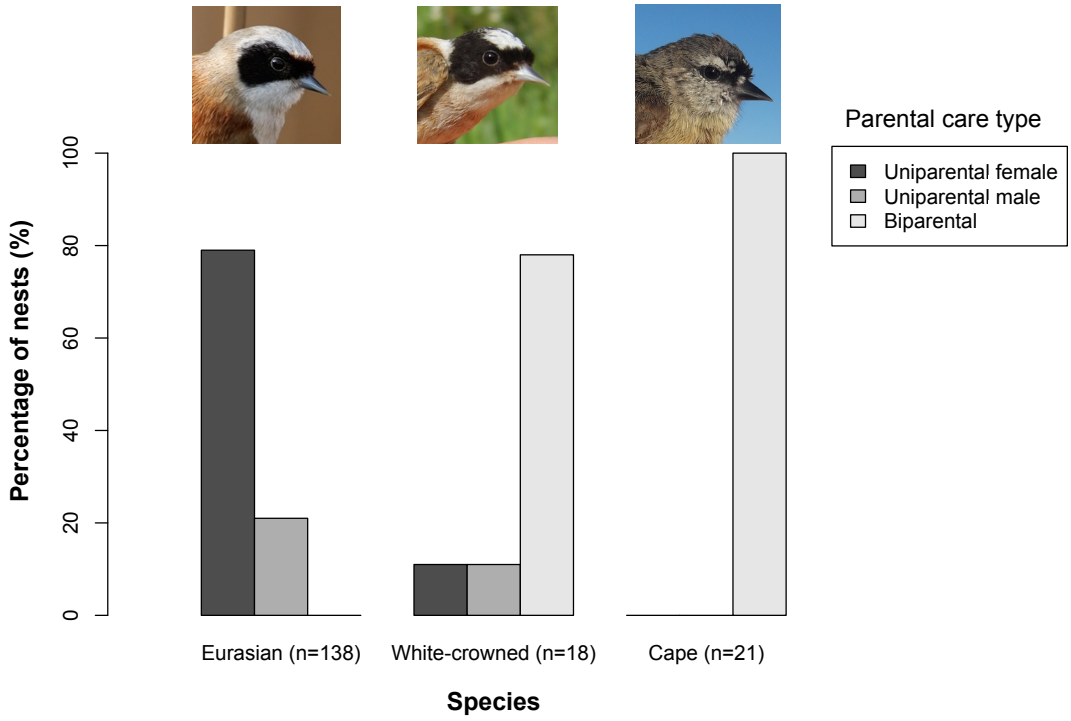

Figure 2.2. Mode of parental care observed in three species of penduline tit. The European penduline tit (EPT) shows a significant difference in parental care system compared to the White crowned (WCPT) and Cape penduline tits (CPT), exhibiting uniparental care provided in the majority of cases by the female (Pearson $\chi^2 = 191.11$, df = 4, $p < 0.001$). Biparental care predominates in the other two species.

Parentage analysis

Three of the 22 microsatellites (CAM20, TG01_000 & Remiz14), genotyped for the parentage analysis in the Cape penduline tit population, were very difficult to score either possessing many alleles differing in size by only 1bp or amplifying inconsistently and so were excluded from further analysis. Two markers (Remiz01 & Remiz09) showed predicted null allele frequencies of ≥0.2 and deviated significantly from Hardy-Weinberg equilibrium, they were thus excluded from further analysis. This meant that 17 microsatellite markers were used to assign parentage (see Table 2.2 for microsatellite details). These gave a high resolution for assigning parentage in this population with a combined non-exclusion probability for a parent pair of 0.00000005 according to CERVUS. Thus if we have sampled the parents they will be identified with high accuracy.

Of the 13 nests where the social mother and father were known, 2 contained EPY (15.4% nests contain EPY). In these 2 nests all EPY were the result of EPP thus a total of 3/42 (7.1%) nestlings were the result of EPP (see Figure 2.3). If we then include all nests where the social father is known, 11.1% (2/18) contain offspring resulting from EPP with a total of 4.6% (3/65) of nestlings the product of EPP. When analysing all offspring with known social mothers 1.4% (1/72) of young are found to mismatch with their social mother and this one case is likely the product of egg-dumping as the individual also mismatches with the father allocated to the other 4 young in its nest. Thus 4.5% (1/22) of these nests contain offspring resulting from egg-dumping.

Two of the 21 microsatellites used to genotype all individuals in the White-crowned penduline tit parentage analysis were difficult to score due to 1bp allele differences and unclear profiles (TG01-000 & CAM15), thus they were excluded from further analyses. The predicted null allele frequencies were also high (≥0.2) for 2 of the markers and these were excluded from the parentage analysis (CAM24 & Remiz-09). Subsequently 17 microsatellites markers were used to assign parentage in the White-crowned penduline tit population (See Table 2.2 for details). They provided a robust method for correct assignment

with the parent pair combined non-exclusion probability calculated at 0.00000000774 in CERVUS.

None of the 4 nests, where both social parents were known, contained EPY. Thus 0% of the 22 nestlings were the result of EPCs. This is also likely to be the case for the other nest where only the social mother was known. All 7 nestlings were assigned to the social mother and the same male (KA46290) with 100% likelihood. Thus no evidence for EPP or EPM (0/29 offspring) was found in the White-crowned penduline tit population (see Figure 2.3 for comparison with European and Cape penduline tit).

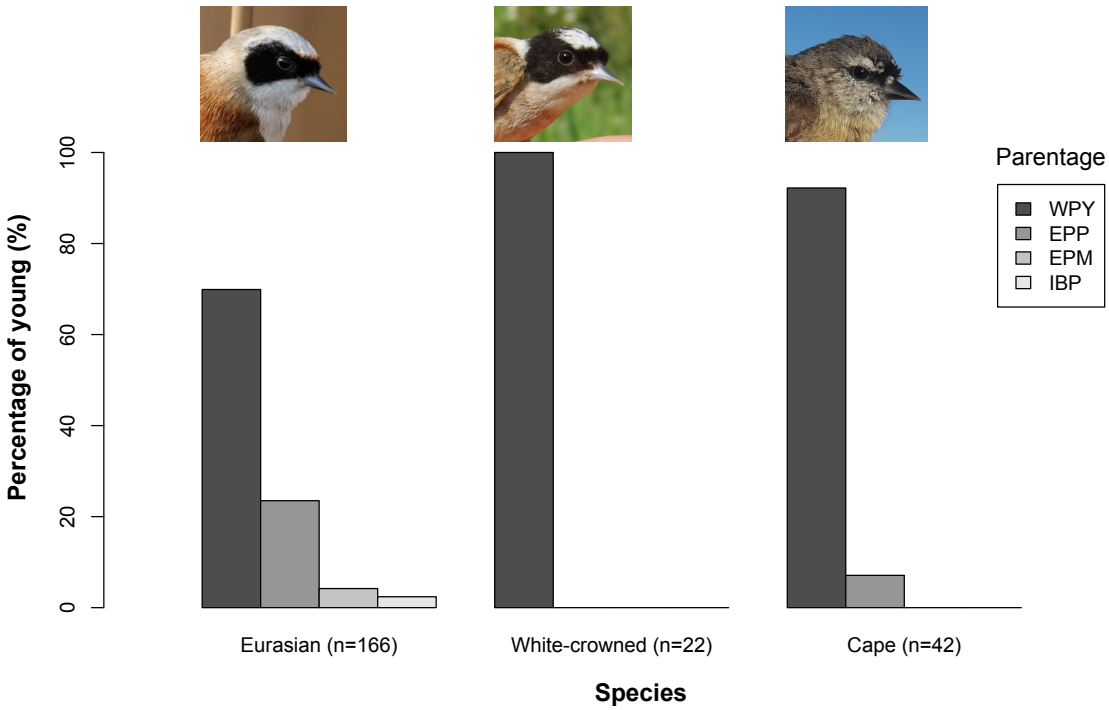

Figure 2.3. Parentage analyses in three penduline tit species (*Remizidae*). Infidelity is significantly greater in the European penduline tit (EPT) compared to its two relatives, the White crowned (WCPT) and Cape penduline tits (CPT) (Pearson $\chi^2 = 16.38$, df = 6, p = 0.012). The EPT has large numbers of offspring arising from extra pair paternity (EPP) compared to the majority of passerines however the WCPT and CPT have very low levels with almost all offspring found to be within pair young (WPY). Evidence for the existence of extra pair maternity (EPM) and egg dumping is also greater in the EPT compared to its two relatives. All data are based on offspring in nests where both social parents had been identified.

Discussion

Parental care is provided by both males and females for a more equal time period in the White-crowned and Cape penduline tits than in their close relative, the European penduline tit. Parental care was biparental through to fledging for the majority of nests in both species with the White-crowned penduline tit exhibiting a few nests with uniparental care by either the male or female during nestling provisioning (see Figure 2.2). These observations are consistent with prior knowledge of these two species which has suggested a more cooperative parental care system than the European penduline tit (Harrap & Quinn, 1996). The greater degree of synchrony between pairs in their parental care behaviour has also previously been documented in the Cape penduline tit compared to the European penduline tit (van Dijk, Pogány, *et al.*, 2010).

This study also reveals the polymorphic nature of 17 microsatellites in the two study species. This has not only allowed us to conduct a parentage analysis but provides a set of markers, which could be used for further genetic analyses in these species. The sexing marker (Z-002) was also successful in both species and allowed us to reveal the equal sex ratio of offspring in both study populations. Sex ratios have been revealed to fundamentally affect mating systems (Liker *et al.*, 2014) and thus factors which cause unequal sex-ratios have important consequences for the mating success of individuals in a population. Our results reveal that sex ratios in the nest are not different from parity and so would not be responsible for a skewed adult sex ratio.

In line with our main prediction that paternal care is associated with reduced infidelity we have found much lower levels of EPP in the biparental White-crowned penduline tit and Cape penduline tit compared to the uniparental European penduline tit (see Figures 2.2 and 2.3). The EPP levels of the European penduline tit were previously found to be much higher than the majority of passerine birds (a level of 23.5% (van Dijk, Mészáros, *et al.*, 2010)). However EPP levels in the two penduline tit species in this study were much lower than the average found in passerines (estimated to be ~15% by (Griffith *et al.*, 2002)). The EPP rate is likely to be between 4.5-7.1% in the Cape

penduline tit and no evidence of EPP was found in the White-crowned penduline tit. We have to be aware that our sample sizes are small thus there are large opportunities for error however at present they indicate much lower levels of EPP in these two species compared to the European penduline tit. Obviously further sampling would greatly increase the accuracy of the current estimates in all three species.

Our study raises questions about the evolution of parental care and EPP in these species. Although we provide evidence that they seem to be associated, what is the driver behind these associations? The large differences in such closely related species could allow us to tease apart the causes and consequences of these differing parental care and mating strategies. Some researchers are acknowledging that parental care and mating systems are intimately linked and to study either in isolation is unlikely to lead to resolution of the causes and consequences underlying these behaviours (Alonzo, 2010). The ability of comparative research to test some of the current theories is helping to answer long standing questions (Pagel & Meade, 2006; Cornwallis *et al.*, 2010).

This study outlines the diversity in the breeding systems found within the Penduline tit family. All three species exhibit differing parental care systems and we now show that levels of infidelity differ, from very high to very low compared to the majority of other passerines. This strengthens their position as a group for comparative research on the evolution of mating and parental care systems. Increasing our knowledge of other members of this group could shed even more light on these fascinating topics. Anecdotal evidence of cooperative care in some species raises the possibility that even greater diversity exists within this small family. This group thus provides an exciting opportunity for comparative evolutionary research.

Chapter 3

Parental care and latitude predict sexually dimorphic plumage evolution in the penduline tits (*Remizidae*)

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Abstract

The origin and maintenance of sexual dimorphism is thought to evolve under a variety of situations. Most hypotheses fall into two main categories 1) sexual selection, and 2) ecological selection for gender specific niche divergence. This chapter investigates the presence of these types of selection pressures on sexual dimorphism in the penduline tit family. Sexual dimorphism in birds is most commonly found in body size and plumage traits. We thus calculate sexual dimorphism indices for each of these traits and compare the predictive ability of two ecological variables (rainfall and latitude) and the level of sexual conflict over care. Ecological selection on niche divergence predicts that sexual dimorphism evolves to decrease foraging competition between the sexes and is thus likely driven by reductions in food availability. If sexual selection drives sexual dimorphism we would expect dimorphism to be greatest in the species with the highest disparities in sex specific parental care levels. We explore the variation in parental care across the penduline tit family and compare the evolution of sexual dimorphism between uniparental and biparental species. The results provide little evidence of sexual size dimorphism and no support for greater levels of sexual dimorphism being associated with environments with reduced food availability (low rainfall and low latitude). Instead plumage dimorphism is positively associated with latitude and evolves much more rapidly in species with reduced parental care, driven by changes in male plumage. The results suggest a complex interaction between parental care, sexual selection and latitude in the evolution of the penduline tit family.

Introduction

Sexual dimorphism, here defined as the differences between the sexes in secondary sexual characters, is present to varying degrees in sexually reproducing organisms (Lande, 1980; Fairbairn *et al.*, eds, 2007). It has garnered the attention of naturalists for centuries and its all-pervasive nature was instrumental in the development of Darwin's theory of sexual selection (Darwin, 1871; Kottler, 1980). It has been especially well studied in birds due to the often showy nature of plumage and vocal displays present in one sex but not the other (Owens & Hartley, 1998). However the selection pressures that lead to and maintain sexual dimorphism have been found to be highly variable across species. The main hypotheses fit into two main categories, driven by either sexual or ecological pressures.

Sexual selection hypotheses encompass those ascribing sexual dimorphism to on-going sexual conflict. Sexual conflict is thought to be a key force in the evolution of sexually selected traits (Parker, 1979; Chapman, Arnqvist, *et al.*, 2003). A conflict arises when the optimum level of a trait differs between the sexes, causing antagonistic co-evolution, where in one sex a trait value conveys an advantage but to the other the same trait value is less advantageous or is even detrimental. It is thought that the outcome of this on-going conflict manifests itself as sexual dimorphism, which has the ability to mediate or resolve the conflict (Bedhomme & Chippindale, 2007; Bonduriansky & Chenoweth, 2009).

Ecological pressures are thought to explain some examples of sexual dimorphism (Shine, 1989). In species with breeding territories, intersexual competition between the pair is limited if there is sexual dimorphism in feeding behaviour. For example sexual dimorphism in beak size is often attributed to niche separation between the sexes with males and females evolving different diets (Slatkin, 1984). More modern approaches are now coming to the realisation that both ecological and sexual pressures work in concert to produce the sexual dimorphism we observe in the natural world (Krüger *et al.*, 2014). By

studying individual cases of sexual dimorphism we can build up an understanding of the key pressures that have led to its evolution.

The European penduline tit exhibits obvious sexual dimorphism in plumage traits and is known for its unusual mating system, dominated by sequential polygamy by both sexes. This leads to large numbers of deserted nests (~30%), where neither parent stays to provide parental care (Pogány *et al.*, 2008). This high level of clutch desertion has led to detailed research on the underlying reasons for this aberrant behaviour (Bleeker *et al.*, 2005; Szentirmai *et al.*, 2007; van Dijk, Mészáros, *et al.*, 2010). It is thought that conflict over care explains this unusual behaviour; both male and female benefit from deserting and starting a new clutch if their current partner remains to care (van Dijk *et al.*, 2012). However, parental care is biased towards females with 60% of nests cared for by single females compared to 10% of nests cared for by males. A recent study has found evidence that sexual dimorphism in a facial plumage trait is less marked in a biparental species with reduced conflict over care, suggesting that this sexual dimorphism is associated with sexual conflict over care.

We aim to increase knowledge of the parental care systems in the penduline tit family, by behavioural field studies and test the influence of environmental variables and parental care on sexual dimorphism across the penduline tits. Due to their large geographic range and differing levels of sexual conflict the penduline tit family (*Remizidae*) provide a convenient system in which to test the differing roles of ecological and sexual selection pressures on the evolution of sexual dimorphism. Here we develop and test *a priori* predictions about the evolution of sexual dimorphism under differing selection pressures. If foraging niche pressures are driving sexual dimorphism we predict that sexual dimorphism will be present in traits associated with foraging ability (wing and beak morphology) (Seddon *et al.*, 2013). We also predict that greater dimorphism will be associated with less productive environments. This study associates low productivity with environments lacking rainfall and also with those at lower latitudes. Both rainfall and latitude are known to have large impacts on food availability in birds (Lack, 1954; Martin, 1987; Preston & Rotenberry, 2006). It is predicted that the fewer daylight hours, at lower

latitudes, provides a large constraint to brood provisioning. Studies linking higher rainfall levels with increased productivity, especially at lower latitudes also suggests this could have a large effect on food availability (Sinclair, 1978; Lepage & Lloyd, 2004; Oppel *et al.*, 2013).

However, if sexual conflict over care is driving sexual dimorphism we predict that traits associated with mate attraction (plumage) show greater sexual dimorphism in species with increased conflict over care. The non-caring sex is predicted to be under increased levels of sexual selection due to increased choosiness by the caring sex. Thus rapid evolution in plumage sexual dimorphism is predicted in uniparental species driven by changes to the non-caring sex (usually males in the penduline tit).

We thus aim to describe the detailed parental care behaviour of a range of penduline tit populations and explore the evolution of sexual dimorphism within this group. We test the effect that parental care and environmental differences have on the evolution of sexual dimorphism in both body size and plumage traits. This group has a wide distribution, across 4 continents, spanning a large range of environments and exhibiting large variety in parental care behaviour, and thus provide a suitable system in which to test ideas on the evolution of sexual dimorphism.

Methods

Penduline tit specimens used in this study are from the museum collection at the British Natural History Museum and from fieldwork conducted throughout Eurasia and in South Africa during the years 2003-2008. All 12 monophyletic species within the penduline tit family (*Remizidae*) have been sampled (species according to phylogeny in Chapter 1). Of the 41 subspecies described by (Harrap & Quinn, 1996), 20 have been obtained for morphometric analysis from across the global distribution of the penduline tit family.

Phylogeny

The phylogeny used to study the evolutionary rates of sexual dimorphism in the penduline tits was produced using the *BEAST program as outlined in Chapter 1. This is a Bayesian method, which infers species trees using multiple gene sequences. This phylogeny was created using 5 gene sequences from all 12 species of the penduline tits including subspecies and 3 passerine outgroups, the *Paridae*, *Corvidae* and *Tyrannidae* (see Figure 3.1).

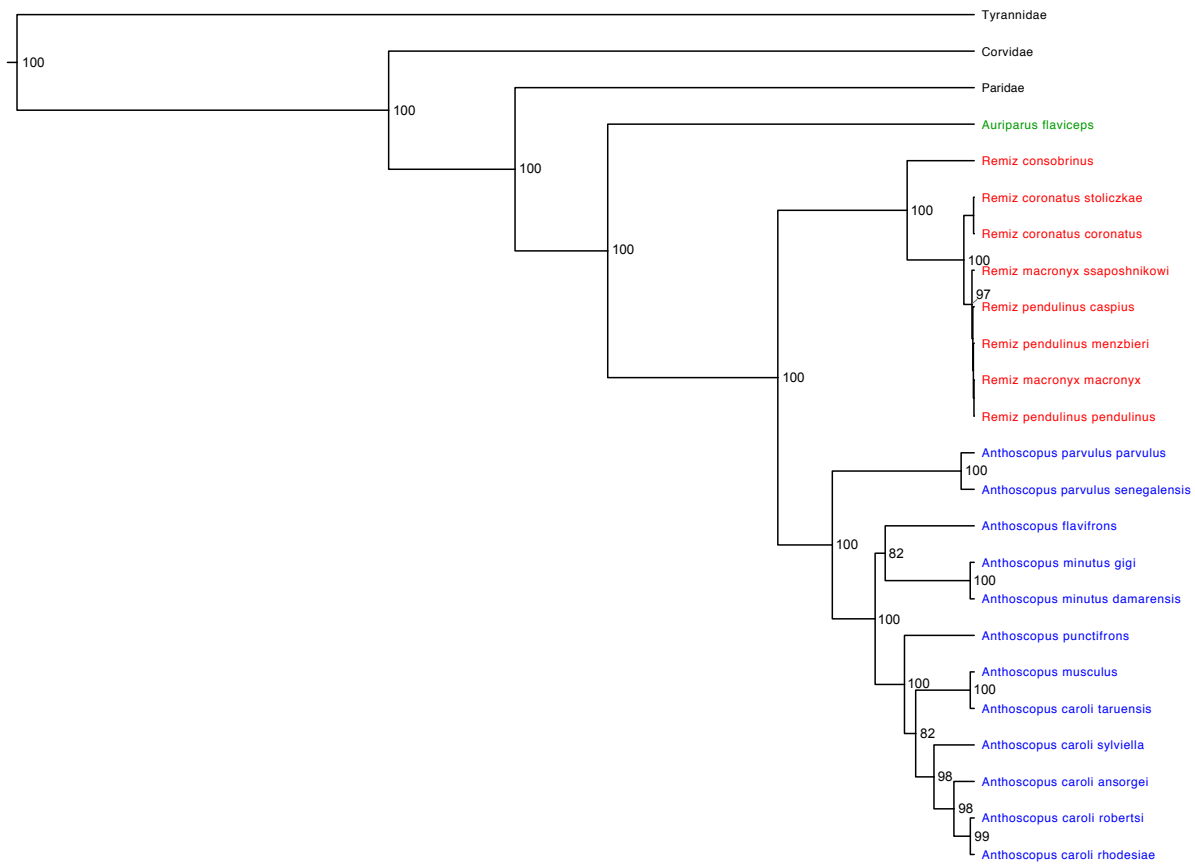

Figure 3.1. Phylogeny inferred by the *BEAST coalescent approach detailed in Chapter 1. Node labels denote the posterior probabilities, only those over 80 are shown. Tip labels are colour coded according to genera (Red = *Remiz*, Blue = *Anthoscopus* and Green = *Auriparus*).

Sexual dimorphism

For 20 of the 21 subspecies used in the 5-gene phylogeny we collected morphometric data from both sexes, using a combination of museum specimens (British Natural History Museum) and individuals mist-netted in wild populations. These included three body size traits (right wing length, beak length and beak height) which are often associated with ecological selection pressures in passerines (Grant & Grant, 1993; Dawideit *et al.*, 2009), and three plumage measurements (area of melanin based face-mask, contrast between the face mask and crown feathers and the colour saturation of mantle plumage) generally associated with sexual selection pressures in passerines with evidence for female preference for large mask size in the European penduline tit (Andersson & Iwasa, 1996; Pogány & Székely, 2007; Kingma *et al.*, 2008). All museum measurements were taken by ADB and the live specimen measurements by RvD, SB, AP and ADB. The sex of every individual measured was determined by dissection for the museum samples at time of capture as noted on the specimen labels and for the live specimens behaviour and plumage traits were used to differentiate the sex of mating pairs.

Wing length was measured from the carpal joint to the tip of the longest primary with the wing chord flattened and straightened. Beak length was measured on the right side of the bird from the anterior edge of the nostril to the tip of the upper mandible and the beak height was measured using vernier calipers at the point of feathering on the upper mandible.

The size of the melanin based head ornament was calculated for a subset of individual penduline tits from each population. The melanin based head ornament consists of a black mask starting at the beak and extending backwards around the eyes and head (see Figure 3.2). All birds had three photos taken of both the right and the left side of their head, with a Kodak R27 grey card and a 100mm ruler in the background. The grey card was used to set the grey point of every photo to control for differences in light conditions. All photos were taken at ~10cm distance from the bird. These photos were then

standardized using the freely available photographic software GIMP 2.6 (http://www.gimp.org/) or Photoshop v 7.0. The number of pixels in a 1cm^2 area of the photo was calculated using the ruler. The area of the melanin plumage trait was measured using the 'fuzzy select' tool, which selects all pixels of a similar colour to the one highlighted, thus selecting all the black pixels in close proximity. The area of the eye was included in this selection and any aberrant pixels, such as the beak were excluded from the selection using the 'free select' tool. The number of pixels in the selected region was calculated, and this along with the number of pixels in a 1cm^2 region allowed us to calculate the area of the melanin ornament in cm^2. This was repeated on each photo giving three measures for each side of the head from which the means were calculated.

The contrast in brightness between the mask and crown feathers was also calculated using GIMP or Photoshop. The average red, green and blue (RGB) values of the mask pixels were calculated in the photographic software and then a 0.2mm^2 area of the crown feathers just above the eye were selected and the average RGB values also recorded for this area. We quantified contrast in brightness between mask and crown using the CIELAB colour-difference equation in BabelColor® Color Translator and Analyzer v 3.1.1 using the RGB values obtained from the photographic software.

The intensity of colour in the mantle feathers was estimated by calculating the degree of colour saturation in the mantle. Saturation, a measure of intensity and the distribution of wavelengths being reflected, quantifies the purity of a colour, a higher saturation score indicating a brighter more intense colour. Three photos were taken of the back of each bird at ~10cm distance. Once again a grey card was used as background to control for variation in light conditions, although these were maintained as much as possible. The photos were analysed in GIMP or Photoshop as before and the photos standardized using the grey card to set the grey point. A 0.5cm^2 area was selected in the centre of the mantle feathers and the mean RGB values for the selected region obtained. The Saturation level (S_{HSV}) was calculated using the following formula:

Saturation (S_{HSV}) = 100 × (MAX(R,G,B)-MIN(R,G,B))/(MAX(R,G,B))

This was conducted for all birds, replicated twice and the mean for each bird was calculated from the three separate measurements.

Two Sexual Dimorphism Indices (SDI) were calculated for each subspecies, one using the three bodysize traits and one for the three plumage traits. We initially used the SDI advocated by Smith 1999 which is calculated as follows:

SDI = ln(Mean value of larger sex/ Mean value of smaller sex)

This allowed us to gain a sexual dimorphism measure for each of the 6 traits. We then normalised these measures across the subspecies to gain a standardised sexual dimorphism index (z) for each trait, where z = (trait value for a given species – mean trait value across all subspecies)/(standard deviation of trait value across all subspecies). To calculate a subspecies sexual dimorphism index for both plumage and morphological traits we took the mean of the three relevant z scores. The mean measurements and SDIs for each subspecies for the 3 size traits and 3 plumage traits can be found in Appendices 3.1 and 3.2 respectively.

Figure 3.2. Examples of the melanin based facial mask in 2 penduline tit species, A) White-crowned penduline tit male (*Remiz coronatus*) B) White-crowned penduline tit female and C) Cape penduline tit (*Anthoscopus minutus*). Photos taken by REvD (A & B) and ADB (C).

Parental care classification

We classified the parental care strategies of each species as either conflicting (uniparental) or cooperative (both parents care for the young through to fledging). Data for each species was gained by behavioural observations in the field (5 sub-species) or a literature search. Thus abandonment by one parent occurring before or during incubation was defined as a uniparental population. Observations were made at a minimum of 18 nests for each species studied in the field however the literature searches focused on evidence with fewer observations or were inferred modes of care. Observations were made on colour-ringed birds for all populations studied in the field. As uniparental care can be provided by either the male or female, for the species in which we obtained detailed data on parental care modes we also calculated the % of males and females that care for their young through to fledging. This allowed us to test the predictive ability of parental investment and environmental variables on the patterns of sexual dimorphism across the species via phylogenetic generalized least squares (PGLS) analyses.

Breeding latitude and rainfall

Three localities within each subspecies breeding range were selected. Two of the selected localities were on opposite range margins of the breeding range and the other was situated randomly within the core central range. Breeding ranges were determined using the data held by birdlife international and

geographic information from (Harrap & Quinn, 1996). For each locality both the latitude was recorded and the mean annual precipitation obtained from meteorological websites. The averages of these three localities were then used to represent the typical environmental conditions in the breeding range of each subspecies.

Correlations with sexual dimorphism

We tested the correlations between sexual dimorphism and three potential explanatory variables, the two environmental variables (latitude and annual rainfall) and the amount of male care, measured as the % of males who provide parental care through to fledging. We performed phylogenetic generalized least square (PGLS) analyses in the software environment R using the "caper" package. We used the lambda parameter to decide whether to control for phylogenetic signal when performing the analyses. Lambda models the rate of evolution of traits evolving under a Brownian motion model of evolution. A lambda value of 1 indicates a strong phylogenetic signal in the data and a lambda of 0 signifies a lack of phylogenetic signal. We tested the relationship of both the sexual dimorphism indices (plumage and size) against the three explanatory variables (% of males who care, latitude and rainfall).

Evolutionary rate of sexual dimorphism

For the two sexual dimorphism measures (plumage and size) we estimated their rate of evolution in the Penduline tit family following a phylogenetic comparative method in the R package, MOTMOT (Thomas & Freckleton, 2012). This used a maximum likelihood approach to compare the rate of evolution of traits in different parts of the phylogeny. For each analysis we split the phylogeny into two and compared the rates of evolution of the plumage and size measurements between each. The phylogeny was split via mode of care (conflict versus cooperative) and latitude (high (>30°) versus low (<30°). Using our behavioural observations and available literature we defined each species as either exhibiting conflicting or cooperative care systems. Cooperative care here

being defined as any care system in which both parents were involved in the care of young through to fledging. The rate of evolution of sexual dimorphism for both of the sexual dimorphism indices, were then compared between the two groupings using the methods outlined in (Thomas *et al.*, 2006). We assumed a Brownian rate of evolution for each trait and compared a model with one rate of evolution across the entire phylogeny with a model that allowed different rates of evolution between the two groups. A maximum likelihood approach was then used to test for a significant difference between the explanatory power of each model.

We used the phylogeny produced by the coalescent method in *BEAST and initially tested its reliability by running 10,000 simulations. The simulated data was produced by modelling a Brownian rate of evolution on a randomized ancestral trait to produce a likely value for each (sub)-species branch tip. The differences in the evolutionary rate between the two parental care groups were then calculated for each simulation to test for any deviations from the expected NULL hypothesis of no differences. All 10,000 simulations agreed with the NULL hypothesis and had a mean, mode and Median of THETA = 1.03, indicating no bias within the phylogeny.

Historic modes of care were inferred using the MacClade program, which uses parsimony to assign historical traits based on the current modes of care. Past latitudes were denoted using the bio-geographical analysis conducted by (Tietze & Borthakur, 2012), which showed tropical Africa as the most likely origin of the penduline tit clade. In order to work out whether the observed sexual dimorphism was driven by changes in male or female traits we also performed the same analyses using the male and female means for the normalised body size and plumage measurements.

Results

The sexual dimorphism indices calculated for each subspecies can be found in Table 3.1 along with the estimated environmental variables and observed/inferred modes of care. The values from which these were calculated can be found for both the body size and plumage traits in Appendices 3.1 & 3.2 respectively, along with the sample sizes for each subspecies. Uniparental care was found to varying degrees across the *Remiz* genus, but one subspecies exhibited substantial biparental care. In the *Anthoscopus* genus, biparental or cooperative care was documented or inferred across all species (Harrap & Quinn, 1996; Hockey *et al.*, eds, 2005; Cockburn, 2006).

None of the three variables (parental care, latitude or annual rainfall) explained the pattern of sexual size dimorphism found within the *Remizidae* (Table 3.2, Figure 3.3). However sexual plumage dimorphism exhibited a slight positive correlation with latitude (PGLS, $t = 3.2412$, $df = 4$, $p = 0.0316$, Table 3.2, Figure 3.3), although this was not significant after applying a Bonferroni correction. No significant correlation between sexual plumage dimorphism and rainfall was observed and the proportion of male care did not explain the pattern of plumage sexual dimorphism (Table 3.2, Figure 3.3). However the sample size was reduced in the last analysis as only 6 subspecies had detailed nest data, and it is worth noting that the results do show a negative trend between the amount of male care and the degree of sexual dimorphism (Table 3.2, Figure 3.3).

When inferring the rates of evolution of sexual plumage dimorphism we observed significantly faster rates of evolution in the uniparental compared to the cooperative subspecies (Uniparental $\theta = 11.1$, $\chi^2 = 7.99$, $p = 0.0047$, Table 3.3, Figure 3.4). This rapid evolution is likely driven by selection on male plumage, which showed a much faster rate of change in the uniparental males (Uniparental $\theta = 6.5$, $\chi^2 = 4.33$, $p = 0.038$, Table 3.3, Figure 3.4). Subspecies at tropical latitudes (<30°) exhibited significantly slower rates of sexual plumage evolution than those in temperate regions (Low latitude $\theta = 0.066$, $\chi^2 = 8.29$, $p = 0.004$, Table 3.3, Figure 3.5). Sexual size dimorphism did not evolve significantly

differently between either of the parental care groups or between the subspecies at different latitudes (Table 3.3, Figure 3.5).

Table 3.1. Summary of data for each subspecies of penduline tit used in this study, including sexual dimorphism indices, environmental variables and parental care systems.

Genus	Species	Subspecies	SDI Plumage	SDI Bodysize	Latitude	Annual precipitation (mm)	Care system	% nests with males caring	n nests	Nest reference
Remiz	*pendulinus*	*menzbieri*	1.094492375	0.629344035	36	376	Conflict	-	-	-
Remiz	*pendulinus*	*pendulinus*	0.218290782	-0.528800759	47	568	Conflict	19	404	Pogany et al. 2008
Remiz	*pendulinus*	*caspius*	0.150424188	-0.411824413	47	547	Conflict	-	-	-
Remiz	*macronyx*	*macronyx*	-0.808723027	-0.148265172	42	125	Conflict	-	-	-
Remiz	*macronyx*	*ssaposhnikowi*	1.841666939	2.010319257	45	371	Conflict	-	-	-
Remiz	*coronatus*	*coronatus*	0.651134933	-1.054341425	38	315	Cooperative	84	18	This study
Remiz	*coronatus*	*stolickzkae*	1.039964812	0.250195021	49	283	Conflict	29	14	This study
Remiz	*consobrinus*	*consobrinus*	0.646393885	0.03790056	47	445	Conflict	0	17	This study
Anthoscopus	*parvulus*	*parvulus*	NA	NA	12	538	Cooperative	-	-	-
Anthoscopus	*parvulus*	*senegalensis*	0.181671725	1.97216339	12	868	Cooperative	-	-	-
Anthoscopus	*minutus*	*gigi*	-0.713592459	-0.214030351	32	365	Cooperative	100	21	This study
Anthoscopus	*minutus*	*damarensis*	-0.86606947	-0.070486799	23	517	Cooperative	-	-	-
Anthoscopus	*flavifrons*	*flavifrons*	-0.394012277	-0.163027751	3	1478	Cooperative	-	-	-
Anthoscopus	*punctifrons*	*punctifrons*	-0.476572322	-0.262987416	14	415	Cooperative	-	-	-
Anthoscopus	*musculus*	*musculus*	-0.683573436	-0.165311227	4	619	Cooperative	-	-	-
Anthoscopus	*caroli*	*taruensis*	-0.480047325	0.073776495	5	1005	Cooperative	-	-	-
Anthoscopus	*caroli*	*sylviella*	-0.394123326	0.504960251	6	947	Cooperative	-	-	-
Anthoscopus	*caroli*	*ansorgei*	0.056746849	-0.048004465	10	1240	Cooperative	-	-	-
Anthoscopus	*caroli*	*robertsi*	-0.055665076	-0.514222848	15	977	Cooperative	-	-	-
Anthoscopus	*caroli*	*rhodesiae*	-0.216889313	0.008510525	9	1131	Cooperative	-	-	-
Auriparus	*flaviceps*	-	-0.791518457	0.139288638	31	249	Cooperative	100	20	Taylor 1970

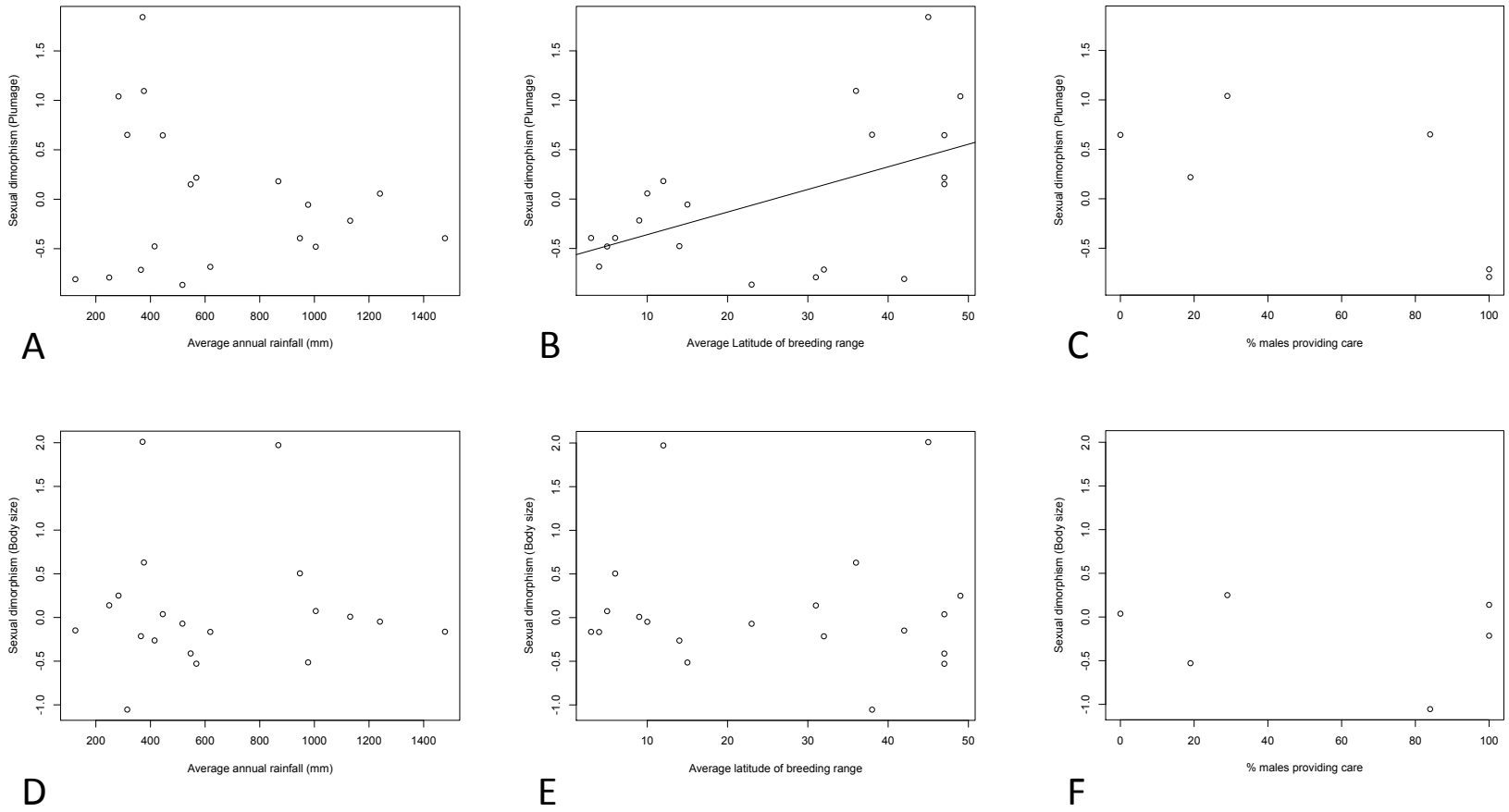

Figure 3.3. Relationships between plumage sexual dimorphism (A, B & C) and size sexual dimorphism (E, F, & G) with two environmental variables (latitude and rainfall) and parental care (% male care). No significant relationships were observed between dimorphism and any of the three variables although a slight positive trend was observed between Latitude and Plumage sexual dimorphism (B).

Table 3.2. Results of phylogenetic least squares analyses comparing the relationships between the two sexual dimorphism indices (body size and plumage) and the parental care system, and breeding environment of all studied penduline tit subspecies. A Bonferroni correction was applied to control for multiple testing and thus a statistically significant relationship was only inferred given a p value of < 0.0083.

Trait	Slope (±SE)	t-value	P	$\lambda^{\#}$	r^2
A: Body size SD					
Male care	-0.0023298 (0.005402)	-0.4313	0.6885	0 (1,0.00047262)	-0.1945
Latitude	0.0070573 (0.03026)	0.2332	0.827	0 (1, 0.11772)	-0.2332
Annual Rainfall	-0.0011455 (0.0019951)	-0.5742	0.5966	0 (1, 0.00000052858)	-0.1548
B: Plumage SD					
Male care	-0.012101 (0.0061067)	-1.9816	0.1186	0 (1, 0.049762)	0.3692
Latitude	0.080662 (0.024886)	3.2412	0.0316	0 (1, 0.017254)	0.6553
Annual Rainfall	-0.002182 (0.0012691)	-1.7193	0.1607	0.956 (0.055422, 0.0075533)	0.2812

#The numbers in brackets after the phylogenetic scaling parameter (λ) indicate whether the λ parameter was significantly different from 0 (first position) or 1 (second position) in likelihood ratio tests.

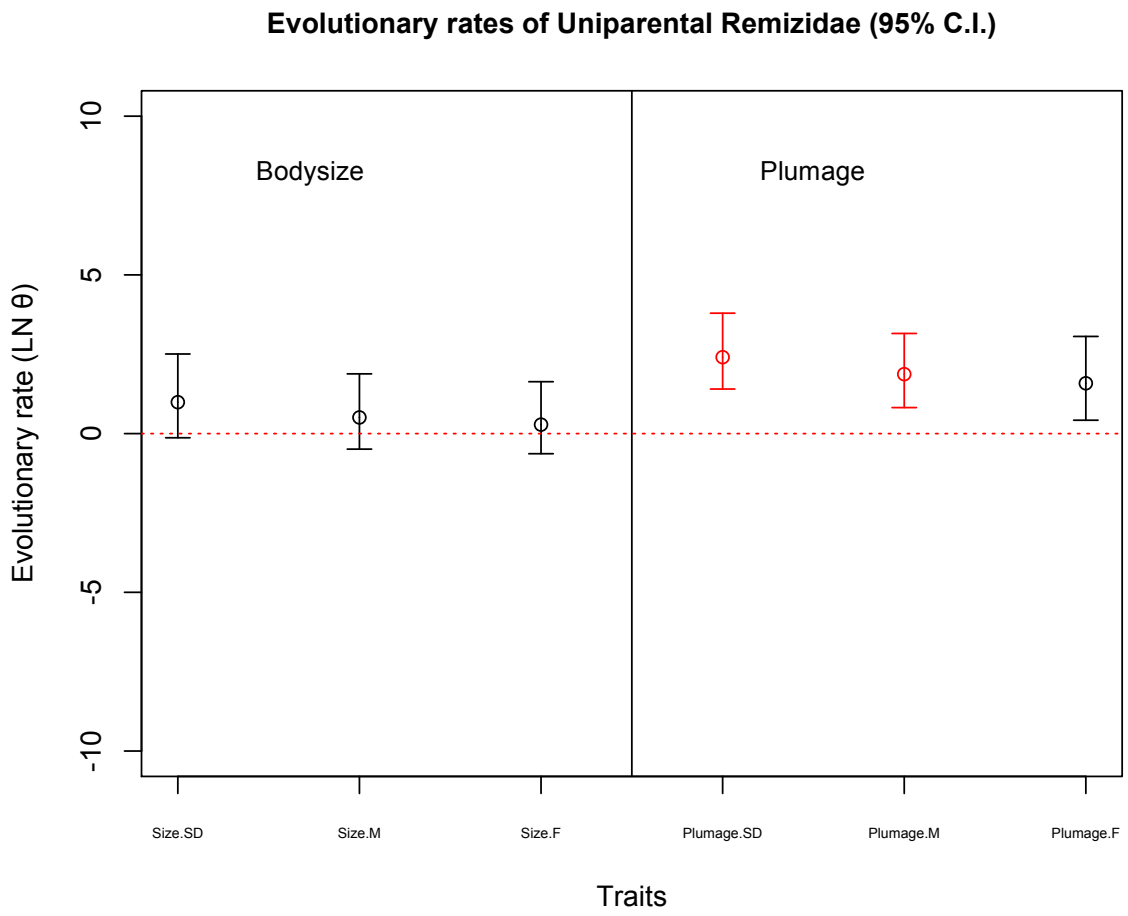

Figure 3.4. Comparison of the evolutionary rate of body size and plumage traits in the uniparental and biparental species. The biparental species rates are standardized at 0 (red dashed line) and the graph shows the uniparental rates. All traits are evolving faster in the uniparental species but only sexual dimorphism in plumage and male plumage are evolving significantly faster (highlighted in red and see Table 3.3 for detailed results)

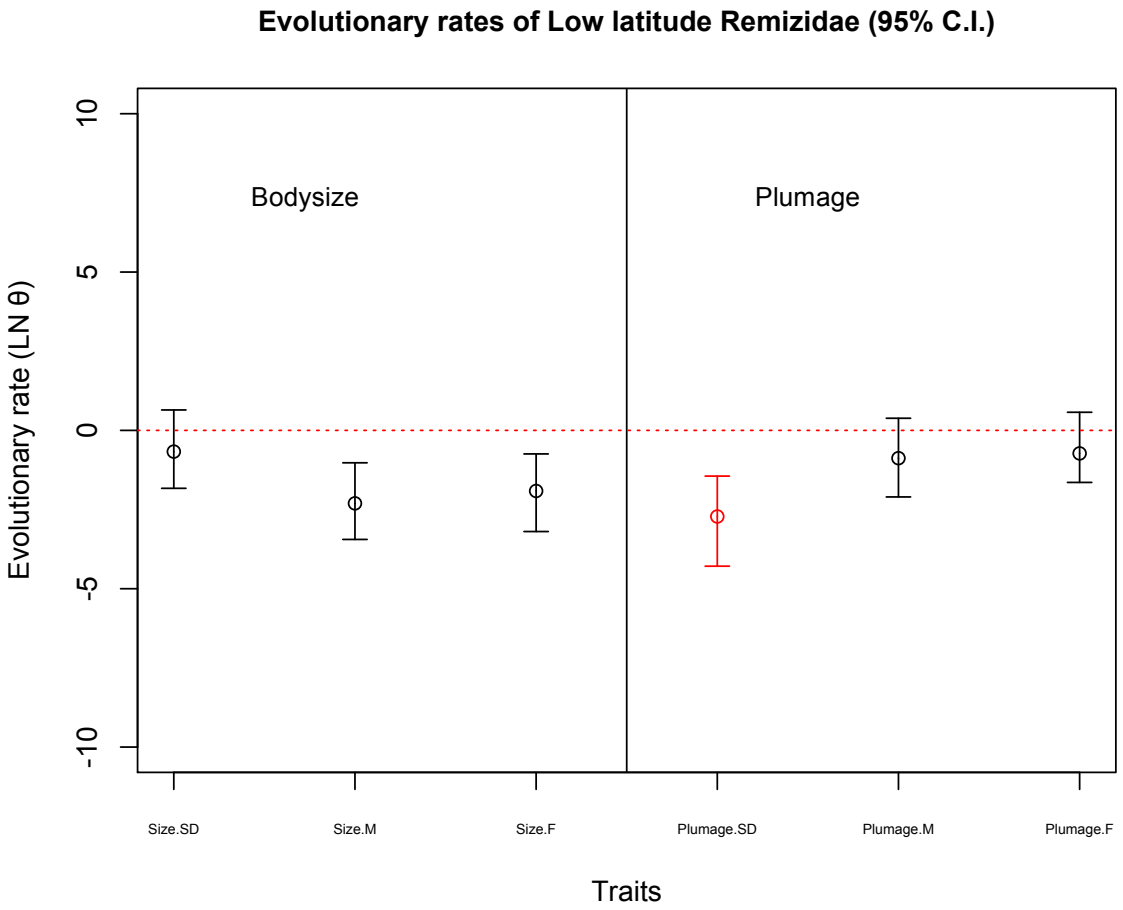

Figure 3.5. Comparison of the evolutionary rate of body size and plumage traits in the high latitude (>30°) and low latitude (<30°) species. The high latitude species rates are standardized at 0 (red dashed line) and the graph shows the low latitude rates. All traits are evolving faster in the high latitude species but only sexual dimorphism in plumage is evolving significantly faster (highlighted in red and see Table 3.3 for detailed results).

Table 3.3. Summary of MOTMOT analyses comparing the evolution of 6 traits between two parts of the penduline tit phylogeny. The phylogeny was split by either parental care behaviour, latitude or rainfall before analysis and one part of the phylogeny was constrained to an evolutionary rate (θ) of 1. * or ** denote significant differences in the evolutionary rate between the two parts of the phylogeny for the respective trait.

Trait	θ	χ^2	p	λ
Parental care	Biparental care held at 1			
A: Body size SD	2.689641	0.9808261	0.3219949	0
B: Male Body size	1.66383	0.2341632	0.6284539	0
C: Female Body size	1.326443	0.06297874	0.8018484	0
D: Plumage SD	11.10225	7.994095	0.004693014**	0
E: Male Plumage	6.503738	4.325917	0.03753619*	0
F: Female Plumage	4.878575	2.802676	0.09410713	0
Latitude	High latitude held at 1			
A: Body size SD	0.5116082	0.3501693	0.5540173	0
B: Male Body size	0.09980887	0.9267538	0.3357079	0.971052
C: Female Body size	0.1475886	2.579402	0.1082626	0.9755536
D: Plumage SD	0.06590455	8.290662	0.003984947**	0.5449049
E: Male Plumage	0.4155954	0.9110236	0.339843	0.7370524
F: Female Plumage	0.4832895	3.748696	0.05284873	0.6827141

Discussion

This is the first study to infer the evolutionary history of traits within the penduline tit family (*Remizidae*). The results suggest that uniparental care evolved once in this group, within the *Remiz* genus soon after their split from the *Anthoscopus*. We have observed one transition back to a biparental care system within the *Remiz coronatus coronatus* subspecies. Thus diversity in care was observed in the *Remiz*, however we were unable to gain detailed behavioural observations across *Anthoscopus*, this would be a priority for future work as a detailed understanding of the diversity within this group would strengthen the comparative approach.

The niche differentiation hypothesis for sexual dimorphism was not supported by the results. Sexual dimorphism in body size traits was not associated with any of the variables tested (latitude, rainfall or parental care). Helpers at the nest have been observed in some penduline tit species at low latitudes so perhaps this behaviour has evolved in response to reduced food availability rather than differing diets between the sexes. Differing foraging behaviour rather than body size might also lead to diet differences, thus further work on foraging behaviours or detailed analysis of diet from faecal samples could be used to assess whether there are sexual differences in foraging niches in the future.

No significant relationship between sexual dimorphism in plumage traits and the three variables (Latitude, Rainfall and care) was revealed. Unexpectedly, a positive trend hints at greater plumage dimorphism with increasing latitude. As the uniparental species were found to inhabit higher latitudes it is not possible to distinguish whether the sexual plumage dimorphism is caused directly by environmental variables associated with latitude or perhaps indirectly by environmental effects on parental care behaviours. A greater number and diversity of species is required to disentangle these competing hypotheses. A greater number of species with detailed parental care data is required or a study across populations within one species, such as the White-crowned

penduline tit (*Remiz coronatus*), could be informative. The contrasting levels of male care in the two populations of this species indicates a diversity in parental care that could be used to separate the competing hypotheses for the evolution of sexual dimorphism.

A more refined scale within a single species could also better tackle the influence of rainfall and latitude on the flexibility of parental care behaviours. Rainfall and latitude for each subspecies were determined in a very broad way that does not control for differences in range size or differing climate variability within each subspecies. A future approach could incorporate simultaneous measures of climate and parental care behaviour in multiple populations within species and across years. A better understanding of the flexibility of parental care behaviour is vital to exploring the evolutionary effect of environmental selection pressures.

As uniparental care has only arisen once in the history of the penduline tits work is needed to expand the number of avian groups for future comparative analyses in order to incorporate additional transitions to uniparental care. Experiments manipulating food availability or an in-depth study of the reasons for the variability observed within the White-crowned penduline tit could disentangle the competing variables. This study used two environmental variables that are particularly important for food availability (Lack, 1954; Martin, 1987; Preston & Rotenberry, 2006). Latitude determines the number of daylight hours during the breeding season, as this increases with latitude it could be the case that increased productivity and foraging time makes it possible for one bird to provide enough food for the brood (Evans *et al.*, 2009), assuming that other constraints limit an associated increase in clutch size. Thus the reason that sexual dimorphism correlates with latitude could be because of a latitudinal effect on parental care. This could explain why rainfall, also associated with increased productivity (Sinclair, 1978; Lepage & Lloyd, 2004; Oppel *et al.*, 2013), does not show the same correlation with sexual dimorphism.

The results are consistent with the prediction that loss of male care leads to increased sexual selection pressure from the caring sex. Rapid evolution of plumage dimorphism was found in the uniparental species compared to the cooperative parental care groups. We also show that this relationship is driven by selection on male plumage, consistent with increased sexual selection driving the plumage dimorphism and not selection on females associated with a change in parental care demands. We also found this same rapid evolution in species at higher latitudes, expected if parental care and latitude are correlated. However as mentioned previously we are unable to tease out the causative agents for these observed patterns. The same relationship was not found with sexual size dimorphism, this is consistent with the prediction that size traits are more likely associated with environmental selection pressures in passerines (Miles & Ricklefs, 1984; Grant & Grant, 2002; Dawideit *et al.*, 2009).

However we are unable to rule out the possibility that other variables associated with latitude could be driving our results. Extensive future work will be required to determine the exact selection pressures which have lead to conflict over care in the penduline tits but we now know that it evolved early in the evolutionary history of the *Remiz* genus and is currently maintained to varying degrees throughout the *Remiz* group with one subspecies having reverted to biparental care. In depth behavioural studies on further African penduline tits will be critical to ascertain the variation in the degrees of cooperative parental care systems in the *Anthoscopus* genus.

Our results in the penduline tits complement a recent study on the evolution of sexual dimorphism in passerines, which revealed rapid diversification of male plumage traits under higher sexual selection pressure (Seddon *et al.*, 2013). The penduline tit family are consistent with this hypothesis, which would predict rapid diversification of lineages with high levels of sexual selection, as sexually selected male traits have large effects on pre-mating isolation (Panhuis *et al.*, 2001). This is a key area for future investigation as the role of sexual selection in speciation has much theoretical backing but to date empirical evidence is sparse (Ritchie, 2007).

To summarize we have found that rapid evolution of sexual dimorphism, driven by selection on male plumage traits, is associated with the transition to uni-parental care within the penduline tits (*Remizidae*). These results suggest an intricate relationship between sexual selection, parental care and the environment. Future research revealing the detailed parental care and mating systems of additional members of this family would aid additional comparative analyses in addition to experimental work on parental care behaviours in order to establish causation for the relationships we have observed.

Chapter 4

Sperm morphology and mating systems in the penduline tits (*Remizidae*) and allies

Alexander D. Ball, Nina Ockendon, Ákos Pogány, Gergely Zachar, Carlos Ponce, Tamás Székely & Steve Dorus

Abstract

The ability of sexual selection via mate choice and intra-sexual competition to drive the evolution of elaborate behaviours and sexual ornaments is well established. However in species with multiple mating by females, copulation does not necessarily convey fertilisation success as sexual selection continues within the female reproductive tract. Sperm are the obvious candidates for post-copulatory selection and in many species sperm length is thought to correlate with fertilisation success primarily due to its impact on sperm swimming ability. However in birds, evidence for this is inconclusive and it is thought that due to long periods of storage before fertilisation, traits that affect longevity or successful storage may be of greater importance. Here we use 12 passerine species with differing levels of female promiscuity to test the impact of post-copulatory selection on three sperm traits. We test whether directional selection for an increase in these traits or stabilizing selection on optimum trait values are the most important factors in passerine sperm evolution. We focus on three sperm traits that are predicted to influence different aspects of sperm function, 1) **Total sperm length** has been found to closely associate with sperm storage tubule (SST) length in the female reproductive tract and is thus predicted to impact storage success 2) **Mid-piece length** has been found to determine energy availability, and 3) **Flagellum to Head ratio** has been found to correlate with sperm swimming velocity. By focusing on these three traits we attempt to reveal whether sperm storage, energy levels or speed are under selection during sperm competition. Previous studies have found familial relationships that fail to provide over-arching explanations across passerines, we thus focus our analyses on two sister families, the *Remizidae* and *Paridae*.

The results reveal that within male variance in total sperm length is negatively correlated with levels of sperm competition. We found no significant relationships between mid-piece length or flagellum to head ratio and sperm competition levels. This suggests that stabilizing selection on total sperm length perhaps for increased levels of sperm storage rather than sperm speed or longevity plays a greater role in fertilisation success during bouts of sperm competition in these passerines.

Introduction

Sexual selection is known to have a huge influence on the evolution of many traits, the most well studied being those related to competition and attraction of mates (Darwin, 1871; Andersson, 1994; Andersson & Iwasa, 1996). These include the use of colourful displays to woo the opposite sex or the evolution of weapons to defend access to mates. However in species with internal fertilization sexual selection can act even after mating. If females gain multiple mates in a single reproductive bout then fertilization success of a male is not guaranteed by successful copulation, instead the outcome of sperm competition and cryptic female choice will be the deciding factor (Parker, 1970; Thornhill, 1983; Eberhard, 1996; Birkhead, 2000). Thus selection will favour male traits that can ensure fertilisation even after mating. Post-copulatory mechanisms which increase a male's fertilisation success when in competition with other male ejaculates have been observed in a variety of taxa (Birkhead & Pizzari, 2002; Poiani, 2006). This includes the modification of chemicals in seminal fluid which can have profound effects on female re-mating behaviour or form copulatory plugs that compromise the ejaculates of additional males (Martan & Shepherd, 1976; Dixson & Anderson, 2002; Chapman, Bangham, *et al.*, 2003; Fry & Wilkinson, 2004). However the most direct selection pressures are likely to affect sperm morphology and it is this post-copulatory sexual selection, which is thought to have created the extreme diversity in sperm; the sperm cell possesses the most diverse morphology of any cell type (Pitnick *et al.*, 2009).

The intensity of selection on sperm morphology is going to be determined in a large part by the degree of female promiscuity. Thus research in this area has focused on groups with large disparities in the re-mating behaviour of females, such as primates, rodents and fruit flies (*Drosophila* Spp.) (Smith, 1984). In the last couple of decades the differing levels of extra-pair paternity (EPP) have been documented across bird species (Petrie & Kempenaers, 1998; Griffith *et al.*, 2002). The evidence from numerous genetic parentage studies has revealed that for the majority of birds multiple mating by males and females is an ever present possibility, found to occur in ~86% of species (Griffith *et al.*, 2002). The

variation in EPP levels is also large; in some species as many as 72% of young are the result of infidelity, however other species have been found to be truly monogamous (Griffith *et al.*, 2002). This has led to a focus on post-copulatory research in birds, with studies attempting to reveal the impact that differing mating behaviours have on sperm morphology (Lüpold, Calhim, *et al.*, 2009; Immler *et al.*, 2011).

The sperm morphology of passerine birds follow a distinctive pattern setting them apart from other vertebrates (Jamieson, 2007). They are formed of three sections, a corkscrew shaped head containing the paternal genetic material to its posterior, a mid-piece and the tail. The final two sections make up the flagellum, the mid-piece being defined by the presence of a mitochondrial sheath that spirals around the flagellum at its anterior (see Figure 4.1). Although almost all passerines studied to date share this conserved plan the proportions of these different sections show extreme variability and there are also very large differences in the overall length of sperm between species (Briskie & Montgomerie, 1992). A key exception to this general sperm plan can be found in the Bullfinch, *Pyrrhula pyrrhula*. This species lacks the spiral shaped mitochondria and exhibits a rounded head more like the sperm found in reptiles and mammals (Birkhead *et al.*, 2006, 2007). This variation has been attributed to the very low risk of sperm competition in this species, thus the selection pressures maintaining the typical passerine sperm morphology have been lost (Durrant *et al.*, 2010). This hints at a relationship between post-copulatory sexual selection and sperm morphology. However more general relationships between promiscuity and sperm morphology have been found in phylogenetic comparative studies (Briskie *et al.*, 1997; Kleven *et al.*, 2008).

Sperm length has been hypothesised to increase under higher levels of sperm competition and this does seem to be the case in mammals where selection favours sperm which can swim faster and fertilize an egg before others can try (Gomendio & Roldan, 1991). However, in birds where sperm are stored for long lengths of time before fertilisation (Birkhead & Moller, 1992), perhaps sperm speed is of less importance. Indeed a relationship between sperm length and

sperm competition appears to be variable across bird families (Immler & Birkhead, 2007; Immler, Gonzalez-Voyer, & Tim R Birkhead, 2012). The relationship between sperm length and speed is also highly debatable (Humphries *et al.*, 2008). Recent studies suggest that sperm velocity is best explained by the flagellum to head ratio, as the head creates substantial drag compared to the thrust generated by the flagellum (Humphries *et al.*, 2008; Lüpold, Calhim, *et al.*, 2009; Mossman *et al.*, 2009). However, one sperm trait that does appear to correlate with sperm competition across the majority of passerines has been recently documented, both (Kleven *et al.*, 2008) and (Calhim *et al.*, 2007) show that the variance in total sperm length decreases in species with higher levels of EPP.

The variance in sperm length both within a male and within a population has been found to correlate with the degree of sperm competition (Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010). In species with high levels of EPP, and thus higher rates of sperm competition, all sperm within a male and within the population are very similar in length however in monogamous species sperm length is much more variable, differing within and between males to a much greater degree (Lifjeld *et al.*, 2010). This suggests that an optimum sperm length in each species gives the best chance of fertilization success. Thus under high levels of sperm competition the more sperm that are near to the optimum sperm size perhaps increases the likelihood of fertilizing a female's eggs. This selection is perhaps driven by the length of a female's storage tubules, with sperm of the optimum length being stored for a longer period or in greater numbers. Indeed (Briskie *et al.*, 1997) found a close association between female sperm tubule length and male sperm length across passerines.

The idea that sperm storage is a determining factor in sperm morphology has also been explored from the perspective of the high duration that sperm remain in storage before successful fertilisation in birds (Birkhead & Moller, 1992). This has lead to research into the ability of sperm to maintain function and motility through time (Pizzari *et al.*, 2008). The ability to maintain function through time is likely to depend on the energy reserves of the individual sperm

cell and the efficiency of their use (Pizzari & Parker, 2009). A recent study found that sperm with longer mid-pieces have increased concentrations of ATP, although this did not translate into increased sperm velocity it provides evidence that sperm with longer mid-pieces have greater energy availability (Rowe *et al.*, 2013).

Here the aim is to explore the relationship of three sperm traits and their variance, with levels of sperm competition, inferred via levels of EPP, across the penduline tits (*Remizidae*) and closely related passerines. We focus on three traits that are likely to influence different aspects of sperm competitive ability, 1) Total sperm length is closely associated with SST length across passerines (Briskie & Montgomerie, 1992), 2) Mid-piece length is associated with sperm energy availability (Rowe *et al.*, 2013) and 3) Flagellum to head ratio is closely associated with sperm velocity (Lüpold, Calhim, *et al.*, 2009). We thus aim to determine whether traits that influence sperm storage, energy availability or sperm velocity provide competitive advantages during post-copulatory sexual selection in the penduline tits and allies. By analysing the within male (CVwm) and between male variance (CVbm) in each of these three traits we also attempt to reveal whether stabilising or directional selection is likely to be acting on these traits. If they are under stabilising post-copulatory selection then lower variance would be predicted under higher levels of sperm competition, however if directional selection is occuring we would predict a correlation with the average trait value and EPP across the species. In addition, if any of the traits are closely associated with levels of EPP it could provide the ability to infer mating systems from the sperm morphology of males. This method would be both less invasive and less labour intensive than the current parentage studies that are currently used to assess mating systems and levels of sperm competition in bird species.

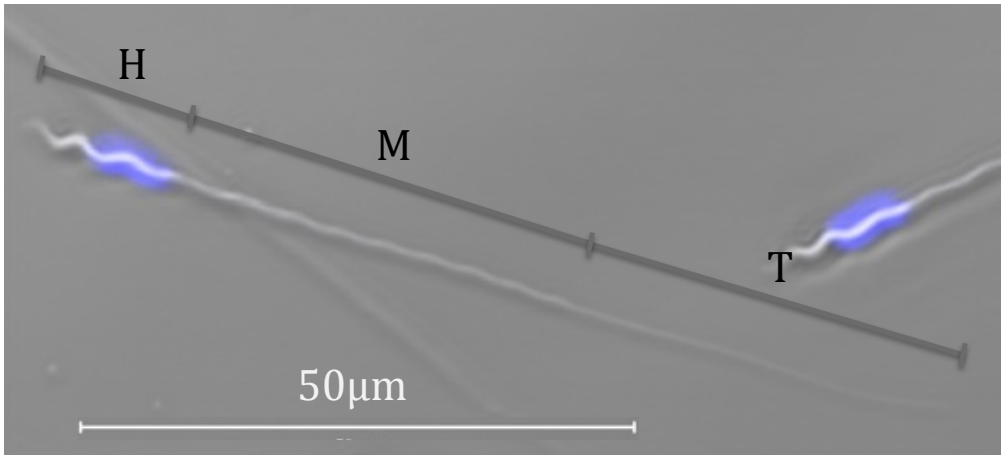

Figure 4.1. An example of typical passerine sperm morphology, highlighting the three main components, the head (H), mitochondrial mid-piece (M) and tail (T).

Methods

Species selection

A total of 12 passerine species were used in this study, two of which were the European and Cape penduline tit species, *Remiz pendulinus* and *Anthoscopus minutus* respectively. All species had extra pair paternity data available from at least 1 population (see Table 4.1 for sources) and exhibited substantial interspecific variation in these levels (5.2% – 31.4% of offspring were extra-pair young (EPY) across the species). Males of the same species were sampled from the same locality in order to calculate the between male population level variances in sperm morphology (CVbm) in addition to the within male variance (CVwm). The data for six of the *Paridae* were obtained from (Lifjeld *et al.*, 2010), and the other six species were all measured as part of this study (see Table 4.1 for details).

Field sampling

Sperm samples were collected in the field either by faecal sample collection or by dissection of the seminal glomera (sperm storage organ at the distal end of the ductus deferens). All samples were collected from adult males in the breeding season captured by mist net and in most cases using species-specific song playback. An example fieldwork report can be found in Appendix 4.1 for the Cape penduline tit. Faecal samples were collected as described by (Immler & Birkhead, 2005) which involved placing a male bird in a cotton bag containing a plastic tray. After 5 minutes the translucent liquid around any resulting faecal sample was pipette into an eppendorf tube containing ~300ul of 5% formalin fixative. Seminal glomera samples were collected posthumously from males that were sacrificed under licence for alternative studies (Ministerial Order no. 1470/2011). The distal end of the seminal glomera was dissected within 5 minutes of death and placed in an eppendorf tube containing 1ml of 10% formalin. All tubes were kept in the dark at room temperature until use, this

period ranged from 1-18 months, as sperm can be kept for years using this method (Briskie & Birkhead, 1993; Immler & Birkhead, 2005).

Sperm measurement

Sperm were observed using a Zeiss LSM510Meta confocal laser-scanning microscope. Firstly 10ul of the formalin solution was extracted from the bottom of the sample collection tube. It was placed in the centre of a glass bottomed microwell dish and left to air-dry in a fume hood for ~40 minutes. Once dry 10ul of a 0.6umol DAPI solution was added to the sample. Sperm were then initially located using a 20x Phase 2 air objective under white light. Once a suitable sperm had been located, free of debris and of typical morphology (see Figure 4.2 for an example of typical sperm in each of the 6 species and (Chenoweth, 2005; du Plessis & Soley, 2011) for examples of abnormal sperm morphology in birds), it was viewed using a 488nm argon laser. Photos were then taken with the digital camera and saved as .tif files.

Measurements were taken from the sperm photos using the program ImageJ v1.46 (Abràmoff *et al.*, 2004). The scale of each photo was set using the 50um scale bar created in LSM510 software and the multi line tool was used to measure three components of the sperm's length, the head, midpiece and tail to the nearest tenth of a micron. A diagram depicting the three components is shown in Figure 4.1. Ten sperm were measured for each male and each sperm was measured three times with the average measurement used in further analyses.

The within male variation (CVwm) in total sperm length was calculated for each male using the ten measured sperm as:

$$CV(wm) = \frac{\sigma}{\bar{x}} \times 100$$

Where σ is the standard deviation and $\bar{x}$ is the mean of the ten measured sperm. The species level CVwm was the mean CVwm of the respective males. The

between male variation (CVbm) in total sperm length was also calculated for each species, this was calculated using the standard deviation and mean as above but a correction for sample size was introduced due to the differing numbers of males measured per species. The calculation corrected for sample size was:

$$CV(bm) = \left[\frac{\sigma}{\bar{x}} \times 100\right] \times \left[1 + \left(\frac{1}{4n}\right)\right]$$

Where σ and $\bar{x}$ are the standard deviation and mean respectively of the average sperm size in males of the relevant species and n is the number of males measured.

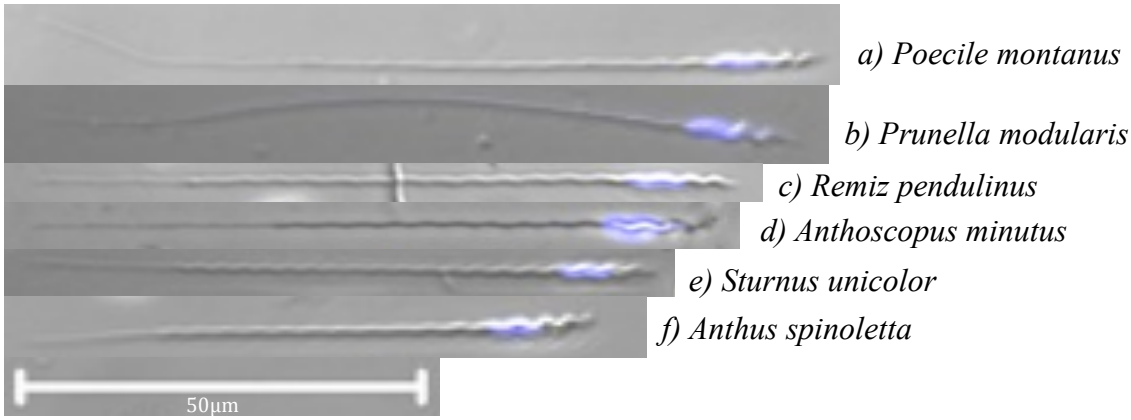

Figure 4.2. Examples of the diversity in the sperm morphology of the six species measured in this study, all scaled to the 50µm scale bar at the bottom.

Table 4.1. Summary of Extra Pair Paternity (EPP) and phylogenetic sequence data for all species with sperm morphometric data.

Species	English name	Family	Locality	Country	EPP (% young)	Cyt-B sequence*	ODC sequence*	EPP source
Anthus spinoletta	Water-pipit	*Motacillidae*	Harghita	Romania	5.2	U46773	GU816919§	(Reyer *et al.*, 1997)
Cyanistes caeruleus	Eurasian blue tit	*Paridae*	Oslo	Norway	11	DQ474041	KF183742	(Krokene & Lifjeld, 2000; Johannessen *et al.*, 2005)
Cyanistes teneriffae	African blue tit	*Paridae*	Tenerife	Spain	15.3	DQ474060	KF183740	(Garcia-del-Rey *et al.*, 2012)
Lophophanes cristatus	European crested tit	*Paridae*	-	Norway	11	AF347954	KF183791	(Lens *et al.*, 1997)
Parus major	Great tit	*Paridae*	Oslo	Norway	8.5	AY495412	KF183747	(Johannessen *et al.*, 2005)
Periparus ater	Coal tit	*Paridae*	Lingen	Germany	31.4	AF347959	KF183786	(Dietrich *et al.*, 2004)
Poecile atricapillus	Black-capped chickadee	*Paridae*	Ontario	Canada	11.8	AF284066	KF183811	(Otter *et al.*, 1998; Mennill *et al.*, 2004)
Poecile montanus	Willow tit	*Paridae*	Harghita	Romania	6.7	AF347944	KF183809	(Lampila *et al.*, 2011)
Prunella modularis	Dunnock	*Prunellidae*	Harghita	Romania	22.6	AY228080	EU680756	(Terry Burke *et al.*, 1989)
Anthoscopus minutus	Cape penduline tit	*Remizidae*	Little Karoo	S. Africa	7.1	Chapter 1	Chapter 1	Chapter 2
Remiz pendulinus	European penduline tit	*Remizidae*	Danube delta	Romania	23.5	Chapter 1	Chapter 1	(van Dijk, Mészáros, *et al.*, 2010)
Sturnus unicolor	Spotless-starling	*Sturnidae*	Madrid	Spain	12.7	HM633385§	EU551928	(Cordero *et al.*, 2003; García-Vigón *et al.*, 2009)

* Outgroup Cyt-B and ODC sequence accession numbers used in the phylogeny construction were Tyrannidae (AF453812 & DQ435489), Corvidae (JQ864491 & FJ358080), *Ficedula hypoleuca* (HM633303 & EU680728), *Turdus merula* (DQ910961 & EU154863), *Acrocephalus Agricola* (AJ004246 & FJ883127) and *Acrocephalus arundinaceus* (AJ004253 & FJ883128) respectively.
§ The sequence of the closest available relative was used, as the focal species had not previously been sequenced at both loci.

Phylogenetic inference

To control for the effect of shared evolutionary history phylogenies were inferred using all the species in this study using the genetic sequence of the Cytochrome B (Cyt-B) mitochondrial gene and the Ornithine decarboxylase (ODC) autosomal gene. Available gene sequences were located on NCBI for all species except the penduline tit species, which were previously sequenced as reported in this thesis (see Chapter 1). Additional species were included to aid phylogenetic resolution including representatives from 2 outgroups, the passerine orders Tyrannidae and Corvidae, these were represented by the Brown-crested flycatcher (*Myiarchus tyrannulus*) and the Carrion crow (*Corvus corone*) respectively (see Table 4.2 for sequence details).

The gene sequences were aligned in the program Mega v 5.2.2. The ClustalW alignment algorithm was used with its default parameters. This created an alignment of length 841bp and 662bp for the Cyt-b and ODC sequences respectively. To create ultrametric trees the program BEAST was employed, this infers phylogenies using Bayesian inference and a Markov chain Monte Carlo (MCMC) approach to estimate the evolutionary relationships among genetic sequences. The most suitable evolutionary model was chosen using the MrModelTest v 2.3 program prior to the BEAST analysis. The analysis was run for 200,000,000 generations, with each 1000th run stored in the data file. A burn-in fraction of 25% was removed from the start of the file before inferring the most likely phylogeny. The final tree was visualised in FigTree v 1.4.0 and is shown in Figure 4.3.

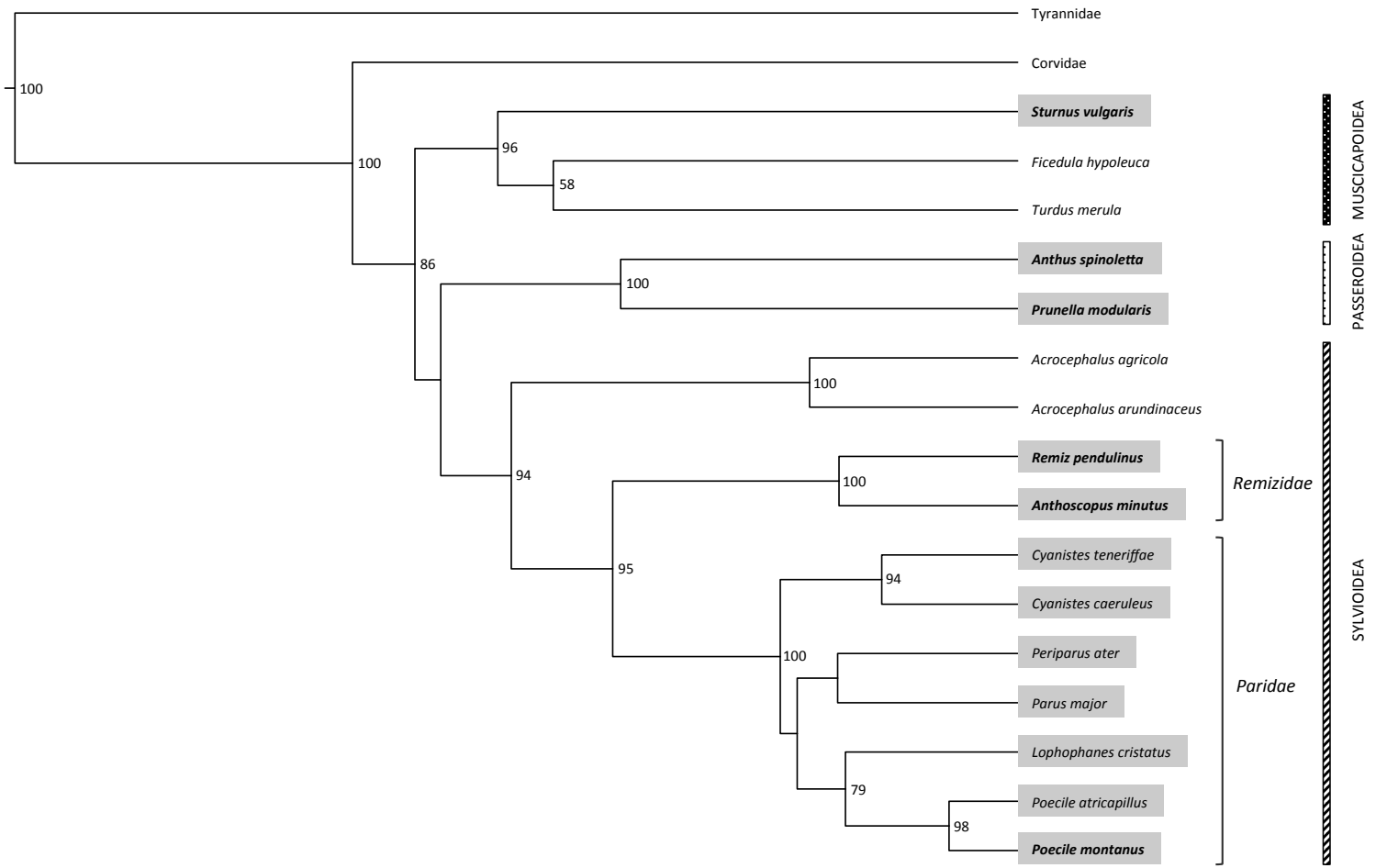

Figure 4.3. Phylogeny incorporating the twelve passerine species analysed in this chapter (highlighted in grey boxes). The six species with sperm traits obtained as part of this study are highlighted in bold. The phylogeny was inferred using the Cyt-B mitochondrial gene and the autosomal gene ODC (introns 6-7) using the BEAST software and visualised in FigTree v1.4. The super-families are indicated on the right along with the sister families, *Remizidae* and *Paridae,* that were the main focus of this study. All posterior probabilities ≥50 are indicated at the branch nodes. .

Comparative analysis

The relationships between EPP and the three sperm traits were analysed using the PGLS function in the R package 'Caper' (Orme *et al.*, 2013). This uses a generalized least squares approach to model relationships whilst controlling for phylogeny using a covariance matrix. The sperm data was log transformed before analysis to create a more normal distribution and the EPP data was arc sin square root transformed, due to higher frequencies of species with lower rates of EPP. Only the six passerine species measured in this study had data for the mid-piece length and the Flagellum to head ratio, and thus the additional six *Paridae* species were only used in the total sperm length analysis. A Brownian model of evolution was assumed for all traits using the phylogeny shown in Figures 4.3. A multivariate analysis incorporating the average trait value, the CVwm and the CVbm was conducted for each of the 3 sperm traits to test their association with the levels of EPP. The slope of the regression lines were tested for a significant difference from zero using a t-test; a summary of the data used in the analyses can be found in Table 4.2.

Results

In the multivariate analysis including the 12 species with measurements on total sperm length a significant negative relationship between EPP and within male variance (CVwm) was observed (Table 4.3, Figure 4.4). No significant relationships between EPP and either the mean Total sperm length or the between male variance (CVbm) were observed (Table 4.3, Figure 4.4).

Only the six species measured during the course of this study (see Table 4.2 for species details) had measurements for midpiece length and its CVwm and CVbm. This was also the case for the Flagellum to Head ratio data. No significant relationships were found between EPP and either midpiece length or Flagellum to Head ratio including between their respective CVwm and CVbm measures (Table 4.3, Figures 4.5 & 4.6).

Table 4.2. Summary of the obtained sperm measurements predicted to influence the outcome of sperm competition in passerine birds. Only measurements on total sperm length were available for the six species where sperm data were obtained from the literature.

Species	n	Total Sperm length				Midpiece length				Flagellum : Head ratio				Sperm data source
		Mean (µm ±SE)	Stdev	CV(wm)	CV(bm)	Mean (µm ±SE)	Stdev	CV(wm)	CV(bm)	Mean (±SE)	Stdev	CV(wm)	CV(bm)	
Anthus spinoletta	4	68.3 (1.38)	2.8	2.98	4.28	40.7 (0.33)	0.65	2.89	1.71	4.34 (0.15)	0.29	5.94	7.2	This study
Cyanistes caeruleus	21	104.6 (0.55)	2.5	2.13	2.38	-	-	-	-	-	-	-	-	Lifjeld et al. (2010)
Cyanistes teneriffae	9	114.4 (1.03)	3.1	1.67	2.78	-	-	-	-	-	-	-	-	Lifjeld et al. (2010)
Lophophanes cristatus	8	85 (0.78)	2.2	2.47	2.63	-	-	-	-	-	-	-	-	Lifjeld et al. (2010)
Parus major	10	98.9 (0.95)	3	2.34	3.06	-	-	-	-	-	-	-	-	Lifjeld et al. (2010)
Periparus ater	10	91.7 (0.76	2.4	2.03	1.81	-	-	-	-	-	-	-	-	Lifjeld et al. (2010)
Poecile atricapillus	10	85.7 (0.6)	1.9	2.72	2.31	-	-	-	-	-	-	-	-	Lifjeld et al. (2010)
Poecile montanus	2	92.2 (3.13)	4.4	3.58	5.39	53.8 (1.48)	2.09	6.32	4.36	5.93 (0.26)	0.37	8.4	7	This study
Prunella modularis	5	96.6 (1.25)	2.8	2.68	3.04	63.2 (1.54)	3.44	4.5	5.72	5.69 (0.16)	0.36	7.22	6.7	This study
Anthoscopus minutus	2	83.6 (0.57)	0.8	4.81	1.09	46 (1.7)	2.4	8.52	5.86	5.39 (0.13)	0.18	8.61	3.77	This study
Remiz pendulinus	4	86 (1.89)	3.8	2.43	4.69	43.9 (1.54)	3.08	6.54	7.44	5.31 (0.19)	0.37	7.41	7.49	This study
Sturnus unicolor	3	79.1 (0.65)	1.1	2.75	1.54	46.3 (1.54)	2.67	4.76	6.25	5.33 (0.04)	0.07	6.3	1.5	This study

Table 4.3. Phylogenetic generalized least squares analysis of the correlation between the three sperm traits and the degree of Extra-pair paternity (EPP).

Sperm trait	Slope (± SE)	t-value	*p*	r^2
A Sperm storage – 12 species				
Total Sperm length	-0.0469 ± 0.312	-0.1503	0.8842	
CVwm	-0.3149 ± 0.118	-2.6674	0.0285*	
CVbm	-0.0298 ± 0.056	-0.5326	0.6088	
combined				0.2981
B Sperm energy reserves – 6 species				
Midpiece length	-0.0172 ± 0.2874	-0.0597	0.9578	
CVwm	-0.3798 ± 0.1664	-2.2819	0.1500	
CVbm	0.3602 ± 0.1098	3.2819	0.0816	
combined				0.654
C Sperm speed – 6 species				
Head:Flagellum ratio	1.4246 ± 0.9278	1.5354	0.2645	
CVwm	-1.0375 ± 0.7401	-1.4017	0.2960	
CVbm	0.0950 ± 0.1119	0.8488	0.4854	
combined				0.0222

CVwm = Within male Coefficient of variation
CVbm = Between male Coefficient of variation

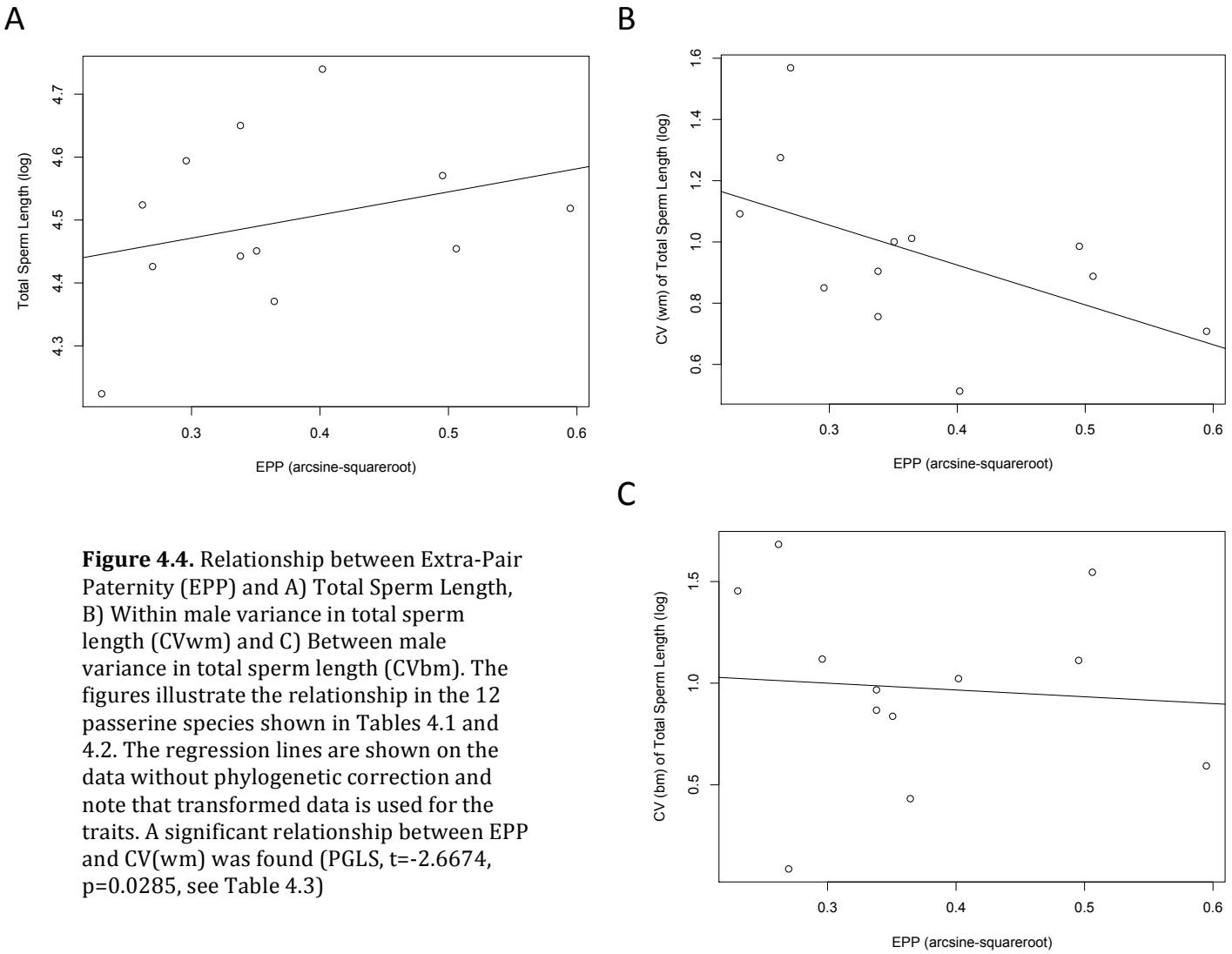

Figure 4.4. Relationship between Extra-Pair Paternity (EPP) and A) Total Sperm Length, B) Within male variance in total sperm length (CVwm) and C) Between male variance in total sperm length (CVbm). The figures illustrate the relationship in the 12 passerine species shown in Tables 4.1 and 4.2. The regression lines are shown on the data without phylogenetic correction and note that transformed data is used for the traits. A significant relationship between EPP and CV(wm) was found (PGLS, t=-2.6674, p=0.0285, see Table 4.3)

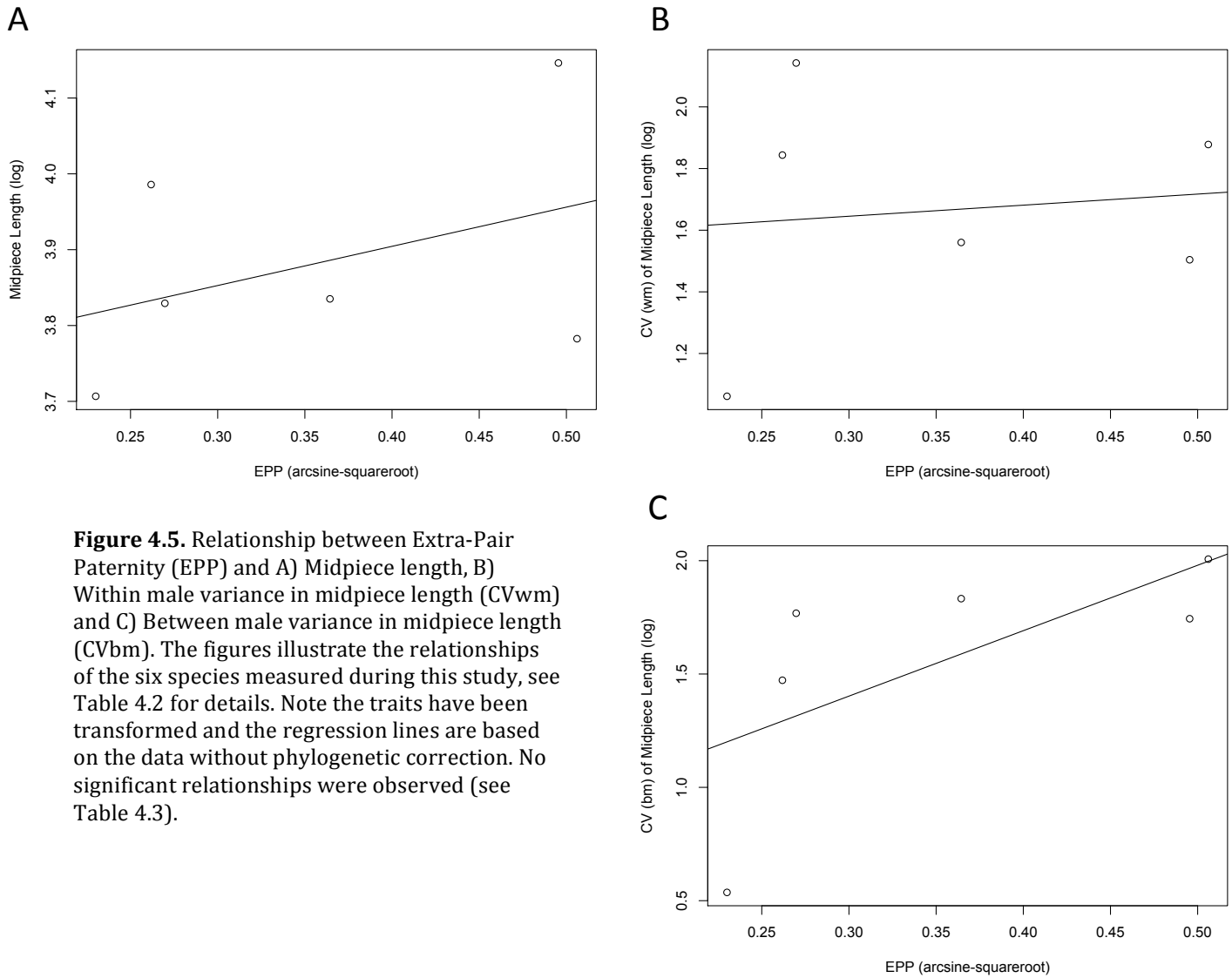

Figure 4.5. Relationship between Extra-Pair Paternity (EPP) and A) Midpiece length, B) Within male variance in midpiece length (CVwm) and C) Between male variance in midpiece length (CVbm). The figures illustrate the relationships of the six species measured during this study, see Table 4.2 for details. Note the traits have been transformed and the regression lines are based on the data without phylogenetic correction. No significant relationships were observed (see Table 4.3).

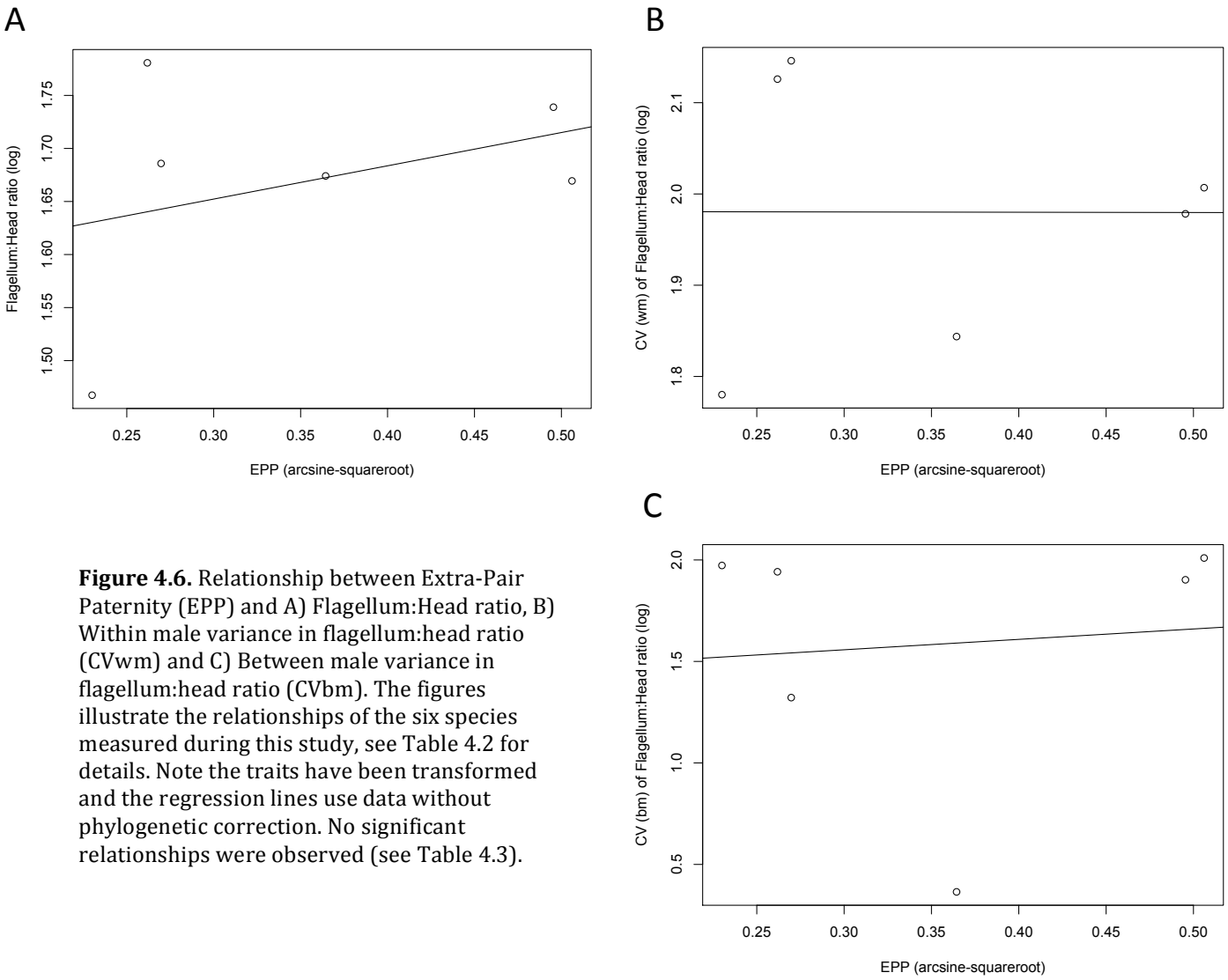

Figure 4.6. Relationship between Extra-Pair Paternity (EPP) and A) Flagellum:Head ratio, B) Within male variance in flagellum:head ratio (CVwm) and C) Between male variance in flagellum:head ratio (CVbm). The figures illustrate the relationships of the six species measured during this study, see Table 4.2 for details. Note the traits have been transformed and the regression lines use data without phylogenetic correction. No significant relationships were observed (see Table 4.3).

Discussion

The results support a strong negative relationship between the level of promiscuity and the CVwm in total sperm length across the passerines tested. Studies to date have found that the strongest predictor of sperm length across passerines is the length of female sperm storage tubules (SSTs). Thus the results suggest that sperm storage in the female reproductive tract could be the largest selection pressure in species with higher levels of promiscuity. However if this was due to stabilising selection driven by the size of the female storage tubules a negative relationship between EPP and CVbm would also be predicted. We failed to observe this although a significant caveat is the low sample size used to obtain CVbm measures in some species. This could indeed be the case given that previous studies have observed a negative relationship between EPP and both CVwm and CVbm of total sperm length (Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lüpold, Linz, *et al.*, 2009; Lifjeld *et al.*, 2010). However our current results suggest that the relationship between CVwm of total sperm length and EPP is instead driven by selection on uniform sperm size within ejaculates and not by selection on a species-specific optimum sperm length. If this is indeed the case exploring whether this is driven by female selection for more uniform sperm or increased competitive ability of uniform ejaculates would be an exciting future area of research.

A uniform ejaculate could provide an advantage within the female reproductive tract such as easier storage or easier manipulation by the female. However Cryptic female choice for uniform ejaculates could evolve due to indirect genetic benefits that they signal to the female. Recently sperm morphology has been shown to impact on the development of the resulting embryo (Immler *et al.*, 2014), thus females may be selecting for uniformity of sperm within an ejaculate to control for the negative impacts of malformed sperm. There is an body of evidence that suggests that developmental stability is favoured in mate choice (Møller & Thornhill, 1998; Martín & López, 2000; Penton-Voak *et al.*, 2001; Roulin *et al.*, 2003; Spencer & MacDougall-Shackleton, 2011). Thus an ejaculate which shows low variance in sperm length could signify a male with

high regulatory control over sperm development. This could indicate superior control over the cellular environment e.g by limiting oxidative stress. Oxidative stress is known to impact negatively on spermatogenesis (Aitken & Baker, 2004) and has also been linked to the attractiveness of sexually selected ornaments (Dowling & Simmons, 2009; Helfenstein *et al.*, 2010). The relationship between sperm morphology and cryptic female choice are areas that would greatly benefit from future research.

Our two further analyses were based on reduced sample sizes, as few previous studies have been conducted on the variation in the separate sperm components. We could therefore only use the six species measured during this study. No significant relationships were observed between midpiece length, its CVwm or CVbm and EPP levels across the passerine species. This was also the case for the relationships between EPP and the Flagellum:Head measures. The current results therefore suggest that energy reserves (midpiece length) and sperm velocity (Flagellum:Head ratio) do not play significant roles under differing levels of sperm competition. This is in contrast to many studies that show a competitive advantage of faster sperm in bouts of sperm competition (Birkhead *et al.*, 1999; Gage *et al.*, 2004; Malo, Garde, *et al.*, 2005) but also see (Bennison *et al.*, 2015). There is also controversy over the relationship between sperm morphology and velocity, although (Lüpold, Calhim, *et al.*, 2009) found that Flagellum to head ratio is a strong predictor of sperm velocity across passerine birds many studies have failed to observe a similar intraspecific relationship in a variety of taxa (Gage *et al.*, 2002; Minoretti & Baur, 2006; Lüpold, Linz, *et al.*, 2009). A future study measuring in vitro sperm velocity across the Remizidae and Paridae would provide a better test of the relationship between velocity and EPP in these families.

However a major caveat in this study is our ability to accurately measure CVbm, this was severely reduced for some species due to the low number of males sampled. In future studies looking at greater numbers of males within each species and also focusing on multiple populations of the same species but with varying rates of EPP would be highly informative. As (Lifjeld *et al.*, 2010) found

that the strongest correlate with EPP was CVbm in total sperm length across a range of families, so it could be that our analysis lacks resolution to detect this or there are different mechanisms for post-copulatory selection on sperm in the the Paridae and Remizidae families.

Although no significant relationships were observed in the analysis on midpiece length parameters, further investigation with a greater number of species is required. An interesting trend suggesting a negative relationship between CVwm and EPP but a positive one between CVbm and EPP is especially intriguing. This pattern would be predicted if higher levels of sperm competition selected for differing male strategies, perhaps linked to trade-offs with other fitness related traits (Simmons & Emlen, 2006; Evans, 2010; Dowling & Simmons, 2012), but with continued selection for uniform within male ejaculates. Perhaps uniformity within ejaculates allows simultaneous progression through the female reproductive tract separated from competing male ejaculates. Current knowledge on the mechanisms that allow the movement and progression of sperm within the female reproductive tract is lacking and although there is evidence for cryptic female choice in birds, very little is known about the mechanisms that underlie this choice (Pizzari & Birkhead, 2000; Chaline *et al.*, 2004; Løvlie *et al.*, 2013). Greater insight into the internal mechanisms is imperative if we are to understand the selective processes impacting sperm morphology.

In the future the ability of EPP rates to accurately reflect levels of sperm competition will also need to be addressed. As EPP only gives a measure of successful extra pair copulations (EPCs) many unsuccessful ones may be going undetected. The potential success of an EPC may also vary within a species depending on the history of sperm competition. At present the positive correlation between EPP and testes size supports the use of EPP as a proxy for the level of sperm competition (Pitnick & Markow, 1994; Moller & Briskie, 1995) but see (Calhim & Birkhead, 2006; Griffith, 2007; Lüpold *et al.*, 2011; Ramm & Schärer, 2014). Potentially other traits that indicate sperm

competition risk, such as testes size or female mating rate, could be used in future investigations.

With the data currently available the trait likely to provide the best estimate of EPP within *Paridae* and *Remizidae* species is the variance in sperm length within a male. If future studies can increase the sample size of both males, populations and species then these studies could gain a stronger understanding of the relationship between these two traits as in our analysis CVwm only explains a small amount of the relationship with EPP. There are obviously other factors at play but if sperm morphology can be used to predict mating systems in these species then it provides an opportunity to accurately describe species mating systems with less invasive and less time consuming techniques (Immler & Birkhead, 2005).

Chapter 5

Sexually selected plumage trait predicts sperm morphology in the European penduline tit (*Remiz pendulinus*)

Alexander D. Ball, Carlos Ponce, Ákos Pogány, Tamás Székely & Steve Dorus

Abstract

Sexually selected ornaments have fascinated and perplexed biologists for centuries. In birds, male plumage ornaments often provide an advantage in mate attraction. However, increased attractiveness does not necessarily convey higher fitness in species in which females mate multiply. Revelations of the pervasive nature of promiscuity has focused work on the relationships between mating success and fertilisation success. Sperm competition theory predicts a trade-off between mate acquisition and fertilisation success depending on the risk of sperm competition. However the "fertility insurance" hypothesis predicts that females mate multiply to guard against infertile partners, predicting that females select additional mates based on honest signals of their fertility. Here we test the relationship between a variable plumage ornament in the European penduline tit (*Remiz pendulinus*) and a sperm trait selected by high levels of sperm competition, within male variance in sperm length (CVwm). Our results reveal a positive relationship between this pre-copulatory plumage trait and this post-copulatory sperm trait. This suggests that attractive males are not only likely to gain more mates but they are also likely to have higher fertilisation success. This result raises questions about the maintenance of variation in these two traits under high sexual selection pressures while also providing support to the fertility insurance hypothesis. However a greater understanding of the genetic and environmental control of these traits is required to further elucidate the reasons underlying this relationship.

Introduction

Sexually selected traits are often split into two main groups, pre-copulatory and post-copulatory traits. This classifies traits by whether they are used to compete for and attract a mate or used to gain fertilisation success once sperm are within the female reproductive tract either via sperm competition or cryptic female choice. As the pervasive nature of promiscuity has become clear the increasing relevance of post-copulatory sexual selection has moved to the fore (Birkhead, 1998, 2000; Griffith *et al.*, 2002). The theory of sperm competition theory predicts a trade-off between the level of resources allocated to pre and post-copulatory traits depending on the risk of sperm competition (Parker, 1998). However an alternative theory, the phenotype-linked fertility hypothesis, predicts that pre-copulatory traits are honest indicators of fertility that are used by females to guard against infertile partners (Sheldon, 1994).

These two hypotheses predict different relationships between pre and post-copulatory traits depending on a complex suite of variables. Sperm competition theory predicts that males will invest in competitive sperm traits when likely to experience higher levels of sperm competition (Parker *et al.*, 1997; Parker, 1998). Substantial evidence for this comes from studies on ejaculate allocation under differing levels of sperm competition risk; such that males will transfer larger ejaculates when in the presence of rival males (Gage & Barnard, 1996) or if the likelihood of mating with previously mated females is higher (Simmons *et al.*, 1993; Pizzari *et al.*, 2003). Therefore a relationship between pre-copulatory and post-copulatory traits is only predicted if pre-copulatory traits influence the degree of sperm competition. This could be the case if females are monopolised by a pre-copulatory trait that decreases their propensity or ability to remate (Lüpold *et al.*, 2014) or if females preferentially select ejaculates of attractive mates via cryptic female choice (Dean *et al.*, 2011). The phenotype-linked fertility insurance hypothesis predicts that a positive relationship exists between sexually selected pre-copulatory traits and the fertilizing ability of males (Sheldon, 1994) reviewed in (Birkhead & Pizzari, 2002). Females can therefore increase their fecundity or guard against infertile partners by

choosing males based on honest indicators of their fertility. However evidence for a relationship is mixed with studies finding both negative and positive relationships as well as no relationship at all (Birkhead & Fletcher, 1995; Pizzari *et al.*, 2002; Locatello *et al.*, 2006; Navara *et al.*, 2012; Mautz *et al.*, 2013). Explanations for this stem from the underlying genetic architecture and condition dependence of the traits (Pizzari *et al.*, 2002; Evans, 2010). As antagonistic pleiotropy could explain the high variance in many sexually selected traits, this underlying conflict could lead to negative relationships or the relative costs of pre and post-copulatory traits could vary across populations, selecting for variations in the acquisition and allocation of resources that influence the predicted relationship between traits (van Noordwijk & de Jong, 1986; Roff & Fairbairn, 2007).

As the direction of the relationships between pre and post-copulatory traits has fundamental implications for the evolution of sexually selected traits (Danielsson, 2001; Andersson & Simmons, 2006; Mautz *et al.*, 2013) initial research has been directed towards describing these relationships in a range of taxa (Malo, Roldan, *et al.*, 2005; Parker *et al.*, 2006; Calhim *et al.*, 2009; Klaus *et al.*, 2011; Lifjeld *et al.*, 2012; Rahman *et al.*, 2013). To date, support for the phenotype-linked fertility insurance hypotheses has been mixed (Mautz *et al.*, 2013). However, recent support for sperm competition theory was revealed by a study that found the direction of the relationship between pre and post-copulatory traits depends on the ability of males to monopolise matings (Lüpold *et al.*, 2014). The study found that when mating is dominated by scramble competition and females are able to mate multiply then males allocate resources to both pre and post-copulatory traits and a positive relationship is observed. However if males are able to monopolize matings with a female then resources are directed increasingly towards pre-copulatory traits that allow this monopolisation and a negative relationship between pre and post-copulatory traits is observed. This study helps to explain the often contradictory relationships that have been found between pre- and post-copulatory traits in the studies to date (Mautz *et al.*, 2013). However a greater diversity of research is required as currently the majority of studies have focused on these

relationships in mammals where there is greater knowledge of the post-copulatory traits that convey fertilisation success.

In birds, work has been focused on the post-copulatory traits driven by sperm competition as soon as biologists discovered the pervasiveness of Extra-Pair Paternity (EPP) within a group previously held up as a model of chaste monogamy (Birkhead, 1998; Griffith *et al.*, 2002). A substantial body of work has been conducted on sperm length, which in mammals is found to positively relate to the strength of sperm competition via its presumed effect on sperm swimming speed (Gomendio & Roldan, 1991). However the affect that sperm length has on sperm velocity has been questioned (Humphries *et al.*, 2008) and in birds the broad relationship between sperm length and sperm competition seems less clear; throughout birds it seems that only in some groups is sperm length significantly related to sperm competition (Immler & Birkhead, 2007; Immler, Gonzalez-Voyer, & Tim R. Birkhead, 2012). However, a few studies have recently revealed a relationship between the variance in sperm length and the degree of extra pair paternity within a population (Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010). This suggests that stabilising selection on an optimum sperm length is greater in species undergoing high levels of sperm competition. This points to sperm competition selecting for an optimum sperm size in males as the key to fertilisation success. However it is not clear what is driving the need for optimum sized sperm and also what the costs of maintaining uniform sperm length are? However it has shown a consistent association with EPP across passerines, suggesting it plays a fundamental role in sperm competition. In Chapter 4 it was shown that within male variance in sperm length (CVwm) was associated with sperm competition (measured as degree of EPP) in the penduline tits and allies, and Chapter 3 documented the rapid evolution of plumage ornaments in this family. We thus aim to use the European penduline tit to study the relationship between these pre and post-copulatory sexually selected traits.

The European penduline tit is an interesting species for this analysis given that it has a mating system dominated by sequential polygamy by both males and

females (Persson & Ohrstrom, 1989; Pogány *et al.*, 2008). Within these fleeting pair bonds infidelity also occurs, with up to 23.5% of offspring the result of extra pair paternity (van Dijk, Mészáros, *et al.*, 2010). Females have also been shown to prefer males with a larger melanin based plumage trait (a black eye-mask). This ornament is sexually dimorphic and has been shown to evolve rapidly in the penduline tit family (Chapter 3). In Chapter 4 the variance in sperm length within males (CVwm) was shown to vary between species in relation to the degree of sperm competition. Thus males evolving under higher levels of sperm competition exhibit reduced variation in sperm length, suggesting it is a trait that provides an advantage in sperm competition. Thus the European penduline tit possesses a sexually selected plumage ornament and a sperm trait known to correlate with the level of sperm competition, providing us with the opportunity to test competing hypotheses about the relationship between pre and post-copulatory sexually selected traits.

The aim of this study is to test the predictions made by sperm competition theory and the fertility insurance hypothesis on the relationship between pre-copulatory (mask size) and post-copulatory (CVwm) traits in the European penduline tit. These relationships fundamentally rely on the underlying genetic variance of each trait, the degree of condition dependence of the traits and the costs associated with mating. Thus it is acknowledged that predictions of the relationship between pre and post-copulatory traits are highly complex (Engqvist, 2011; Mautz *et al.*, 2013). If the mating behaviour or sperm selection of females is influenced by the attractiveness of their partner, with females less likely to engage in extra pair copulations or more likely to store sperm when they have mated with an attractive male, attractive males will experience lower rates of sperm competition. Sperm competition theory would thus predict that attractive males would invest less in competitive sperm traits than un-attractive males (Parker, 1998). We would therefore expect to find a negative relationship between mask size and sperm variance within males. However, if females engage in extra pair copulations for alternative reasons, such as avoiding infertility (fertility insurance hypothesis) or in order to maximise sperm competition (sexually-selected sperm hypothesis) (Keller & Reeve, 1995;

Pizzari & Birkhead, 2002) then both attractive and unattractive males will benefit from maximising their competitive ability in sperm competition. The relationship between pre and post-copulatory traits will then depend on the genetic architecture and environmental control of the traits. If they are condition dependent but low cost then a positive correlation would be predicted, with males in top condition being able to maximise both mate attraction and sperm competitive ability. However with costly traits a negative relationship could indicate a trade-off between the allocation of resources to either pre or post-copulatory traits with relatively few individuals able to maximise both.

Methods

Field sampling

Fieldwork was conducted in two populations of the European penduline tit, one in the Danube delta, Romania (Grid ref: 49°10' N, 29°25' E) and the other along the banks of the Jarama river, near Madrid, Spain (Grid ref: 40°18' N, 03°32' W). Samples were collected during the breeding seasons, June 2011 and April 2013 in the Romanian and Spanish populations respectively. Two populations were used to maximise the sample size.

Males were captured in mist nets placed within ~10m of their nest, which typically hang from tree branches about 2m-6m above water. Song playback and dummy models were set up on the opposite side of the net to the nest. European penduline tit males are highly territorial in the breeding season and males are typically captured within ~15 minutes of set-up (see (van Dijk, Szentirmai, & Székely, 2007) for typical penduline tit fieldwork techniques).

Once birds were caught they were placed in a cotton bag containing a small plastic tray covered in wire mesh. After ~5 minutes the tray was removed from the bag. The liquid around any faecal sample present in the tray was pipette into an eppendorf containing 500ul of 5% formalin fixative. Sperm can be collected in this way during the breeding season as the seminal glomera (sperm storage organ) is located at the distal end of the reproductive tract very close to the cloaca and thus sperm is usually shed in the faeces of most bird species (Immler & Birkhead, 2005). Avian sperm can also be obtained by cloacal massage or by dissecting the seminal glomera. One bird died during capture and thus the distal end of the seminal glomera was dissected in order to obtain a sperm sample, which was stored in 1ml of 10% formalin. Sperm can be stored in formalin for many years without damage (Briskie & Birkhead, 1993), and all samples were used within 2 years of collection.

Morphometrics were taken including wing, tarsus and beak length as well as weight. A small blood sample (30-50ul) was extracted from the brachial wing vein and stored in either 1ml of 100% ethanol or 1ml of Queen's lysis buffer. Finally photos were taken of each side of the birds' head and of the back. Three photos from each angle were taken and a Kodak grey card, to control for differing light conditions, was included in the background of every photo in addition to a metal ruler. All photos were taken in the shade to limit differences in light conditions between each photo.

Plumage trait measurements

We measured three plumage traits in each male, mask size, the contrast between crown and mask feathers and the saturation of the mantle feathers. Each of these traits were measured from the photos taken in the field using image software Gimp Version 2.6.11. Before making any measurements the light conditions of each photo were standardised using the Kodak grey card. The grey point of the photo was set using a 100x100 pixel area of the Kodak grey card in close proximity to the plumage trait. The following methods were then used to measure each trait (also see Figure 5.1):

i) Mask size – The 'Fuzzy select tool' was used to select the black area of the mask including the eye. The number of pixels in the selected area was used to measure its size when compared to the number of pixels in $1cm^2$ of the photo. The mask size was measured thrice on both sides of the head, and the average of the six measurements calculated.

ii) Crown-Mask contrast – The average pixel colour in the selected mask area was determined by calculating the average RGB values of the pixels. A $\sim 0.5cm^2$ section of the crown, just above the mask, was then selected and average pixel colour determined as above. The contrast between these two colours (mask and crown) was calculated in the BabelColor® Color Translator Analyzer program Version 3.1.1 using the CIELAB colour comparison algorithm using the ΔE value as a

measure of the degree of contrast between the average colour of the mask and the average colour of the crown. Again this was performed on all 6 photographs of each bird's head (3 of the left side and 3 of the right).

iii) Mantle saturation – A 0.5cm^2 section in the central section of the mantle feathers was selected using the "rectangular select" tool. The average RGB values were recorded for this section of the mantle and saturation calculated using the following formula:

$$SATURATION = 100 \times \frac{MAX(R,G,B) - MIN(R,G,B)}{MAX(R,G,B)}$$

Saturation describes the intensity of a colour, from high saturation signifying very colourful and a low value signifying a washed out colour nearer to the grey-scale.

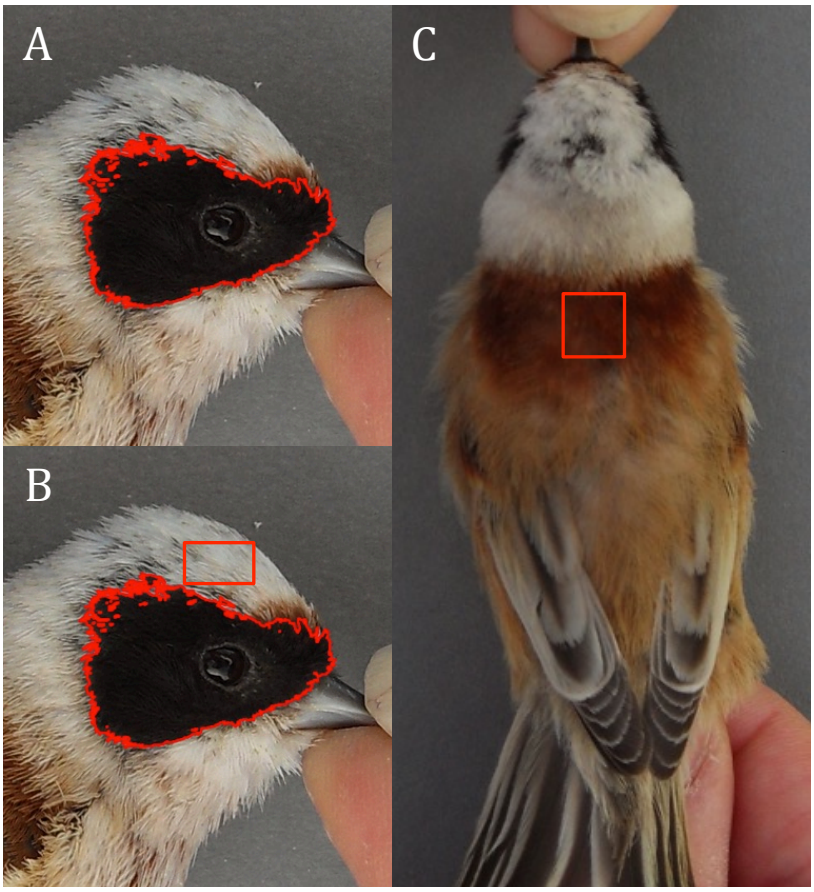

Figure 5.1. Plumage measurements taken for each male bird included A) Mask size, B) Contrast between the mask and crown feathers and C) The saturation of the Mantle feathers.

Sperm morphology

Sperm were observed under a Zeiss LSM510Meta confocal laser-scanning microscope. A 10ul sample from the bottom of the eppendorfs containing faecal or seminal glomera samples was pipetted onto a glass bottomed micro-well dish. These were left to air-dry in a fumehood for ~45 minutes. The sperm cells were then re-suspended in 10ul of a 0.6umol DAPI solution, in order to stain the nuclei. Sperm were initially located using a 20x Phase 2 air objective under white light. Once a suitable sperm had been located, free of debris and of normal morphology (sperm with two heads, two tails or crooks were avoided), it was viewed using a 488nm argon laser. Photos were taken with a digital camera and the images saved as .tif files.

The graphical program ImageJ Version 1.46r was used to visualise the sperm photos and perform measurements. The length of the three main components of passerine sperm were measured, the head, midpiece and tail, to the nearest micron. All three measurements were combined to calculate the total length of each sperm (see Figure 4.1 for delineation of the sperm components). At least 8 sperm were measured from each male and each sperm was measured three times and the average of these used to calculate within-male variance in sperm length for each male (CVwm).

Statistical methods

All traits were compared between the two populations of penduline tits using Welch's t-tests to ascertain if there were any significant differences in morphology between the Romanian and Spanish birds. We assumed unequal variances due to the lack of knowledge of the diversity within these two populations on either side of Europe. They could be under different sexual selection pressures given the rapid evolution of sexually selected traits within this group (as documented in chapter 3).

An analysis of covariance (ANCOVA) was performed to assess the relationship between the pre-copulatory trait (mask size) and the post-copulatory trait (CVwm of total sperm length) whilst controlling for population. The maximal model was initially applied before subsequently removing the non-significant variables in a step-wise fashion. This would lead to the model with the highest likelihood by routinely comparing models via anova after a variable was removed. All analyses were performed and graphs produced using the R statistical environment.

Results

Plumage comparison

Spanish penduline tits had significantly larger masks than their Romanian counterparts (Welch two sample t-test; t = -3.67, df = 12.8, p < 0.01). They also had significantly greater mantle saturation (Welch two sample t-test; t = -3.648, df = 12.85, p < 0.01) and the contrast between the Mask and Crown feathers was also more pronounced in the Spanish birds, although not significantly different (Welch two sample t-test; t = -2.164, df = 8.79, p-value = 0.059). See Figure 5.2 for plumage comparisons.

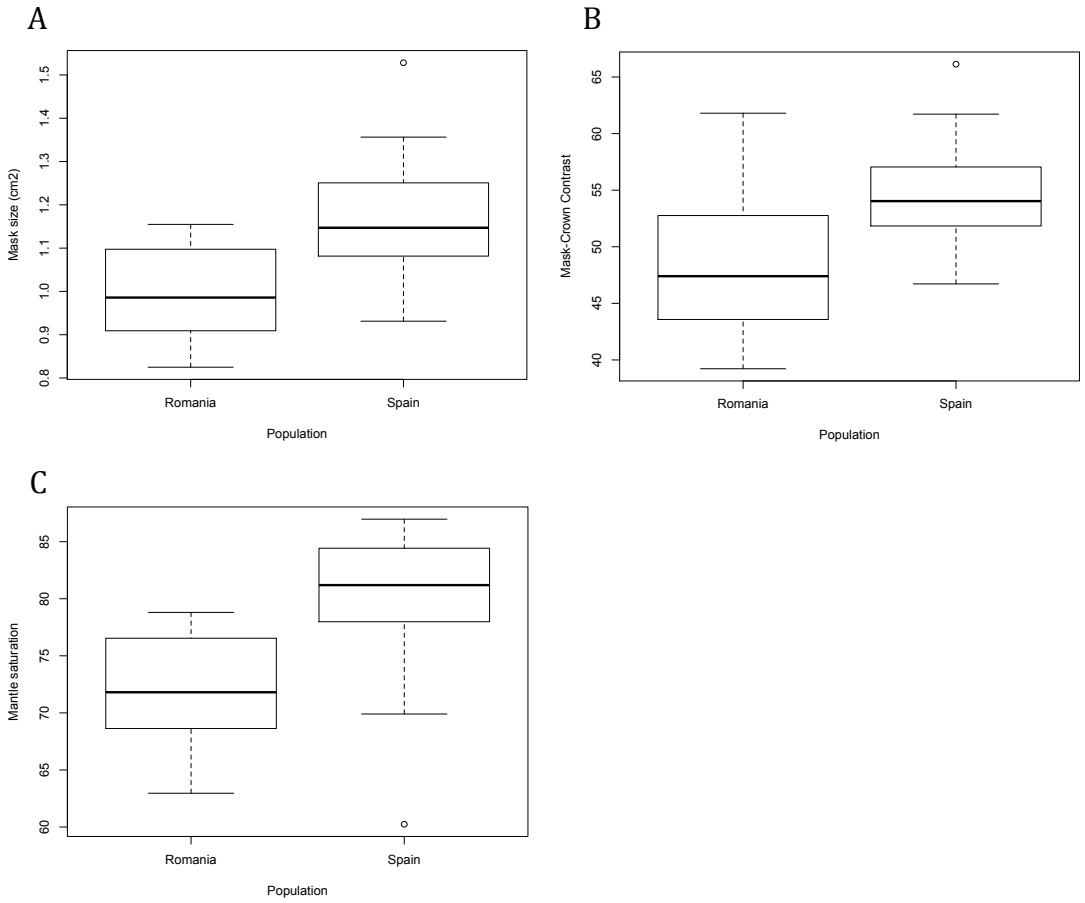

Figure 5.2. Plumage comparison between the two penduline tit populations showing the greater ornamentation in the Spanish population. The Spanish males had significantly greater A) Mask size and C) Mantle saturation than Romanian males (Mask size: Welch two sample t-test; t = -3.67, df = 12.8, p < 0.01, Mantle saturation: Welch two sample t-test; t = -3.648, df = 12.85, p < 0.01). However there was not a significant difference between B) Mask-Crown contrast (Welch two sample t-test; t = -2.1636, df = 8.79, p = 0.059)

Sperm morphology

The required number of sperm (8-10 per male) were only obtained from 50% (4/9) of the Romanian birds and 19% (5/27) of the Spanish birds. These samples did not reveal any significant difference between the within male variance in sperm length (CVwm) of the two populations (Welch two sample t-test; t = -1.703, df = 5.46, p-value = 0.14) or a significant difference in total sperm length between them (Welch two sample t-test; t = -1.927, df = 4.02, p-value = 0.126). Although both traits verged towards higher values in the Spanish population (see Figure 5.3).

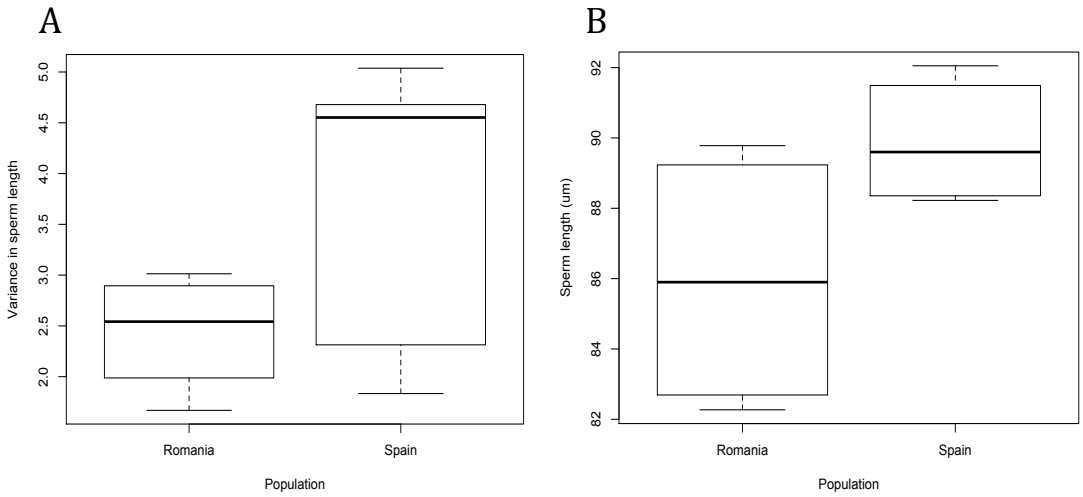

Figure 5.3. Comparison of sperm morphology between two populations of European penduline tits, *Remiz pendulinus*. A) There is no significant difference between the populations in within male variance in sperm length (CVwm) (Welch two sample t-test; t = -1.703, df = 5.46, p-value = 0.14) or B) Sperm length (Welch two sample t-test; t = -1.927, df = 4.02, p-value = 0.126).

Correlation between within male sperm variance (CVwm) and mask size

In the maximal model to explain the observed differences in the within male variance in sperm length (CVwm) we included population, mask size and mantle saturation. These were included as these two plumage traits are both sexually dimorphic across Eurasian penduline tit species (see chapter 3) and there is evidence that mask size is a sexually selected trait driven by female choice (Pogány & Székely, 2007). We included population as a variable since we observed significant differences between the two populations in both these plumage traits. The maximal model also incorporated interactions between each of the three explanatory variables.

Maximal model: $y_{romania} = a_{romania} + b_{romania}\, x + c_{romania}\, z$

$y_{spain} = a_{spain} + b_{spain}\, x + c_{spain}\, z$

Where y is the dependent variable (CVwm) and the explanatory variables are x (mask size) and z (mantle saturation). The inclusion of mantle saturation did not significantly affect the model fit and was thus removed. The interaction between mask size and population did not significantly affect the model fit and was also removed. All other variables significantly affected the model's ability to explain the observed pattern in sperm variance. Thus we found that the most likely model was:

Minimal adequate model: $y_{romania} = a_{romania} + bx$

$y_{spain} = a_{spain} + bx$

Therefore we found that within male sperm variance is negatively correlated with mask size. There is also a significant effect of population with the Spanish males exhibiting larger masks but higher variance in sperm length than the Romanian males (see Table 5.1 and Figure 5.4).

Table 5.1. The minimal adequate model to explain the variation in within male sperm variance (CVwm) of 9 male penduline tits: Sperm variance (wm) ~ Country + mask size

Term	estimate	S.E.	t	P	Effect size
Intercept	9.122	2.443	3.734	0.00969	
Country	2.601	0.752	3.459	0.01348	2.943
Mask	-6.796	2.447	-2.777	0.03212	0.589

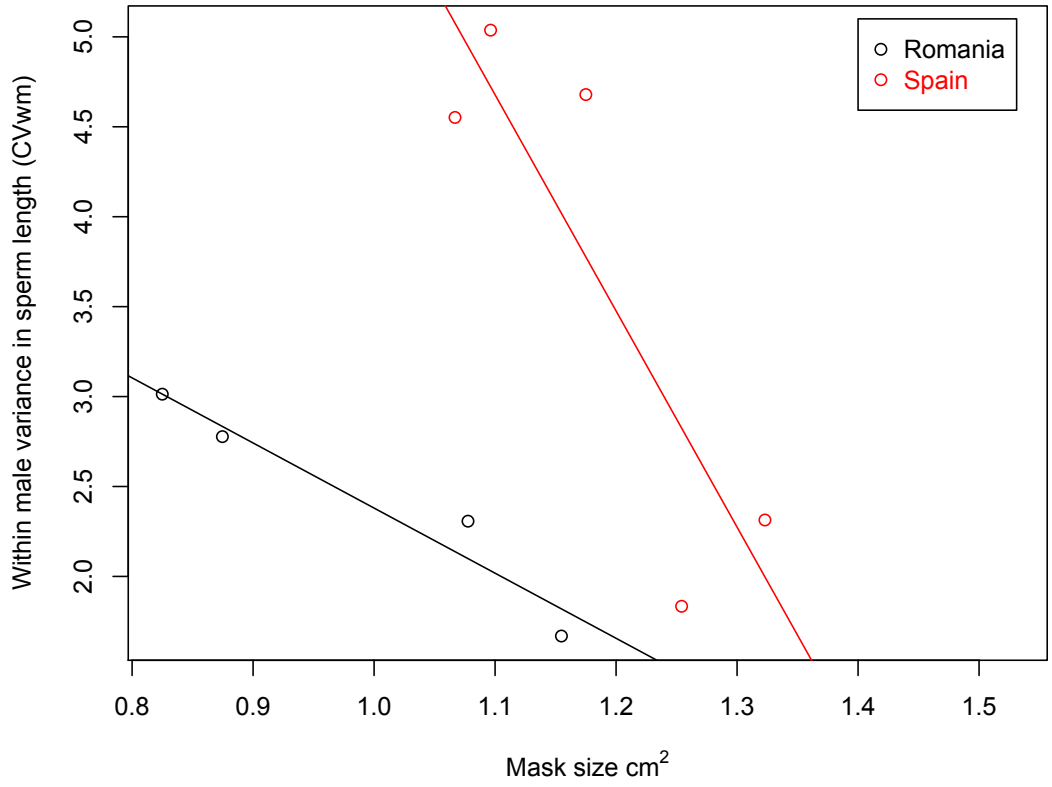

Figure 5.4. Relationships between a pre-copulatory (mask size) and post-copulatory (CVwm) sexually selected trait in two populations of the European penduline tit, *Remiz pendulinus*. There is a significant negative relationship CVwm and mask size. Although in the Spanish population variance in sperm length is higher, mask size is greater (see Table 5.1).

Discussion

The two populations studied are at separate ends of the European distribution of the European penduline tit, from Madrid, Spain in the west to the Danube delta, Romania in the east. However both populations are currently classified within the same subspecies, the nominate subspecies *Remiz pendulinus pendulinus*. The other 3 subspecies as described by Harrap and Quinn occur to the east of Europe (Harrap & Quinn, 1996). However we have found significant differences in morphology between male plumage in our two populations. This is not surprising given the ~2700 km separating the two localities. However as far as we are aware no previous description has been noted about the variation in the penduline tits across Europe. Here we describe the greater ornamentation of the Spanish males compared to their Romanian counterparts. The Spanish males exhibit larger masks, a trait preferred by female penduline tits in a Hungarian population (Pogány & Székely, 2007; Kingma *et al.*, 2008), and their mantle feathers are more colourful. The mantle feathers are also likely to be a sexually selected trait, showing pronounced sexual dimorphism in all Eurasian species of penduline tit (see chapter 3). The reasons for these observed differences are intriguing and would benefit from further investigation. They could be driven by genetic or environmental differences between the populations. Extensive ringing data has revealed that the Spanish population is resident year round (Carlos Ponce, pers comm), perhaps the loss of migration pressures have allowed increased resource allocation to ornamentation or are perhaps driven by increased female choosiness by Spanish females. However, as melanin based traits are thought to be under a large degree of genetic influence (Griffith *et al.*, 2006) it is perhaps driven by genetic differences between the populations. From our work on the evolution of plumage and body size traits (see chapter 3) we know that these sexually selected plumage traits have undergone rapid evolutionary change across the Eurasian penduline tits. This pattern if driven by sexual selection, could mean that there are increasingly "choosy" females in Spain, it would be interesting to see how parental care and the mating system varies in this Spanish population,

as little variation has been found elsewhere in Europe (van Dijk, Brinkhuizen, *et al.*, 2010).

Unlike the pre-copulatory sexually selected traits no significant differences were found between sperm length in the two populations. However the sample sizes are very small due to the low success of sperm acquisition. The small success rate of obtaining sperm samples from the Spanish population is especially surprising but perhaps has something to do with the timing of sample collection. In Spain the faecal samples were collected in the first few weeks of the breeding season where as in Romania, samples were collected towards the middle of the season. Variation in sperm numbers could change throughout the season, perhaps based on depletion due to differing mating rates, which is known to have a large effect on the number of sperm in the seminal glomera (sperm storage organ) in some passerines (Birkhead, 1991; Sax & Hoi, 1998). Increased sample collection throughout the season in both populations would be useful also in determining if sperm traits are maintained in males through time, as has been found in other studies on passerines (Birkhead & Fletcher, 1995; Laskemoen *et al.*, 2013). The comparison of sperm length would also benefit from increased sampling as a slight trend towards longer sperm in the Spanish population is suggested by the current results (Figure 5.3).

The current evidence supports the hypothesis that females could use pre-copulatory traits to determine the post-copulatory traits of a male, as male mask size, a sexually selected pre-copulatory trait, where by females prefer males with larger masks, is negatively correlated with sperm variance within a male. As we have confirmed for the *Paridae* and *Remizidae* in chapter 4 and has been found across passerines (Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010), species exhibiting higher levels of sperm competition (measured as increased levels of EPP) have lower variance in sperm length both within and between males in the population. The supposition is that males with more sperm of an optimum length for storage in the female reproductive tract gain higher fertilisation success. Thus male mask size could indeed function as an honest fertility signal as predicted by the phenotype-linked fertility hypothesis.

However we cannot rule out alternative explanations, as perhaps they co-vary due to a shared condition dependence that females select due to its direct fitness benefits, although this seems unlikely as penduline tits spend very short periods of time together post-mating (van Dijk, Szentirmai, Komdeur, *et al.*, 2007). Alternatively selecting males in good condition could provide indirect genetic benefits to offspring; signalling the presence of disease resistant genes (Hamilton & Zuk, 1982; Whittingham *et al.*, 2015) or more efficient cellular mechanisms (Hill & Johnson, 2013).

The results lack support for within-male trade-offs between the two sexually selected traits, which would predict a negative relationship. This provides support that similar risks of sperm competition are faced by all males; attractive males are unlikely to reduce the re-mating rate of their partners and thus maintain investment in both pre and post-copulatory traits. Evidence from extra-pair paternity studies supports this assertion as males with larger mask do not have significantly different levels of extra-pair young in their nests compared to smaller masked males (van Dijk, Mészáros, *et al.*, 2010). However this is also contradictory to both the sperm competition and phenotype-linked fertility hypotheses. If large masked males have a fertilizing advantage, as suggested by their lower variance in sperm size, then we would predict that they would have lower levels of EPP within their nests unless the mates of attractive males actually increase their levels of infidelity. This could perhaps occur if attractive males are paired with attractive females that gain larger numbers of extra-pair suitors. However the degree of sexual dimorphism in plumage traits suggests selection is driven by female not male choice. Further studies focusing on male mate preferences and female fecundity along with their relationship to female extra pair copulations could help resolve this paradox.

Currently very few studies have explored the presence of a relationship between pre and post-copulatory traits in birds. The few that have been completed have provided differing results. In the pied flycatcher two studies found completely contrasting results, Calhim et al. found a positive relationship

between sperm length and 2 traits involved in female preference in this species, plumage blackness and breeding date (Calhim *et al.*, 2009). Females preferred darker males and those that obtain a territory earlier. However with an increased sample size Lifjeld et al. found no relationship between either of these traits and sperm length, leading them to reject a link between pre-copulatory traits and sperm morphology (Lifjeld *et al.*, 2012). They also found that longer sperm do not necessarily provide a competitive advantage within this species. This is a common finding with studies across passerines struggling to find a consistent pattern between sperm length and sperm competition, in some groups it seems to play a role and in others it does not (Briskie & Montgomerie, 1992; Immler, Gonzalez-Voyer, & Tim R. Birkhead, 2012). However the variance in sperm length within a male is a trait that has shown a consistent relationship with the levels of sperm competition across passerine birds suggesting that it is driven by sperm competition (Calhim *et al.*, 2007; Kleven *et al.*, 2008; Lifjeld *et al.*, 2010). Here we show that there appears to be a relationship between this trait and a plumage trait driven by female choice, however the benefits a female gains by selecting a male with more competitive sperm remains to be elucidated. It is possible that both traits are condition dependent and correlated because of this rather than because of female selection for competitive sperm. If we are to manage to separate the competing hypotheses we first need to understand more about the genetic control of melanin based plumage traits and sperm morphology. Also understanding the alternative roles that melanin plays within the body could provide information on why it consistently underpins sexually selected traits in birds. One link that is gaining increasing attention is the role that oxidative stress plays in the formation of sexually selected traits (Dowling & Simmons, 2009).

It has long been known that sperm are very susceptible to oxidative stress and many mechanisms have evolved to reduce their contact with reactive oxygen species (ROS) both in the testes and the female reproductive tract (Smith *et al.*, 1996; Surai *et al.*, 2000; Helfenstein *et al.*, 2010). Recently evidence has also suggested that oxidative stress plays a role in melanin plumage deposition. In red-legged partridge dosed with diquat dibromide, a chemical which produces

high levels of ROS, the expression of eumelanin based plumage traits were significantly greater (Galván & Alonso-Alvarez, 2009). This suggests the potential for a trade-off between the size of melanin based plumage traits (high ROS levels) and the successful development of sperm (low ROS levels). Although we found that males with larger masks had more consistent sperm length within each population, our other notable finding is that there are differences between the two populations. Although the Spanish birds have larger masks they have higher between male variance in sperm length compared to the smaller masked Romanian males, thus showing the opposite pattern to that observed within each population. Perhaps a large proportion of the Spanish males are experiencing a negative trade-off for their larger masks.

In support of this trade-off is the recent evidence that internal and external levels of melanin are positively correlated throughout the body (Dubey & Roulin, 2014). Thus if high levels of ROS maintain high levels of eumelanin then perhaps this is associated with high levels of ROS throughout the body. One study has even linked increased testes melanisation to higher mutation rates in birds (Galván *et al.*, 2011). Only with increased research on the additional functions of melanin will we perhaps understand why melanin based plumage ornaments are so widespread in birds.

These differences in the relationship of the two populations also raise other interesting questions. Not only does it suggest that these traits are evolving rapidly, which is expected of sexually selected traits (see chapter 3) but why would an increase in mask size occur at the expense of sperm competitive ability in the less attractive males in the Spanish population. Perhaps there is a different balance being struck between the importance of pre and post-copulatory sexually selected traits. It would thus be interesting to compare the levels of sperm competition between the two populations. Is initially attracting a mate more beneficial than competing for fertilisation success in Spain? At present the sample sizes are extremely small but the questions they raise provide challenging areas for further investigation.

Chapter 6

Mating systems do not predict genetic diversity in the penduline tits and allies (Super family: *Sylvioidea*)

Alexander D. Ball, Deborah A. Dawson, René van Dijk, Carlos Ponce, Jin-Won Lee, Ben Hatchwell, Steve Dorus, Tamás Székely & Terry Burke

Abstract

The distribution of genetic diversity within and between species is of key importance to the viability of populations. With increasing numbers of species facing rapidly changing environments, their ability to adapt is of current concern. Many conservation programs have made it a priority to maximise levels of genetic diversity in endangered and declining species, however few studies have looked at the natural levels of genetic diversity found across species. This is in part due to the lack of widespread homologous loci, which would provide unbiased estimates between species. With the recent advent of large numbers of conserved avian microsatellites, it has become feasible to compare genetic diversity across passerine groups. The genetic diversity of 11 species across the *Sylvioidea* super-family is estimated using 15 homologous microsatellites. The results show that genetic diversity as measured by allelic diversity and expected heterozygoisty (He) is highly variable between species. The penduline tits show the lowest levels of genetic diversity, with the Willow warbler and Vinous throated parrotbill exhibiting the highest levels. The results reveal that genetic diversity exhibits a large phylogenetic component but the provide no support for a relationship between genetic diversity and current Extra Pair Paternity (EPP) levels whilst controlling for population size. These results fail to support an influence of mating systems on the retention of genetic diversity in the *Sylvioidea*, providing no evidence that female extra-pair mating has an effect on Ne.

Introduction

A huge amount of work has focused on assessing the biodiversity of the planet (Linnæus, 1758; Heywood, ed, 1995). But as we begin to grasp just how little we know about life on earth, it is disappearing in a wave of anthropomorphic change (Wilson, 1992; Barnosky *et al.*, 2011; IPCC, 2013). One way in which people are trying to limit the impact of this onslaught is through conservation efforts targeted at increasingly vulnerable species (Hoffmann *et al.*, 2010; Foden *et al.*, 2013). However, in order to identify the most vulnerable species an understanding of the factors that make a species vulnerable to change is required.

There are many components that could heighten or lessen a species' ability to adapt to environmental change. However, as selection acts upon underlying genetic differences between individuals, species with higher levels of diversity are thought to be more adaptable to change (Lande & Shannon, 1996; Booy *et al.*, 2000; Frankham *et al.*, 2002). Thus a key attribute for a long-term viable population in a changing environment is a large pool of genetic diversity. A policy for conserving genetic diversity is implemented in many conservation programs by attempting to avoid inbreeding (Ballou & Lacy, 1995; Fred. W. Allendorf & Luikart, 2007). Many studies have shown the negative consequences of inbreeding, resulting in increasingly homozygous individuals that can unmask deleterious alleles (Spielman *et al.*, 2004). But ultimately a lack of genetic diversity leads to a population with less ability to change.

Studies have focused on changes in genetic diversity in a range of species in order to understand the natural mechanisms that affect it. The field of population genetics has driven research into the processes that produce, maintain, and reduce both neutral and selected genetic diversity (Charlesworth *et al.*, 1993; Charlesworth & Hughes, 2000; Fred W. Allendorf & Luikart, 2007). A substantial factor in the production and maintenance of genetic diversity is the effective population size (Ne). This is intimately linked to the number of individuals within the population but is more closely dependent on the number

of breeding individuals that contribute their genes to the next generation. Theory has shown that many mechanisms can impact Ne, such as unequal sex ratios, age specific mortality or variance in breeding success (Hill, 1979; Frankham, 1995). However a trait which has provided contention over its effect on Ne is a species mating system (Karl, 2008; Pearse & Anderson, 2009; Snook *et al.*, 2009).

It is assumed that highly polygynous systems will have decreased Ne as a few males will be able to monopolise the parentage of the next generation, such as in mating systems dominated by male-male competition (Hedrick, 2005; Karl, 2008). However contrasting arguments have been put forward over the effect that multiple mating by females can have on the value of Ne. Some advocate that multiple mating by females provides some males an opportunity to monopolise the production of offspring in the next generation, thus increasing variance in male lifetime fitness and decreasing Ne (Karl, 2008). However, others propose that by multiple mating females are able to select a diverse array of partners increasing the genetic diversity of their offspring but crucially lowering variance in male fitness and increasing Ne (Pearse & Anderson, 2009). In species which form socially monogamous pair-bonds, individuals are restricted to one mate, thus females cannot all have the same partner, the crux of the issue here is centred upon the differing views of how individuals select additional mating opportunities. If females all choose to mate with the same additional male/s then Ne will be drastically reduced by multiple mating however if all females choose different mates based on their genetic complementarity then multiple mating could increase Ne. Recently theory based on random multiple mating has predicted that multiple mating by females within a pair-bond is able to increase or decrease Ne depending on a suite of life history traits within the species (Lotterhos, 2011). It predicts that in short-lived species where females mate with many partners but have few offspring and high variance in female reproductive success, multiple mating by females can increase Ne. However in species where there are long generation times, few multiple mates and low variance in female reproductive success Ne would be lowered by multiple mating compared to a monandrous system, one in which females pair with a

different partner each season but are faithful within the pair-bond. Thus theory predicts that mating systems could impact Ne in a multitude of ways and thus the mating system could potentially affect the long-term genetic diversity of a species. With the recent theoretical work on this topic it is now time for empirical studies to test the relationships between genetic diversity and mating systems.

Birds are a group in which a vast amount of knowledge about mating systems has been documented (Orians, 1969; Dunn *et al.*, 2001; Hasselquist & Sherman, 2001). Avian studies have led the way in sexual selection research and parentage studies since the 1980s have revealed vast differences between species in the promiscuity of females (Griffith *et al.*, 2002). Not only this but the requirement of genetic markers for use in these parentage studies has driven the development of an array of genetic markers. Recently a suite of cross-species markers have been developed that allow the comparison of homologous regions of the genome across passerine birds (Dawson *et al.*, 2010, 2013). These microsatellite markers can therefore provide an estimate of genetic diversity that's comparable across species allowing hypotheses about the distribution of genetic diversity to be tested.

Passerine birds have short generation times and produce relatively few offspring (Slagsvold, 1989; Beauchamp, 2010), however female variance in reproductive success is difficult to quantify, measures of lifetime fitness are difficult to obtain (McGraw & Caswell, 1996; Brommer *et al.*, 2002), although the few studies that have successfully quantified them in passerines show that there can be large variance in reproductive success of females, sometimes exceeding that of males (MacColl & Hatchwell, 2004; McCleery *et al.*, 2004). Within season differences in female breeding success are more readily available and show large variances in many passerine species especially associated with female age (Nol & Smith, 1987; Clutton-Brock & Sheldon, 2010). Our aim is to test whether EPP increases or decreases Ne in passerines by revealing the relationship between genetic diversity and EPP. Thus, this study aims to test whether multiple mating by females is associated with the retention of genetic

diversity within a species. If extra pair mating by females increases Ne then theory would predict that species with higher levels of EPY would retain greater levels of genetic diversity.

Methods

Species sampling

The microsatellite primers used to ascertain a measure of genetic diversity have been shown to exhibit increasing polymorphism in species relative to their genetic distance from the zebra finch, *Taeniapygia guttata* (Dawson *et al.*, 2010, 2013). They were initially designed as conserved avian markers using both the chicken and zebra finch genomes, with preferential use of the zebra finch sequence when mismatches were encountered (see (Dawson *et al.*, 2010, 2013) for detailed methodology). This use of the zebra finch in the design of the primers is thought to explain the observed bias in polymorphism in the species tested so far. Therefore to exclude this bias only species within the same monophyletic super-family (the *Sylvioidea*) were targeted. All members of this super-family have the same genetic distance from both the zebra finch and chicken thus controlling for the observed bias due to the primer design method. This super-family includes the penduline tits and their closest relatives based on the most recent genetic phylogenies of passerine birds, which estimate that this group split from the other passerines around 44.7 mya (Jetz *et al.*, 2012).

Eleven species within the *Sylvioidea* super-family were used in this study. The species were chosen in order to span a large range of extra pair paternity (EPP) rates exhibited within this group. Attempts were made to select closely related species that diverge in their rates of EPP. One population of each species was used for sampling and for all but one of these populations the extra pair young (EPY) rate had been calculated in the population used in our analyses (see Table 6.1. for species, EPY rates and sources). In order to gain a population representative of the species, those thought to have undergone recent bottlenecks were avoided, thus small island populations were avoided and

species with large continental breeding ranges were instead selected. Thirty two adults were caught within each species/population and as far as is known consisted of unrelated individuals (i.e. only breeding adults were sampled). Birds were caught using mist net trapping during the breeding season and a small amount of blood (~50ul) was taken from the brachial vein and stored in eppendorf tubes containing either 1ml of ethanol or 1ml of Queen's lysis buffer. These were stored at either room temperature or 5°C until use.

Table 6.1. Summary of Extra pair paternity (EPP), genetic diversity and breeding range data for the 11 *Sylvioidea* species used in this study. NCBI accession numbers for the Cytochrome (Cyt-B) and Ornithine decarboxylase (ODC-6-7) sequences used in the phylogenetic inference are included. The genetic diversity measures (He and Allelic diversity) were inferred by genotyping 27 individuals at 15 microsatellite loci for each species.

Species	English name	Family	Location	Average He	Average Allelic diversity	EPP	EPP reference	Breeding Area (km^2)	Cyt-B sequence	ODC 6-7 sequence
Acrocephalus schoenobaenus	Sedge warbler	*Acrocephalidae*	Wraysbury, UK	0.3812	3.27	8.4	(Buchanan & Catchpole, 2000)	12,000,000	AJ004243	FJ883144
Acrocepahlus scirpaceus	Eurasian reed warbler	*Acrocephalidae*	Wicken Fen, UK	0.4487	4.2	6.5	(Davies *et al.*, 2003)	13,900,000	AJ004771	FJ883145
Aegithalos caudatus	Long-tailed tit	*Aegithalidae*	Sheffield, UK	0.4281	4.2	2.4	(Hatchwell *et al.*, 2002)	18,600,000	DQ792803	EU680703
Cyanistes caeruleus	Blue tit	*Paridae*	Lancashire, UK	0.3334	3.13	11.7	(Leech *et al.*, 2001)	9,620,000	AF347961	KF183742
Parus major	Great tit	*Paridae*	Strodam, Denmark	0.2311	2.6	9.9	(Otter *et al.*, 2001)	32,600,000	AY495412	EU680749
Periparus ater	Coal tit	*Paridae*	Madrid, Spain	0.3819	3.6	31.4	(Dietrich *et al.*, 2004)	18,700,000	AF347959	KF183786
Phylloscopus trochilus	Willow warbler	*Phylloscopidae*	Sheffield, UK	0.5209	5.4	33	(Wilson, 2000)	15,800,000	Z73492	-
Anthoscopus minutus	Cape penduline tit	*Remizidae*	Koeberg, South Africa	0.2748	2.8	7.1	Chapter 2	1,710,000	Chapter 1	Chapter 1
Remiz coronatus	White-crowned penduline tit	*Remizidae*	Jabagly, Kazakhstan	0.2985	2.53	0	Chapter 2	458,000	Chapter 1	Chapter 1
Remiz pendulinus	European penduline tit	*Remizidae*	Feherto, Hungary	0.2538	2.4	23.5	(van Dijk, *et al.*, 2010)	5,890,000	Chapter 1	Chapter 1
Paradoxornis webbianus	Vinous-throated parrotbill	*Timaliidae*	Yangpyeong-gun, S. Korea	0.5322	5.07	7.7	(Lee *et al.*, 2009)	3,910,000	JX565699	EU680748

DNA extraction

DNA was extracted from every blood sample using the ammonium acetate precipitation method as described in (Nicholls *et al.*, 2000). The method was slightly modified depending on whether the sample had been stored in ethanol or Queen's lysis buffer. Samples stored in ethanol were initially centrifuged for 1 minute at 13,000rpm and then toothpicks were used to extract about $2mm^3$ of the pelleted blood sample. This was subsequently dried and placed into an eppendorf containing 250ul of Digsol buffer and 15ul of Proteinase K. As the blood cells have been lysed in the tubes containing Queen's lysis buffer a centrifugation step is not needed and instead 30-50ul of the gloopy sample is transferred by pipette into the Digsol buffer and proteinase K mixture. All eppendorf tubes were then incubated overnight in a rotating oven at 36°C. After digestion, 300ul of 4M ammonium acetate was added to each tube and then vortexed several times over a period of 15 minutes. Tubes were then centrifuged for 10 minutes (20 minutes for Queen's lysis samples) at 13,000rpm. The supernatant was then aspirated into clean 1.5ml tubes and the pelleted protein discarded. A volume of 800ul absolute ethanol was added to the supernatant and the DNA precipitated by inverting the tubes and mixing very briefly. The tubes were then centrifuged for 10 minutes (15minutes for Queen's lysis samples) at 13,000rpm. The ethanol was discarded leaving the DNA pellet in the tube to which 500ul of 70% ethanol was added. The pellet was rinsed by inverting the tube and briefly vortexed before centrifuging for a further 5 minutes at 13,000rpm. Once again the ethanol was discarded and then the tubes were left to air-dry for ~1 hour. Once dry 100ul (50ul for Queen's lysis samples) of T.E. was added to each tube. The DNA was resuspended by agitating the tubes and placing them in a 65°C water bath for 30 minutes. Samples were then stored at -20°C until quantification.

A FLUOstar OPTIMA spectrophotometer was used to quantify the raw DNA samples. A volume of 2ul of each DNA sample was placed in the well of a BMG black greiner plate along with seven known calf thymus standards (0, 3.24, 6.49, 12.98, 25.95, 51.9, 103.8ng/ul). A volume of 200ul of Hoechst dye was added to

each well and then the florescence was measured in the spectrophotometer. A 10ng/ul concentration was created for each sample using low T.E. and these were stored at -20°C until use.

Microsatellite genotyping

As previously mentioned, the microsatellite primers used in this study were designed to amplify conserved avian markers. They were designed by two slightly different methods, one using the zebra finch expressed sequence tags (ESTs) to search for homologous microsatellites in the chicken genome (named TG#) and the other comparing entire sets of microsatellites found in the entire genome sequences of both species (referred to as CAM#), see (Dawson *et al.*, 2010, 2013) for detailed methodology. Many of them have been found to amplify polymorphic products across the entire spectrum of bird species (Dawson *et al.*, 2010), and provide for the first time the ability to genotype a very large range of bird species across homologous microsatellite loci.

Twenty markers were selected for genotyping in the 11 species. They were chosen based on their position within the zebra finch genome, to avoid potential linkage between markers only those on different chromosomes were used (see Appendix 6.1.). The karyotype of bird genomes is highly conserved (Shetty *et al.*, 1999; Stapley *et al.*, 2008), thus it seems reasonable to assume that markers physically unlinked in the zebra finch are likely to be unlinked within other passerine species.

Microsatellites were amplified via polymerase chain reaction (PCR) using the primers in Appendix 6.1. Half the markers had their forward primer fluorescently labelled with 5HEX and half with 6-FAM. This allowed 2 markers to be run in duplex within a PCR reaction minimising time and cost (see Appendix 6.1. for the duplex sets). All PCRs were run as 2ul reactions with each tube in a 96-well plate containing 1ng of the relevant DNA, air-dried at the bottom of the well. A primer mix containing the 4 relevant primers each at a concentration of 0.2uM was created and 1ul of the mix was added to the

relevant well. Every well then had 1ul of Qiagen mix added followed by 20ul of mineral oil. Plates were then run on a MJ Research model PTC DNA Engine Tetrad thermal cycler. All primer pairs had been designed to anneal at the same temperature and so the exact same PCR protocol was used throughout this study. A 95°C initial 15 minute period to prime the Qmix was used followed by 45 cycles of the following protocol, a denaturing step of 94°C for 30 secs followed by an annealing temperature of 56°C for 90 secs, and then an elongation step of 72°C for 90 secs was used. After all the cycles a final elongation period of 72°C for 10 minutes completed the PCR protocol.

PCR products were diluted 1/160 times in ddH_2O. A Biosystems 3730 DNA sequencer was used to genotype each microsatellite. This is a 48 well capillary sequencer, which uses a laser to measure the presence of fluorescently dyed products. A Rox size standard was used to calculate the lengths of the fluorescent products. The genotypes were visualised in the GeneMapper software where each allele was scored. After scoring all markers in each species, the ones that were difficult to score in any/all species were excluded from further analyses. Using the program Cervus null allele frequencies were estimated and any markers that showed frequencies above 0.2 for any/all species were also excluded from further analyses. This led to the suitability of 15/20 of the microsatellites for the cross-species comparison of genetic diversity (see Appendix 6.1.).

Phylogeny construction

A phylogeny was inferred using *BEAST in the BEAST software package (Heled & Drummond, 2010). This uses a Bayesian approach to infer the most likely evolutionary relationships based on multiple markers. The NCBI repository (http://www.ncbi.nlm.nih.gov/) was searched for published gene sequences of 62 passerines within the *Sylvioidea* super family, representing 12 families and including our 11 study species. Cytochrome-B (Cyt-B) sequences for all 62 species were located and sequences of the Ornithine decarboxylase introns 6-7 (ODC6-7) were found for 43 species (see Appendix 6.2. for all species and NCBI

gene sequence numbers). Both of these gene regions were used to gain a representative of mitochondrial (Cyt-B) and nuclear (ODC6-7) DNA sequences, which in combination produce much greater resolution. Out-group sequences consisted of members of the nearest super-family (*Muscicapoidea*) and representatives of the more basal passerine groups, the *Corvidae* (Carrion crow) and the *Tyrannidae* (Brown-crested flycatcher). Each set of gene sequences were then aligned using the Clustal-W alignment tool in MEGA v 5.2.2. This created alignment lengths for the Cyt-B and ODC6-7 sequences of 944 bp and 700 bp respectively.

The substitution models used for each gene were inferred using the Akaike information criterion (AIC) in MrModel test. The aligned sequences were then input into *BEAST and run for 200,000,000 generations. A burn-in of 25% was discarded and the most likely phylogeny inferred from the remaining trees using Tree Annotator.

Estimating genetic variation

Genetic variation was estimated in two ways, the first using the mean allelic diversity per marker and the second method used the mean expected heterozygosity of each marker based on Hardy-Weinberg equilibrium. These are both complementary measures of genetic diversity with the first being more sensitive to loss of genetic diversity from small population sizes and the second less dependent on large sample sizes. The expected heterozygosity was estimated in Cervus v 3.0.3. The average across all 15 markers was calculated for all 11 species using 27 successfully genotyped individuals from each population (Table 6.1. and Appendix 6.1.).

Relationships between Promiscuity, Population size and Genetic diversity

The rate of EPP was used as a measure of promiscuity for each species/population. Previous studies had calculated the degree of EPP in 10 of the populations/species sampled for this study. For the additional species, the

Coal tit (*Periparus ater*), our samples originated from a mainland European population in Spain and a value of EPP calculated for a mainland population of Coal tits in Germany was used in this study. Few species have EPP calculated from multiple populations, but those that do show only small amounts of variation except when comparing between island and mainland populations (Griffith, 2000; Brommer *et al.*, 2010).

Breeding ranges for all 11 species were obtained from the Birdlife international datazone (http://www.birdlife.org.uk/datazone/home). All species in this study are small (6-18g) mostly insectivorous passerines and thus it is reasonable to assume that breeding range can be used as a proxy for species population size. To check the validity of this assumption we compared the relationship between breeding range and population size (as estimated by Birdlife international) for 25 *Sylvioidea* species for which both data were available (Appendix 6.3.).

To test the prediction that female promiscuity would increase Ne and thus the level of genetic diversity within a population a phylogenetic generalized least squares (PGLS) approach was used. This was implemented in R using the "caper" package. The *Sylvioidea* phylogeny was necessary to control for relatedness between the populations/species by allowing traits to vary via a Brownian model of evolution using the lambda parameter proposed by (Pagel, 1999). A lambda of 0 indicates that the data shows no phylogenetic signal and a lambda of 1 indicates that the data shows complete phylogenetic dependence evolving in line with a Brownian model of evolution. Lambda was estimated during the analysis via a maximum likelihood approach and likelihood ratio tests were used to compare the model fit when either a lambda of 0 or lambda of 1 was used to explain the fit of the data.

The PGLS analyses used breeding range (log transformed due to large variations between species) and promiscuity as explanatory variables for the variation observed in each of the genetic diversity measures in the 11 *Sylvioidea* species.

Model: Genetic diversity = Promiscuity + log(Breeding range)

Results

Phylogeny

A robust phylogeny with a monophyletic *Sylvioidea* clade in close agreement with the most recent passerine phylogenies was inferred (Figure 6.1.) (Alström *et al.*, 2006; Jetz *et al.*, 2012). All members of the same genus group together and the majority of nodes are supported with high probability.

Breeding range

There was a significant positive correlation between breeding range (km^2) and estimated population size (Pearson correlation, t = 2.68, df = 23, p-value = 0.013), (Figure 6.2.). This was used to support the use of breeding range as a surrogate for population size for the 11 species in this study.

Genetic diversity

Genetic diversity varied significantly across the 11 species measured when using either expected heterozygosity (Kruskal-Wallis χ^2 = 20.83, df = 10, p-value=0.0223) (Figure 6.3) or allelic diversity measures (Kruskal-Wallis χ^2 = 23.33, df = 10, p-value = 0.0096) (Figure 6.4). Both genetic diversity measures were highly correlated (Pearson's correlation, t = 11.99, df = 9, p-value < 0.0001)(Figure 6.5). Genetic diversity showed a strong phylogenetic signal, a lambda of 1 was always significantly more likely than a lambda of 0 (Table 6.2). The lowest levels of variation were found in the Penduline tits (*Remizidae*) and True tits (*Paridae*) with higher levels being found in the warblers, Vinous-throated parrotbill and Long-tailed tit (Figures 6.3 and 6.4).

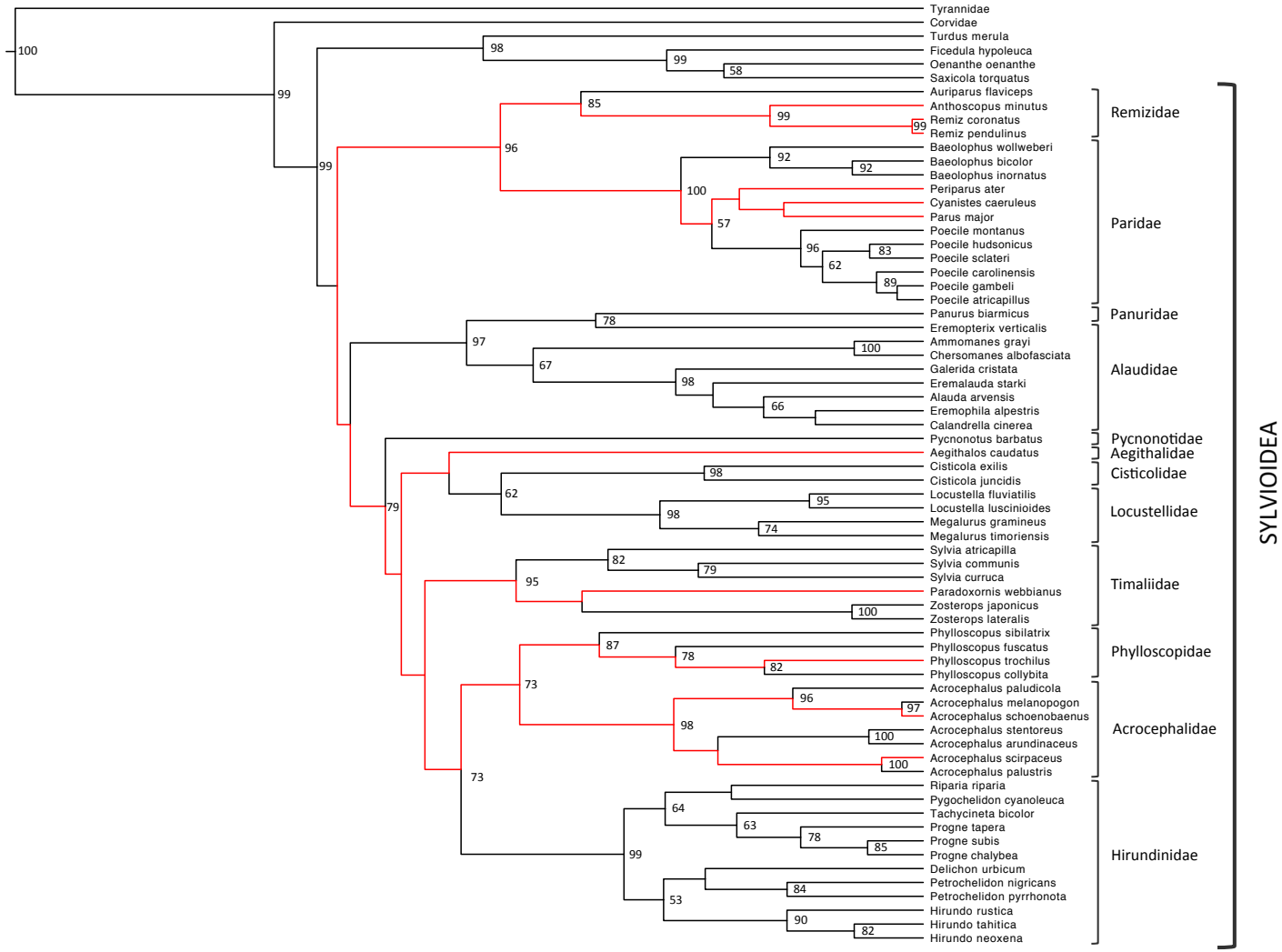

Figure 6.1. Phylogeny of the *Sylvioidea* inferred in *BEAST using a mitochondrial (Cyt-B) and nuclear (ODC-6-7) gene region. All branch nodes with a posterior probability >50 are labelled and the red branches indicate the eleven species compared in this study.

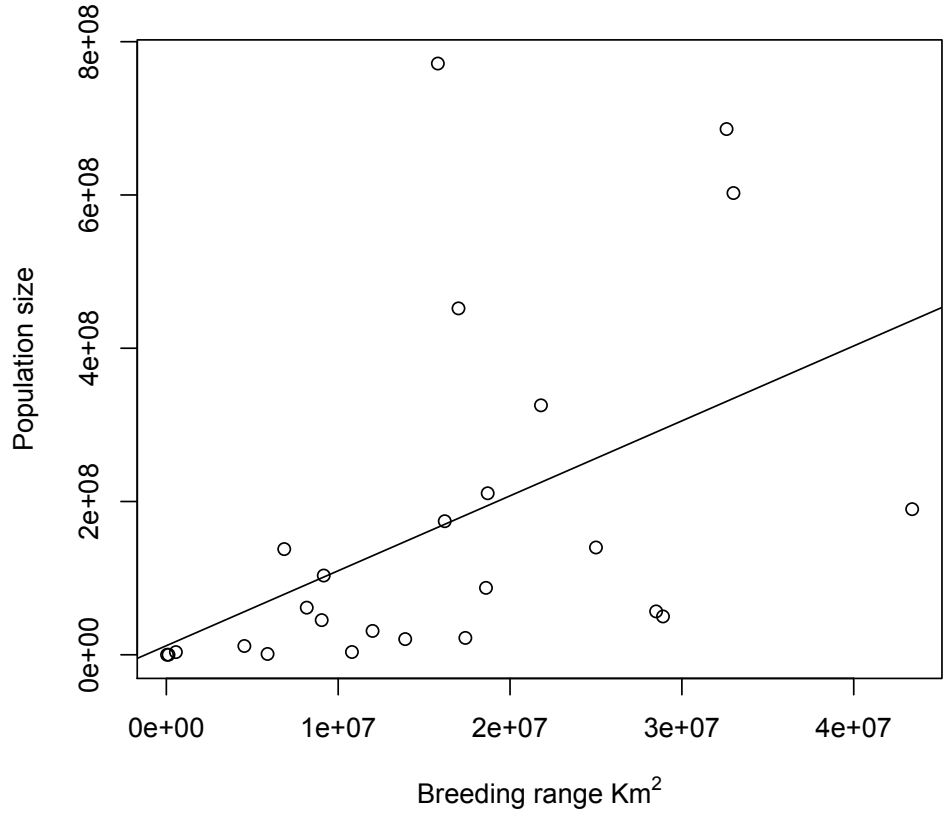

Figure 6.2. The relationship between breeding range and population size for 25 *Sylvioidea* species. The data were obtained from Birdlife international and show a significant positive relationship between breeding range and population size (Pearson correlation, t = 2.68, df = 23, p-value = 0.013).

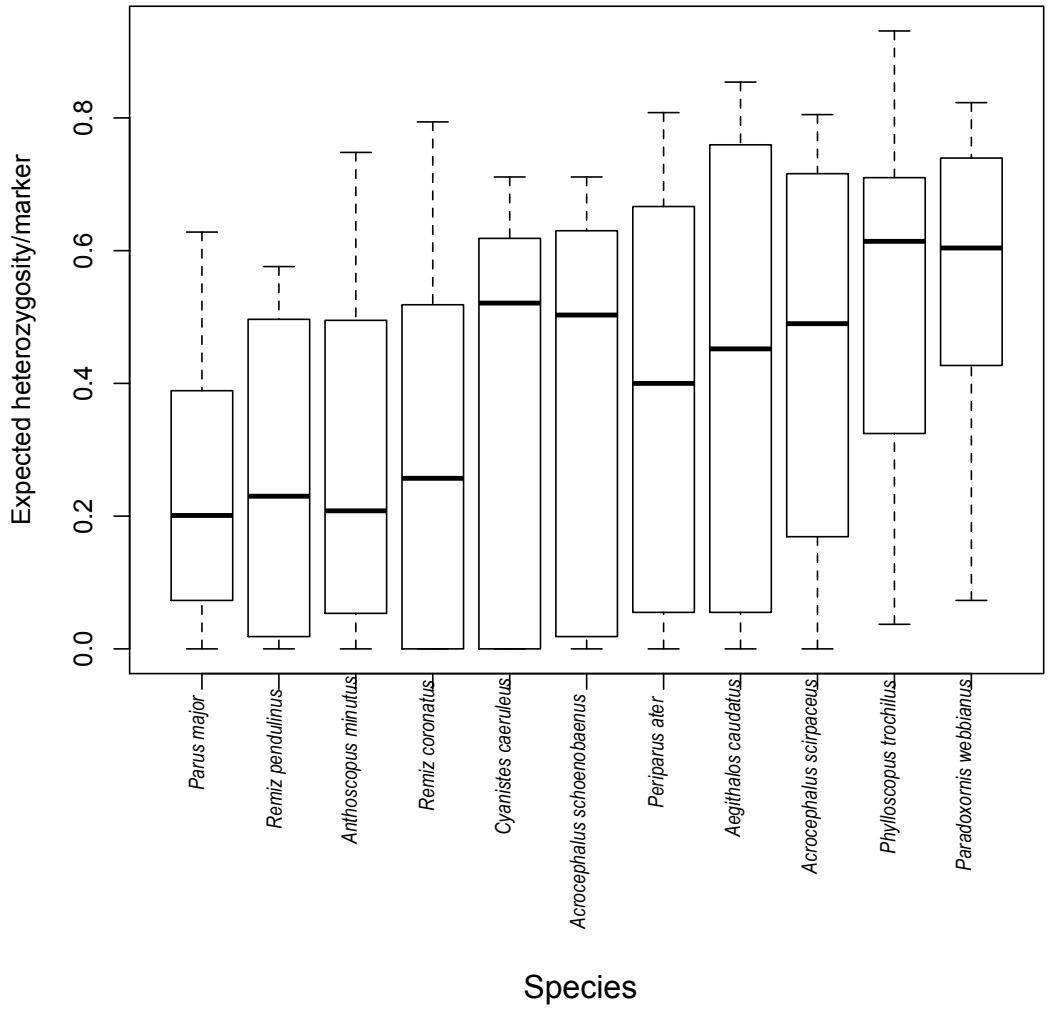

Figure 6.3. Variation in the genetic diversity as measured by expected heterozygosity (He) across 11 *Sylvioidea* species. There is significant variation in He between species at the 15 microsatellite loci (Kruskal-Wallis $\chi^2 = 20.83$, df = 10, p-value=0.0223). Species are ordered along the x-axis according to their mean He.

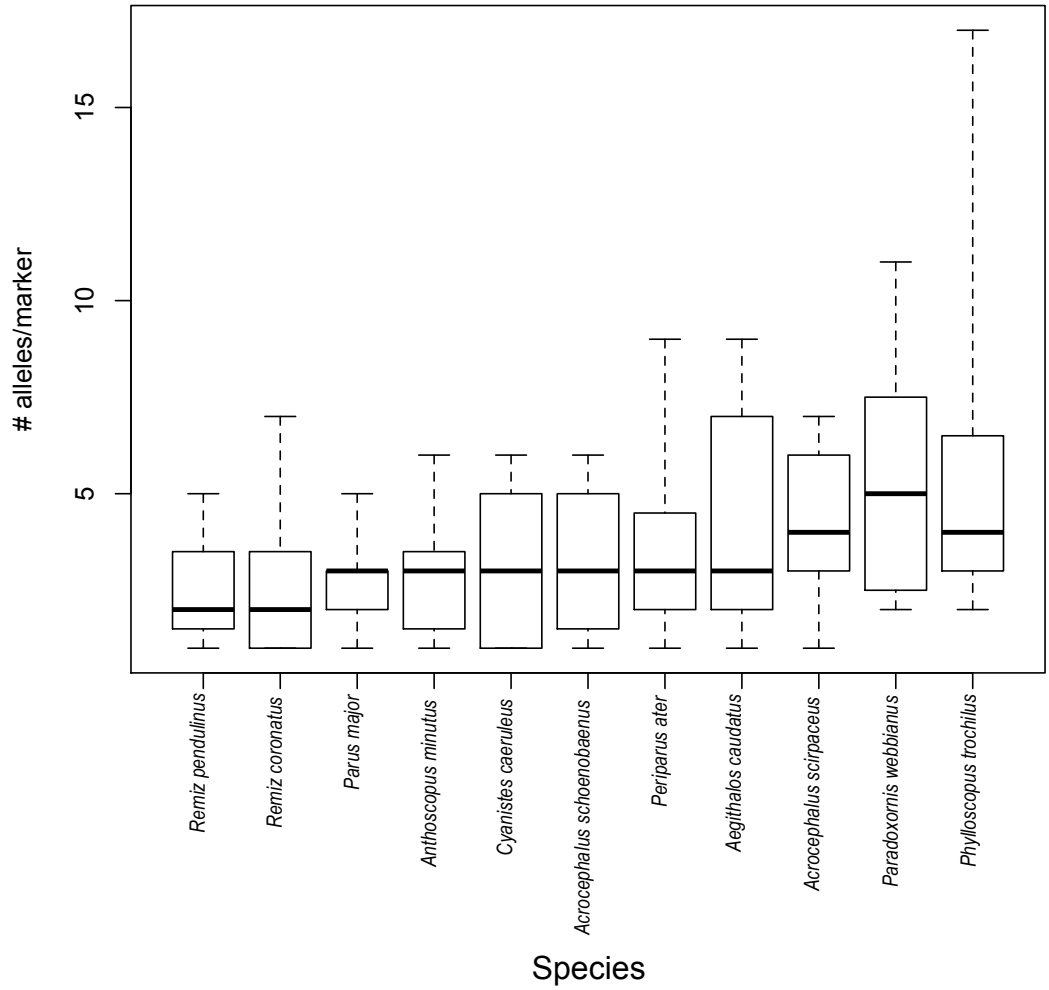

Figure 6.4. Variation in the genetic diversity as measured by allelic diversity across 11 *Sylvioidea* species. There is significant variation in allelic diversity between species at the 15 microsatellite loci (Kruskal-Wallis $\chi^2 = 23.33$, df = 10, p-value = 0.0096). Species are ordered along the x-axis according to their mean allelic diversity.

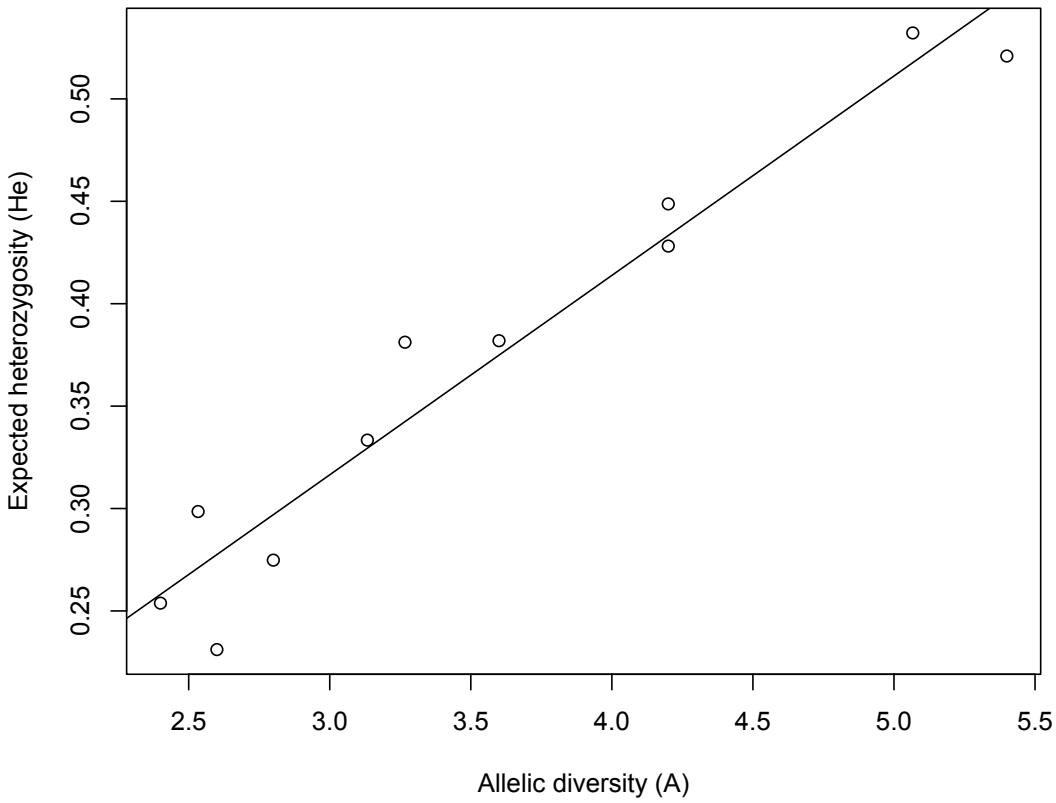

Figure 6.5. The relationship between the two genetic diversity measures used in this study. There is a significant positive correlation between the two measures, Allelic diversity and expected heterozygosity (Pearson's correlation, t = 11.99, df = 9, p-value < 0.0001).

PGLS models

Promiscuity did not explain variation in the genetic diversity observed in the *Sylvioidea* species when measured by either expected heterozygosity (Table 6.2, Figure 6.6.), or allelic diversity (Table 6.2, Figure 6.7.). Breeding area also does not explain any of the variation in genetic diversity to a significant degree (Table 6.2.).

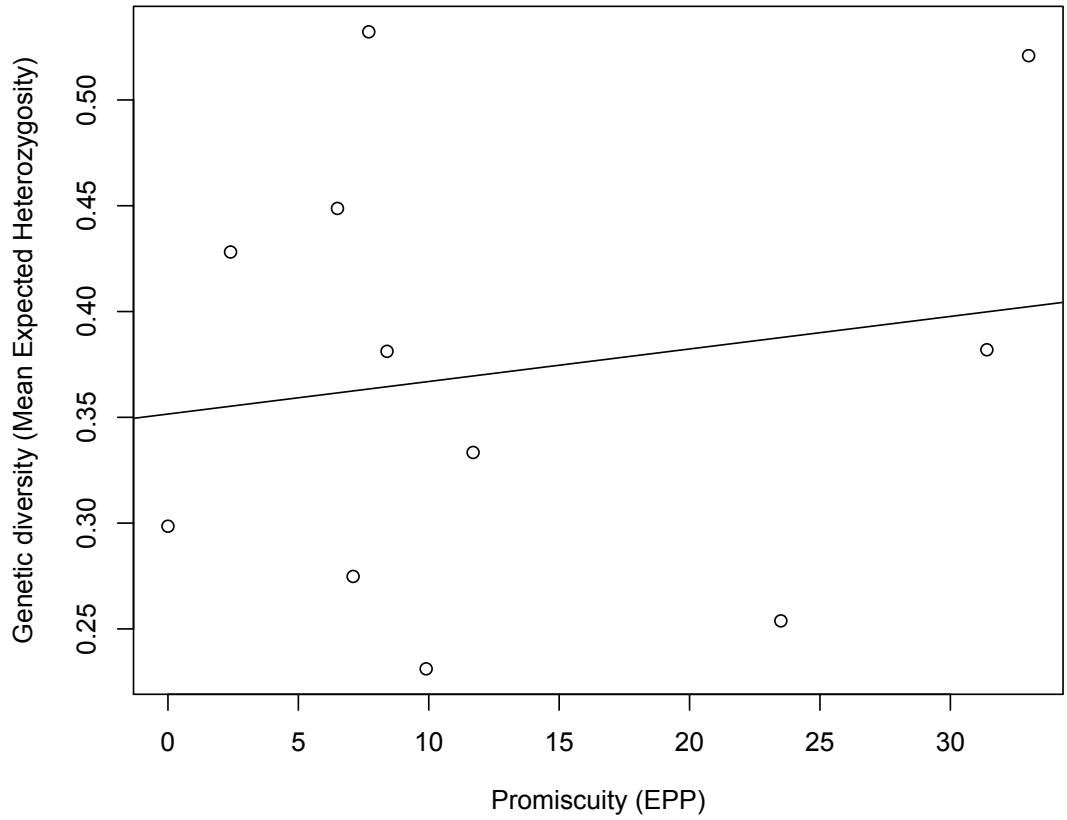

Figure 6.6. Relationship between Promiscuity (EPP) and genetic diversity as measured by mean expected heterozygosity (He) of 15 cross-species microsatellite loci. There is no significant relationship between promiscuity and genetic diversity in the 11 *Sylvioidea* species (see Table 6.2. for PGLS results).

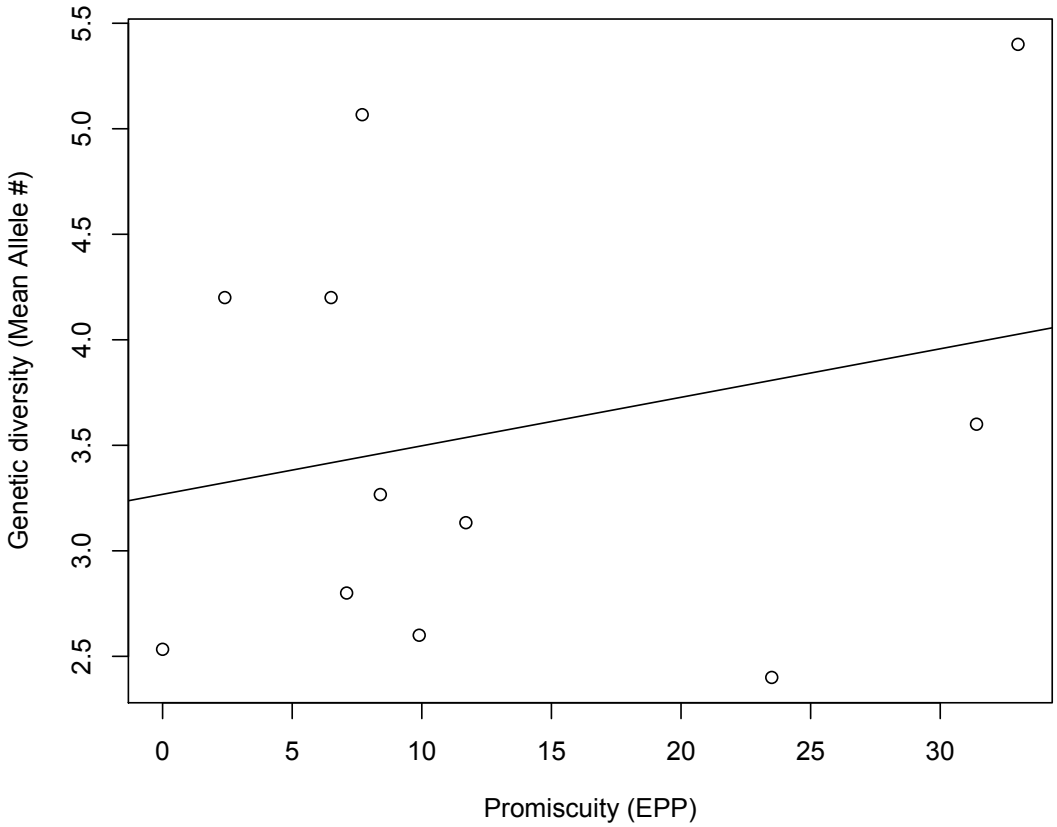

Figure 6.7. Relationship between promiscuity (EPP) and genetic diversity as measured by the mean allelic diversity of 15 cross-species microsatellite loci. There is no significant relationship between promiscuity and genetic diversity across the 11 *Sylvioidea* species (see Table 6.2. for PGLS results).

Table 6.2. Results of the Phylogenetic Generalized Least Square (PGLS) analyses, assessing the explanatory power of breeding range and promiscuity (EPP) on two measures of genetic diversity in 11 species of the *Sylvioidea* super-family. Neither breeding range or promiscuity sufficiently explain the variation in genetic diversity, which possesses a high phylogenetic signal as shown by the lambda values.

Trait	Slope (± SE)	t-value	p-value	Lambda (λ)	r^2
A: Genetic diversity (Mean Expected heterozygosity), separate regressions					
EPP	-0.00131226 (0.00090926)	-1.4432	0.1828	1 (0.029265, 1)	0.0977
Log(Breeding range (km^2))	-0.0171429 (0.0076349)	-2.2453	0.0514	1 (0.007549, 1)	0.2878
B: Genetic diversity (Mean Expected heterozygosity), multiple regression					
EPP	0.0028099 (0.0021216)	1.3244	0.2219		
Log(Breeding range (km^2))	-0.0418608 (0.0200522)	-2.0876	0.0703		
combined				1 (0.0023789, 1)	0.3429
C: Genetic diversity (Mean allele #), separate regressions					
EPP	-0.0013359 (0.0079684)	-0.1676	0.8706	1 (0.0074048, 1)	-0.1077
Log(Breeding range (km^2))	-0.046457 (0.073824)	-0.6293	0.5448	1 (0.0073174, 1)	-0.0643
D: Genetic diversity (Mean allele #), multiple regression					
EPP	0.024214 (0.020972)	1.1546	0.2816		
Log(Breeding range (km^2))	-0.259454 (0.198215)	-1.309	0.2269		
combined				1 (0.0032474, 1)	-0.0263

Discussion

This study uses a recently developed conserved set of microsatellite markers to compare genetic diversity across an avian super-family, *Sylvioidea*. The recent advent of substantial numbers of conserved microsatellite markers has made it possible to compare genetic diversity at homologous loci across a large range of species (Dawson *et al.*, 2010, 2013). This study used 15 avian microsatellites but this number could be substantially increased in further studies and the same approach could be applied to other passerine groups. A large difference between the diversity was found at the 15 microsatellites spread across the passerine genome within the *Sylvioidea* super-family (Figures 6.3 & 6.4). The main explanatory variable for the differing diversity was phylogenetic relatedness, with the penduline tits (*Remizidae*) exhibiting lower levels of diversity than any of the other families. Their sister family, the true tits (*Paridae*) also showed lowered levels of diversity compared to the other families in this study.

This dependence on evolutionary history could indicate that shared life-history traits or shared historic population declines could explain the patterns of diversity. Whilst controlling for this large effect of phylogeny the relationships of two other variables were tested, which were predicted to positively correlate with effective population size (Ne). It was predicted that both larger breeding range and higher promiscuity would positively affect Ne in small passerine birds with short longevity and high variance in reproductive success. As breeding range is positively correlated with population size (Figure 6.2), it was assumed that a larger breeding range equates to a larger effective population size in a species. However, neither of these variables showed significant relationships with genetic diversity (Table 6.2, Figures 6.6 & 6.7). This could suggest that increased promiscuity does not positively affect Ne within the *Sylvioidea*. Current theory predicts that female promiscuity could increase, decrease or have no effect on Ne depending on a suite of life history traits (Lotterhos, 2011). It was predicted that promiscuity in the *Sylvioidea* would increase Ne due to their relatively short generation times and high variance in

female reproductive success. However the number of extra pair mates is often low in many species perhaps lessening the potential effect of promiscuity on Ne (Lotterhos, 2011).

The finding that breeding range also showed no association with genetic diversity is also counter to our predictions. It could be that although breeding range is correlated with population size both of these may fluctuate rapidly through time and provide little indication of the long-term evolutionary history of the population size in each species. This is perhaps supported by the range expansions/contractions of some bird species that have been observed within the last few centuries, including the European penduline tit, a species included within this study (Valera *et al.*, 1993; McCarty, 2001).

A key direction for future research would be to include larger numbers of species. With the many potential influences on Ne a larger number of species would help disentangle the likely combination of variables that have created the current patterns of genetic diversity. The passerines are a specious clade and thus provide a very large resource with which to tackle empirical work testing the influences that shape population and species-specific genetic diversity. Increased data on the dispersal distances within each species could also be a factor in the transfer of genes throughout the global population that is perhaps having an effect on the genetic diversity estimates produced.

In this study only a single population has been used to assess genetic diversity for each species. A mainland population was selected for each species to avoid the potential effects of large sea expanses as barriers to gene flow. However the results could be affected by population level differences in genetic diversity within the studied species. However the strong phylogenetic signal observed in the genetic diversity measures supports the existence of species-specific differences in genetic diversity affected by shared evolutionary history. Obviously further work comparing genetic diversity between multiple populations within species would be valuable, and also allow more accurate species-specific estimates of genetic diversity, at present few species have

multiple populations with estimates of extra pair paternity (but see (Brommer *et al.*, 2010)).

Two previous studies looking at the relationship between promiscuity and genetic diversity have both found positive relationships between the two traits (Petrie *et al.*, 1998; Gohli *et al.*, 2013), however each has tested a different hypothesis. Our results are the first to show no relationship and confirm the need for further studies to test more fully the hypotheses previously put forward. A recent targeted approach has looked at the difference between diversity on the sex chromosomes compared to the autosomes and has shown large impacts of mating systems on sex chromosome diversity (Corl & Ellegren, 2012). Further work is perhaps best approached using multiple strategies to both improve our understanding of within species genetic diversity but also to use the newly acquired genetic resources to increase our understanding of cross-species differences, which have previously been ascertained by comparisons of limited gene regions (Väli *et al.*, 2008; Gohli *et al.*, 2013).

Theory looking at the relationship between Ne and mating systems is still in its infancy. The future ability to test current predictors of genetic diversity will provide both insights into evolutionary mechanisms and is likely to allow a better understanding of how species will be able to maintain genetic diversity in the face of rapid environmental change.

VII. Thesis Conclusions

The evolutionary implications of sexual conflict are being explored using a range of model systems (Rowe *et al.*, 1994; Rice *et al.*, 2006; Székely *et al.*, 2007). The relationships that link mating systems, parental care and sexual selection have and continue to provide a complex puzzle which evolutionary biologists are attempting to piece together (Trivers, 1972; Parker, 1979; Kokko *et al.*, 2006; Kokko & Jennions, 2008). Of key importance to this is the use of the comparative approach on species that exhibit disparities in sexual conflict (Thomas & Székely, 2005; Gonzalez-Voyer *et al.*, 2008; Fitzpatrick *et al.*, 2012). The penduline tits are unusual among passerine bird families in exhibiting species with an array of parental care strategies, from highly cooperative to those with high levels of sexual conflict (van Dijk, Pogány, *et al.*, 2010). A greater understanding of the evolution of the penduline tit family has emerged from the work in this thesis, revealing it to be a diverse group able to provide insights into the far-reaching impacts of sexual conflict.

Evolution of parental care

This work has used the disparities in parental care behaviour of the penduline tits to investigate the evolutionary implications of sexual conflict. As well as confirming the unusual diversity in parental care behaviour exhibited by the penduline tit family (Chapter 2 & 3) the research has revealed that uniparental care evolved once in this family, within the *Remiz* genus, with a single subspecies subsequently reverting to biparental care (Chapter 3). Uniparental care is unusual in birds with only 8% of species exhibiting this care system (Cockburn, 2006). Previous work has shown that the uniparental care system of the European penduline tit is dominated by sexual conflict (Szentirmai *et al.*, 2007; van Dijk, Szentirmai, Komdeur, *et al.*, 2007), however the initial reason for the evolution of uniparental care has been difficult to ascertain and due to the singular evolutionary event within the penduline tits is difficult to test. However the relationships between sexual dimorphism, male care and latitude (Chapter 3) suggest the possibility of an environmental aspect to parental care behaviour.

As latitude is a strong predictor of the length of foraging time during the breeding season it could be that the longer daylight hours at higher latitudes make it possible for one parent to abandon without loss of offspring fitness. In other studies clutch size has been found to increase with latitude suggesting that higher latitudes are conducive to greater levels of resource provisioning for broods (Moreau, 1944; Rose & Lyon, 2013). Considering that penduline tit brood sizes are much smaller than in comparably sized tit species at similar latitudes it seems plausible that a single parent could provision the brood without loss of offspring fitness. This combined with previous work concluding that the penduline tits initially evolved within Africa (Tietze & Borthakur, 2012), suggests that uniparental care could have evolved in response to the expansion of the penduline tits to higher latitudes, outside of Africa. Previous work in shore birds has also found reduced male care at higher latitudes revealing that the evolution of increased migration distances precede transitions to uniparental care (Garcia-Pena 2009). Studying these model systems in concert could potentially provide unifying explanations for the evolution of uniparental care in birds.

Parental care and paternity

In Trivers' extensive work on parental investment he makes clear that parental investment is only likely to evolve if it can be directed at genetic offspring, if parentage is uncertain then parental care would be selected against (Trivers, 1972). So if males are more uncertain of parentage than females, parental investment by females is likely to be greater than that of males. We thus tested the association between extra pair paternity and the provision of male care. A previous genetic parentage study in the European penduline tit found high levels of EPP (24% of offspring) which could explain the higher level of abandonment exhibited by males then females in this species. Although a previous study found no relationship between an individual male's decision to care and his share of paternity, the relationship is likely to act on an evolutionary timescale if species have limited ability to determine parentage (van Dijk, Mészáros, *et al.*, 2010). In the two biparental species studied in this

thesis, much lower levels of EPP were observed, 7.1% in the Cape penduline tit and 0% in the White-crowned penduline tit. This is consistent with a relationship between paternal care and a male's likelihood of parentage acting on an evolutionary time-scale.

However this approach is unable to reveal causation; is loss of paternal care a consequence of increased female promiscuity or did female promiscuity increase once paternal care had been lost? Key to this question is the understanding of why females mate multiply. The fact that we have observed, as have countless other studies, that female promiscuity is present even in biparental species, leads to the supposition that females benefit from it in some way, either directly or perhaps indirectly from genetic benefits. However the impact that it has on other aspects of parental care and mate choice are far-reaching.

Sexual dimorphism and conflict over care

Our findings that sexual dimorphism in plumage traits evolve more rapidly in uniparental species than in species where both parents invest in care (Chapter 3) provides support for increased sexual selection via female choice. Rapid evolution is predicted for traits undergoing intense sexual selection and our results reveal this rapid evolution of sexual dimorphism is driven by changes in male plumage. The hypothesis that the lack of paternal care creates a sex bias in the operational sex ratio, with only the most attractive males able to secure additional mates is consistent with these results. This is also supported by recent research in the European penduline tit which shows that males with larger mask's (i.e. more attractive males) are more likely to abandon their current brood (van Dijk, Pogány, *et al.*, 2010). Thus deserting males likely have higher potential payoffs than caring males. This pattern of increased rates of evolution in sexually dimorphic traits has also been explored in a study on shorebirds revealing that in species with less dependent young evolution of sexual dimorphism is more rapid (Thomas *et al.*, 2006). Thus reduced parental care by one sex appears to increase disparities in sexual selection.

This rapid evolution of sexual dimorphism in traits involved in pre-copulatory mate choice could have implications for pre-zygotic isolation between populations. Theory suggests that in species in which mate-choice by females is the deciding factor in the mating system, gene flow will be restricted, leading to increased divergence of populations and eventually higher levels of speciation (Parker & Partridge, 1998). Another trait likely to affect speciation is post-copulatory isolation between populations. We thus investigated sperm traits within the penduline tits to study the effects of increased sexual selection in the uniparental species on sperm morphology.

Sperm competition and sexual conflict

We discover that in the penduline tit and closely related species within male sperm length is more uniform in species under higher levels of sperm competition (Chapter 4). This suggests that sperm length is under direct selection for an optimal size during competition with other males. This has been found in a couple of other studies in passerines however currently the reasons for such intense selection on sperm length is unknown (Calhim *et al.*, 2007; Kleven *et al.*, 2008). As sperm storage occurs in birds for long periods before fertilisation, in female sperm storage tubules, perhaps optimum sperm length is required to compete for space and increase longevity. Previous work has shown that sperm length is closely correlated with sperm storage tubule length in passerines (Briskie & Montgomerie, 1992), thus this could be the constraining factor in fertilisation success. Having revealed that variance in sperm length is likely a post-copulatory sexually selected trait we explore the relationship between this trait and a pre-copulatory sexually selected plumage trait in the European penduline tit.

The size of the melanin based facial mask is negatively related to variance in sperm length (Chapter 5) suggesting that the males that are most attractive to females are also the most likely to successfully compete for fertilisation with rival sperm. Thus we find no evidence that there is a trade-off between the two

traits within males but our results instead suggest that both pre and post copulatory traits could be indicative of the same benefits to choosy females. Perhaps they are both condition dependent traits or linked to genetic benefits. A recent study has suggested that individuals showing signs of oxidative stress are revealing a lack of efficiency in cellular mechanisms and thus avoided as potential mates (Dowling & Simmons, 2009). Both sperm development and melanin based plumage have both previously been linked to levels of oxidative stress. Thus these hypotheses would benefit from increased studies that explore the true extent of oxidative stress on sexually selected traits.

We also compared the pre- and post-copulatory traits between two populations of the European penduline tit revealing differences between them suggesting that not only plumage traits but also sperm morphology could be evolving rapidly in this species. The Spanish population showed more attractive plumage but higher variance in sperm size, however we found no within male trade-off between the two traits with more attractive males also exhibiting more competitive sperm traits. This suggests the differences could be caused by the differing importance of pre and post-copulatory sexual selection in the two populations. In the Spanish population our result suggests it is more important to attract a mate than compete for fertilisation success than in the Romanian population, where sperm competition is likely to be higher. Our ability to test these predictions in the future would ascertain if this is indeed the case, our results would predict that Spanish females are more choosy but more faithful than the females in Romania who we would predict have higher levels of promiscuity.

Mating systems and genetic diversity

Previous work showing a positive relationship between allozyme diversity and EPP alluding to the strength of mating systems to shape diversity not just of sexually selected traits but of the rest of the genome. A more recent study showed the effect that the mating system has on sex chromosome diversity in shorebirds. These relationships rest on changes in the effective population size

(Ne) created by mate choice. In polygynous systems when few males monopolise the breeding success it is unsurprising that male sex chromosome diversity is reduced. However multiple mating by females could potentially increase or decrease the Ne depending on the mate choice of individuals and as theory has recently shown, a range of life history traits, including longevity, mating rate and variance in female breeding success (Lotterhos, 2011). However unlike previous studies we failed to find a relationship between genetic diversity and EPP. However we are one of the first studies to compare cross species genetic diversity in this way and discovered that across a group of passerine birds with similar life history traits the main predictor of genetic diversity was shared evolutionary history, with the *Paridae* and *Remizidae* exhibiting reduced genetic diversity compared to the warblers and parrotbill in the study. Perhaps the use of current population size is a poor predictor of past population sizes especially with the rapid anthropomorphic changes that have occurred in land-use. Perhaps a within species approach would be more informative, in a species which has populations differing substantially in EPP or a study with a greater number of species pairs could test this question more robustly. Additionally, it could be that in passerines multiple mating does not drastically change the Ne, greater knowledge of variance in female breeding success would help us predict the expected changes in Ne.

VIII. Future directions

As seems typical in research, numerous questions have arisen during this work, far exceeding the questions I initially attempted to explore. Below I outline some of the most intriguing questions which future research could investigate. In addition to extending the scope of the current projects they also encompass ideas in three main areas, 1) exploring the genetic basis of parental care and mating behaviour, 2) investigating the divergence and genetic isolation of populations, and 3) exploring the influence that parasites have on mating system evolution.

One of the most pertinent questions in evolutionary research is the relative importance of genetic and environmental factors. The relative contribution of each of these factors is key to understanding how and why traits vary between species and populations (Champagne & Curley, 2012; Head *et al.*, 2012). Much work has gone into disentangling genetic and environmental factors of a range of traits in multiple species, however the historical focus has been on physical rather than behavioural aspects of organisms (Robinson, 2004). The penduline tits provide us with a unique opportunity to locate the genetic basis of very disparate parental care and mating strategies. By studying parental care and mating behaviours using a large pedigree within a resident population we should be able to ascertain the consistency of individual behaviour across breeding seasons and also gain inheritance measures for a plethora of traits. This approach would eventually allow linkage mapping using species-specific genetic markers to locate the genomic position of the genes that influence parental care and mating behaviours. The genes influencing parental care behaviours in birds remain obscure and whether these will be the same genes across different species and taxa remains to be seen. Discovering the genes involved in these behaviours would also allow us to investigate the interplay between male and female genomes at these regions of sexual conflict. In addition the production of a linkage map would allow the genetic exploration of genes underlying a wide range of traits and provide the basis for comparative genomics with other passerines. Once candidate genes are located they can be

sequenced and compared across the penduline tit species to ascertain their evolutionary history, this would be of increased interest in the White-crowned penduline tit, which we have shown exhibits different care strategies between populations, discovering whether this is due to genetic or environmental factors would be particularly interesting.

The White-crowned penduline tit in which we have documented biparental care in one subspecies and uniparental care in the other also offers the opportunity to experimentally test alternative hypotheses for the control of parental care and mating behaviours as well as allowing us to answer an array of other questions. Why has one population reverted to biparental care? Does the entire subspecies exhibit biparental care? Do the subspecies exhibit mutations in the candidate genes controlling parental care behaviour? Would increased food availability lead to abandonment by one of the parents? Are their clutch size differences, are parents more likely to abandon small clutches? How does provisioning rate differ between the two populations? How does uniparental care affect chick growth? Is promiscuity greater in the uniparental subspecies? The different breeding strategies exhibited within one species could also allow us to disentangle whether increased promiscuity is a result or a cause of uniparental care? If the differences between the subspecies are genetic, are these differences leading to increased or decreased gene-flow between the populations. It would be particularly interesting to study populations that are at the margin between these subspecies.

The study of gene-flow could also expand to other penduline tit species providing cross-species comparisons that would enlighten the process of speciation in this group. Do polygamous mating systems increase gene-flow between populations, limiting divergence and speciation, or does the increased sexual selection lead to more selective mate choice and highly assortative mating, further separating populations? Our understanding of speciation could also be advanced by studying hybridisation where species ranges overlap. Hybridisation is thought to occur in the Middle East between the European penduline tit and Black-headed penduline tit however our results suggest that

these two are in fact the same species. A more detailed investigation into the degree of differentiation between the Black-headed and European populations could shed light on the process of speciation, what are the main genetic differences between them, is there on going gene-flow or have they recently become separate species. Our work has shown that there are differences in plumage traits and sperm morphology between two populations of the European Penduline tit suggesting that divergence at these traits occurs rapidly, but which of these if either is more important to the process of speciation?

Increased collection of sperm samples and plumage measurements from a greater number of populations both within and across species would allow a more robust comparison of the trade-offs between pre and post-copulatory traits and the drivers of their evolution. Currently I have only compared two populations of the European penduline tit but a thorough analysis of the reasons underlying the patterns I have observed would require multiple populations and knowledge of promiscuity (via measurement of EPP) and mate choice for each of them. This could confirm whether sperm competition and mate choice are responsible for the differences in these traits. Experiments could be used to determine the degree of female plumage preference in each population and the fertilising ability of sperm from different populations would ideally be compared. This would allow us to determine whether these traits are currently reinforcing isolation between populations or increasing gene-flow? In line with this work would be the investigation of the coevolution between female sperm storage tubules and sperm length, is it cryptic female preference or sperm competition that has driven the differences in sperm morphology? This question could also be tackled by gaining more detailed information on other penduline tit species, especially the *Anthoscopus* genus, which are likely dominated by monogamous mating systems. If sperm competition has driven the differences in sperm morphology then you would predict that species without sperm competition would have high variance in sperm morphology but similar average sperm lengths to other monogamous species.

Our work looking at genetic diversity has revealed that the least genetically diverse species is the European penduline tit, this is the uniparental species with high conflict over care and high rates of EPP. It would be interesting to ascertain the reasons for the differing levels of genetic diversity between species. Our results suggest it is not caused by the differing levels of promiscuity but we have not investigated alternative explanations, perhaps past population bottlenecks caused by environmental changes explain the differences. Comparing past glaciation events and the sizes of likely refugia could be investigated in the Palearctic species to test whether this relates to current genetic diversity levels. Recent population bottlenecks could also be inferred from large sequencing efforts in a range of species. However an alternative explanation that could explain genetic diversity differences could be differences in pathogen mediated processes. Diversity at key immune regions is thought to provide immunity to a wider array of pathogens through rare allele or heterozygosity advantage (Doherty & Zinkernagel, 1975; Slade & McCallum, 1992). Diversity at immune genes has even been found to explain female mate choice (Brouwer *et al.*, 2010).

There are many other questions that could also provide interesting investigation and although our phylogeny is the most extensive for the penduline tits yet produced there are still missing subspecies. More insights could also be made by increasing the scope of the current sexual dimorphism study by analysing evolutionary rate throughout bird species and not just within the penduline tits. The operational sex ratio is notoriously difficult to estimate but with the rapid expansion of genetic sequencing technologies it could be possible to determine the historic OSR for a wide number of species by comparing the genetic diversity of male and female genetically inherited components, the Z and W chromosomes respectively. Perhaps these could be compared with current ASR estimates to gain comparative sexual selection measures across species (Liker *et al.*, 2013, 2014). The OSR and the ASR have often been treated as one and the same but should be seen as complementary as one can be biased while the other is equal. The ability to determine whether the parental care and mating systems in the penduline tits have been affected by

differences in either the ASR or OSR could provide future insights into mating system and parental care evolution. Hopefully the penduline tits will continue to provide interesting insights into the evolution of sexual conflict and parental care behaviours in future research endeavours.

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X. Appendices

Appendix 2.1. Summary of sample sizes for the parental care observations and parentage analyses. The Cape penduline tit (*Anthoscopus minutus*) was studied over 6 breeding seasons and the White-crowned penduline tit (*Remiz coronatus*) in just one. The nest observations were less detailed in the pilot years of 2002 & 2003 and thus parental care was not observed all the way through to fledging.

SPECIES	YEAR	NESTS			INDIVIDUALS						
		Total monitored	Parental care observations	Used in parentage analysis	Sample type	Adults			Juveniles	Nestlings	Total
						Male	Female	Unsexed			
CAPE PENDULINE TIT *Anthoscopus minutus*	2002	-	-	0	Bloods	2	1	1	8	0	12
					Genotyped	3	1	0	8	0	12
					Parentage	-	-	-	-	-	-
	2003	-	-	12	Bloods	5	7	0	0	39	51
					Genotyped	3	8	0	0	37	48
					Parentage	16	18	0	0	37	71
	2004	8	5	3	Bloods	1	1	5	0	10	17
					Genotyped	4	3	0	0	10	17
					Parentage	18	18	0	0	10	46
	2005	18	8	11	Bloods	3	3	2	4	45	57
					Genotyped	3	3	0	4	41	51
					Parentage	18	18	0	0	41	77
	2006	12	5	6	Bloods	1	4	0	0	21	26
					Genotyped	1	3	0	0	21	25
					Parentage	21	18	0	0	21	60
	2007	11	3	3	Bloods	1	1	0	1	28	31
					Genotyped	2	0	0	1	11	14
					Parentage	22	20	0	0	11	53
	All	49	21	35	Bloods	13	17	8	13	143	194
					Genotyped	16	18	0	13	120	167
WHITE-CROWNED PENDULINE TIT *Remiz coronatus*	2008	25	18	6	Bloods	19	13	0	0	31	63
					Genotyped	19	13	0	0	31	63
					Parentage	19	13	0	0	31	63

Appendix 3.1. Mean measurements and sexual dimorphism index (SDI) for the three body-size traits in the subspecies of penduline tit.

Genus	Species	Subspecies	Wing length (mm)					Beak height (mm)					Beak length (mm)				
			Mean male	n male	Mean female	n female	SDI	Mean male	n male	Mean female	n female	SDI	Mean male	n male	Mean female	n female	SDI
Remiz	*pendulinus*	*menzbieri*	53.66667	3	52.66667	3	0.018809331	4.15	3	4.206667	3	0.013562313	7.996667	3	8.68	2	0.081996699
Remiz	*pendulinus*	*pendulinus*	56.11257	382	55.36216	185	0.013463524	4.274737	19	4.303	10	0.006589874	8.18577	26	8.34125	8	0.018815804
Remiz	*pendulinus*	*caspius*	56.3125	16	54.92857	7	0.024882921	4.327143	14	4.256667	6	0.016421049	8.116154	13	8.102857	7	0.001639681
Remiz	*macronyx*	*macronyx*	55	6	55	1	0	4.656667	6	4.93	1	0.057039032	8.5325	4	NA	0	NA
Remiz	*macronyx*	*ssaposhnikowi*	61	1	58	1	0.050430854	NA	0	NA	0	NA	NA	0	NA	0	NA
Remiz	*coronatus*	*coronatus*	52.25714	35	52.21429	21	0.00082032	3.73	12	3.735714	7	0.001530731	7.108333	6	7.06	3	0.006822706
Remiz	*coronatus*	*stolickzkae*	51.91667	22	52.31111	15	0.007568844	3.885	2	3.58	3	0.081760183	6.935	2	7.06	1	0.017863998
Remiz	*consobrinus*	*consobrinus*	54.42857	14	53.8	10	0.011615733	4.372	5	4.675	4	0.067008592	8.1775	4	8.0975	4	0.009831108
Anthoscopus	*parvulus*	*parvulus*	50	3	NA	0	NA	3.573333	3	NA	0	NA	7.195	2	NA	0	NA
Anthoscopus	*parvulus*	*senegalensis*	NA	0	48.375	4	NA	3.4	1	3.69	3	0.081851026	6.64	1	7.116667	3	0.069327534
Anthoscopus	*minutus*	*gigi*	47.875	8	46.94444	9	0.019628673	NA	0	NA	0	NA	7.075	4	6.95	2	0.017825784
Anthoscopus	*minutus*	*damarensis*	50.25	10	48.625	12	0.032872745	4.068889	9	4.057273	11	0.002858916	7.24	9	7.071818	11	0.023503617
Anthoscopus	*flavifrons*	*flavifrons*	56.125	4	54.75	2	0.024803977	4.355	4	4.46	2	0.023824156	8.513333	3	8.41	1	0.012212049
Anthoscopus	*punctifrons*	*punctifrons*	51.025	20	50	16	0.020292703	4.052353	17	4.074375	16	0.005419661	7.328	15	7.530667	15	0.02728099
Anthoscopus	*musculus*	*musculus*	48.77778	9	47.5	11	0.02654517	3.78875	8	3.717273	11	0.019045814	6.562222	9	6.475	10	0.013380658
Anthoscopus	*caroli*	*taruensis*	49.33333	3	49.25	2	0.00169055	3.883333	3	3.72	2	0.042970137	6.72	3	7.04	2	0.046520016
Anthoscopus	*caroli*	*sylviella*	53	6	NA	0	NA	3.888333	6	3.79	1	0.025614512	7.131667	6	7.5	1	0.050358013
Anthoscopus	*caroli*	*ansorgei*	52	1	53.5	2	0.028437935	4.05	1	4.075	2	0.006153866	7.73	1	7.51	2	0.028873397
Anthoscopus	*caroli*	*robertsi*	51.76471	17	51.54167	12	0.004318036	3.786	15	3.924545	11	0.035940368	7.05353	17	7.12	11	0.009379525
Anthoscopus	*caroli*	*rhodesiae*	51.875	4	53.75	4	0.035506688	3.8525	4	3.8325	4	0.005204956	7.2225	4	7.39	2	0.022926582
Auriparus	*flaviceps*	-	52.47222	18	50.11111	9	0.046041147	4.292632	19	4.245556	9	0.011027276	7.220556	18	7.302222	9	0.011246727

Appendix 3.2. Mean measurements and sexual dimorphism index (SDI) for the three plumage traits in the subspecies of penduline tit.

Genus	Species	Subspecies	Mask size (cm^2)					Mask contrast					Mantle saturation				
			Mean male	n male	Mean female	n female	SDI	Mean male	n male	Mean female	n female	SDI	Mean male	n male	Mean female	n female	SDI
Remiz	*pendulinus*	*menzbieri*	0.9573537	3	0.5246004	3	0.601536086	40.05	3	24.4	2	0.495545541	64.37496	3	55.32869	3	0.1514332
Remiz	*pendulinus*	*pendulinus*	1.278909	173	0.9574296	38	0.289510456	53.91088	38	43.64583	16	0.211224568	77.05911	59	66.99349	18	0.1399773
Remiz	*pendulinus*	*caspius*	1.06505	11	0.8182178	7	0.263648465	18.153	5	36.28	5	0.692430789	61.54465	12	59.49621	7	0.0338503
Remiz	*macronyx*	*macronyx*	1.711713	6	1.775157	1	0.036394246	13.13833	6	16.7	1	0.239874807	62.26592	6	61.92165	1	0.0055444
Remiz	*macronyx*	*ssaposhnikowi*	2.518392	1	1.430459	1	0.565625231	9.663333	1	44.76667	1	1.53312527	76.7581	1	68.4825	1	0.11408
Remiz	*coronatus*	*coronatus*	1.19024	34	0.9660906	21	0.208652628	60.8892	27	46.00579	19	0.280288561	67.26314	32	52.16397	21	0.2542203
Remiz	*coronatus*	*stolickzkae*	1.054738	22	0.6549108	15	0.476548631	54.63258	22	42.06786	14	0.261346379	66.02551	20	52.02496	14	0.2383176
Remiz	*consobrinus*	*consobrinus*	0.6229274	13	0.6569191	10	0.053130896	43.54403	12	20.2875	10	0.763767677	66.99556	12	54.12546	8	0.2133217
Anthoscopus	*parvulus*	*parvulus*	0.1120265	3	NA	0	NA	23.83333	3	NA	0	NA	57.83608	3	NA	0	NA
Anthoscopus	*parvulus*	*senegalensis*	0.1212773	1	0.102448	3	0.168724306	27.35	1	24.16667	3	0.123742118	51.56882	1	62.87969	4	0.198306
Anthoscopus	*minutus*	*gigi*	0.1394123	7	0.1333901	9	0.044157812	20.99214	7	19.02444	9	0.098423614	39.54701	7	41.71842	9	0.0534527
Anthoscopus	*minutus*	*damarensis*	0.1122037	8	0.112594	10	0.003472459	14.295	5	13.65633	5	0.045706675	30.58575	6	29.18962	6	0.046721
Anthoscopus	*flavifrons*	*flavifrons*	0.1249736	4	0.1372694	2	0.093842903	14.77125	4	11.645	2	0.237805821	54.03496	4	50.09804	2	0.0756494
Anthoscopus	*punctifrons*	*punctifrons*	0.1131577	12	0.1143782	11	0.01072808	19.56	5	13.56583	6	0.365932533	42.66857	11	45.45906	9	0.0633496
Anthoscopus	*musculus*	*musculus*	0.1115297	9	0.1238493	9	0.104774581	7.397	5	7.37375	4	0.003148116	25.39554	9	26.86017	10	0.056071
Anthoscopus	*caroli*	*taruensis*	0.071044	2	0.0734406	2	0.033176264	6.3925	2	11.1425	2	0.555641198	23.53033	3	23.20995	2	0.0137092
Anthoscopus	*caroli*	*sylviella*	0.1156542	4	0.1036292	1	0.109785561	13.83625	4	12.475	1	0.103565318	17.9665	6	16.29695	1	0.097531
Anthoscopus	*caroli*	*ansorgei*	0.0986509	1	0.1343497	2	0.308858746	34.15	1	25.1	2	0.30789474	56.84705	1	61.3285	2	0.0758803
Anthoscopus	*caroli*	*robertsi*	0.090228	7	0.0781427	6	0.143803158	9.80625	4	10.605	4	0.078305651	19.74263	13	23.28036	10	0.16483
Anthoscopus	*caroli*	*rhodesiae*	0.0761468	4	0.0747458	7	0.018571096	17.59625	4	13.53625	4	0.262314539	36.27079	4	41.72062	4	0.1399828
Auriparus	*flaviceps*	-	0.1409754	12	0.1402583	5	0.005099684	22.758	5	23.63	5	0.037600359	25.03162	10	26.6971	5	0.064415

Appendix 4.1. South African fieldwork report

The evolution of mating systems in penduline tits

FIELDWORK REPORT
EXPEDITION SOUTH AFRICA 2013

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Photographs by A.D.Ball

Objectives

1) To collect blood samples from a second population of Cape penduline tits (*Anthoscopus minutus*) ~ 500km distant from the current sampled population at Koeberg Nature Reserve (n=168) in order to investigate rate of gene-flow between them. This can then be compared to the rate of gene flow estimated between populations of the European penduline tit (*Remiz pendulinus*) and the White-crowned penduline tit (*Remiz coronatus*).
2) To collect faecal samples from multiple male Cape penduline tits (CPTs) from the same population. In order to obtain sperm samples which will be used in a comparative morphological analysis between a number of passerine species with differing extra pair paternity (EPP) rates.

Hypotheses

To explore the relationship between mating systems and the genetic structure of a species and the morphology of pre and post copulatory sexually selected traits we plan to test the following predictions.

1) Geneflow will be reduced in monogamous compared to polygamous species
2) Variance in sperm length will be higher in monogamous compared to polygamous species
3) Sperm length will be greater in polygamous compared to monogamous species

Methods

I visited 4 fieldsites including the previously sampled site at Koeberg Nature reserve and in addition three potential sites for the intended sample collection (Figure 1). These incorporated four different vegetation biomes including Strandveld, Nama Karoo, Renosterveld and Succulent Karoo (Figure 2).

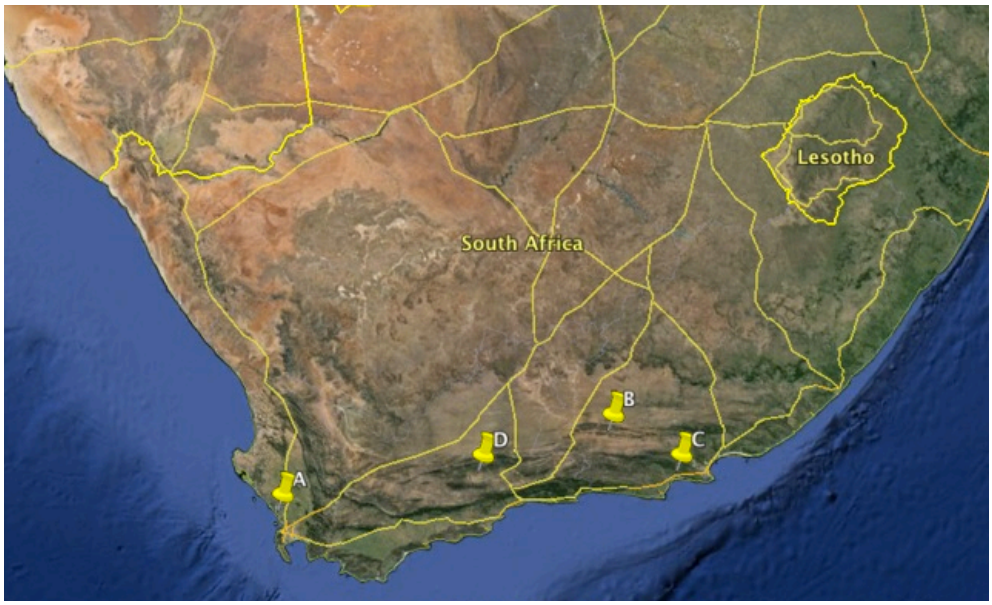

Figure 1. Map of the 4 field-site locations visited in the South African cape.

Figure 2. Photos of the landscape/vegetation at each site

A) Koeberg Nature Reserve – mostly Strandveld, Duneveld and Lowland Fynbos

B) near Steytlerville – Nama Karoo with thorntrees

C) near Hankey – Mostly Thickets and Montane fynbos- Renosterveld

D) near Oudtshoorn – Succulent Karoo - Apronveld

At all sites Cape penduline tits were located by listening for their distinctive "follow my leader" call and watching for their short, weak, bouncy flight. Nests were then located by following observed pairs or listening for a sudden repetitive call that is made by a bird leaving the nest in what seems to be an attempt to locate their partner. A pair's territory can be determined by multiple visits to the area of the first sighting. Birds are nearly always seen in pairs or groups. The few that were observed alone had nests in close vicinity. Nests are usually hidden in the centre of thorn bushes and look very much like the European penduline tit nest but are smaller, less pendulous and lack the entrance spout, instead having a false concave entrance with a flap above concealing the 'velcroed'-shut true entrance (Figure 3).

Birds are captured by setting up a net outside their nest (once its in completed Stage F) an hour before sunrise. Birds become trapped in the net as they leave the nest for the first time that day and can immediately be placed into a cotton drawstring bag. Measurements of the wing, tarsus and beak are taken as well as the weight of each bird. A faecal sample is taken from any male birds by leaving them in a cotton bag with a plastic tray in the bottom (covered in 1cm x 1cm wire mesh). They are left for three minutes and then any watery liquid surrounding faecal matter in the plastic tray is transferred into an eppendorf containing 5% formalin (200ul). A ~30ul blood sample is taken from the brachial wing vein of every bird and stored in 100% ethanol (1ml). Finally photos are taken of both sides of the head and of the back of every bird. All photos are taken with Kodak grey card and a metal ruler in the background. After all measurements and samples are taken the birds are released in close proximity to their nest.

Figure 3. Cape penduline tit nest highlighting the location of the concealed entrance which the birds open and close with their feet and beak.

Results

Observations and Captures

Birds were observed at 2 of the 4 sites (Table 1). These were both in the Karoo habitats.

Site A - The weather at Koeberg was very windy making it difficult to hear any birds, the main reason for visiting this site was to familiarize myself with the habitat of the previous sampling site(van Dijk, Pogány, *et al.*, 2010) .

Site B – This was an ideal environment for CPTs, a valley basin in the Nama karoo with a substantial number of acacia thorn-trees and very short shrubs perhaps due to the Angora goat grazing. A large number of birds were sighted and followed however it was very dry and the usual rains were 2 months late. They were experiencing typical August weather in October, therefore no breeding birds were found. Birds were often sighted in pairs but some were in groups of 4, more typical of winter flocking behaviour. This would be a great sight for catching breeding birds ~ 3 weeks after the heavy spring rain which usually occurs at the end of August but this year was on the 25th October. Nests have been observed previously by the landowners.

Site C – It is very doubtful that Cape penduline tits exist at site C as it is predominantly woodland and thickets with only some fynbos. Weather was ideal for locating birds and still none were observed. Yearly Rainfall is also higher in this area and CPTs prefer dryer semi-arid regions.

Site D – A 2nd good site for CPTs, it is in the north of the wide valley basin south of the Swartberg mountains that makes up the majority of the Little Karoo. A succulent Karoo landscape dominated by small shrubs many of them succulent plants and the occasional small-trees including acacia. A lot of the area is used for Ostrich farming. A few heavy rain showers had occurred from the start of October and the torrential rains arrived on the 25th October. Five nests were found and most birds were in early stages of breeding with only 1 nest containing chicks. Three of the nests appeared quite old (maybe reused from last year) and the other 2 were under construction. Breeding birds have previously been observed in the area by the owners (2 years ago). Six birds were caught at this field-site (Table 2).

Two potential sites for future research have been located (B&D) and we managed to capture 6 breeding adults at site D. Blood samples were obtained from all six individuals and sperm samples were obtained from 2 of the males.

Table 1. CPT observations and captures at each field-site.

Site	GPS	Duration at site (days)	# birds observed	# nests found	# birds caught
(A)Koeberg NR	33°38'S 18°26'E	2	0	0	0
(B)Nr Steytlerville	33°12'S 24°02'E	7	11	0	0
(C)Nr Hankey	33°52'S 25°05'E	2	0	0	0
(D)Nr Oudtshoorn	33°31'S 21°50'E	7	13	5	6

Table 2. Summary of nests found at field-site D.

Nest	Stage	# adults observed	# males trapped	# females trapped	Observations
1	D	2	0	0	Nest building
2	F	2	1	1	Incubating
3	F	2	1	1	
4	F	3	2	0	All 3 adults feeding chicks
5	B	1	0	0	Nest building

Morphometrics

Weight

Females (weight=7.0g±0.1, n=2) were ~1g heavier than males (6.1g±0.15, n=4). This pattern was also found in the Koeberg population but was less dramatic, females weighed 6.8g±0.1 (n=11) compared to males at 6.5g±0.09(n=11).

Wing length

Males had longer wings than females, 48mm±0.9(n=4) compared to 45.75mm±0.8(n=2) respectively. Again the same trend was found in Koeberg but was less dramatic, with males at 48.2mm±0.3(n=11) compared to 47.3mm±0.4(n=11) for females.

Tarsus length

There was no significant difference in tarsus length of males at 16.3mm±0.4(n=4) compared to females at 15.9mm±0.2(n=2). In the Koeberg population there was also no significant difference in tarsus length between males, 16.5mm±0.2(n=11) and females, 16.7mm±0.15(n=11).

Beak length

There was no significant difference in beak length between males, 7.1mm±0.22(n=4) and females, 7mm±0.05(n=2). The beaks of the Koeberg samples were not measured.

There are no significant differences in the overall mean of the 3 body size measurements between the 2 populations.

Behaviour

It appears very similar to that described for the Koeberg population. Unusually there were a trio of birds caring for the young at Nest 4. Two of the three birds were caught at the nest, both male. The microsatellite analysis has revealed that they share ~50% of their alleles revealing that they are probably father and son. As this is likely the first clutch of the year it suggests that helpers at the nest are perhaps retained across breeding seasons.

The analysis of the plumage photos, blood samples and sperm samples are awaiting completion.

Conclusions

Although we have not managed to collect as many samples as planned the 6 blood samples and 2 sperm samples will allow us to perform a basic gene-flow analysis and calculate sperm morphometrics for the Cape penduline tit. This will allow us to perform the envisioned comparative analyses if with less statistical power than hoped.

1) Microsatellite genotyping will allow us to compare rates of gene-flow between 3 penduline tit species, European penduline tit (*Remiz pendulinus*) blood samples have been collected from 3 locations, White-crowned penduline tit (*Remiz coronatus*) blood samples from 2 locations and now for the Cape penduline tit (*Anthoscopus minutus*) blood samples are available from 2 locations.
2) Sperm length is hypothesised to vary with extra pair paternity rates in passerine birds(Kleven *et al.*, 2008; Immler *et al.*, 2011). We plan to test 2 predictions using the Cape penduline tit samples in combination with sperm samples from 8 other passerine species consisting of the European penduline tit, Dunnock (*Prunella modularis*), Water pipit (*Anthus spinoletta*), Willow tit (*Poecile montanus*), Great reed warbler (*Acrocephalus arundinaceus*), Savi's warbler (*Locustella luscinioides*), Paddyfield warbler (*Acrocephalus agricola*) and the Spotless starling (*Sturnus unicolor*). We will test the predictions that sperm length increases under higher EPP rates and that variance in sperm length decreases with increasing EPP.

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Anthoscopus minutus

Appendix 6.1. Summary of genotyping results of the 20 conserved microsatellites tested in 11 species of the *Sylvioidea* super-family. The CAM primers were sourced from (Dawson et al. 2010) and the TG primers from (Dawson et al. 2007). All markers were genotyped in 27 individuals for each species.

Microsatellite information					European penduline tit (*Remiz pendulinus*)				
Microsatellite I.D.	Forward primer	Reverse primer	Duplex sets	ZF chromosome	Allele number	Allele size range	Ho	He	Null allele frequency
CAM06	[5HEX]GTGATGGTCCAGGTCTTGC	CAAGAGGAACAGATGAGGGTC	1	1A	1	282	0	0	-
CAM13	[5HEX]TCAAATACAGCAGCAGGCAG	TTCATTACCAAACAGCATCCAG	2	6	4	216-219	0.556	0.568	0.009
CAM17	[6-FAM]CGGGTTGTAATCAAGAAGATGC	CTGCGGAGCAATTAACGC	1	3	4	201-204	0.481	0.576	0.0718
CAM18	[5HEX]TTAAGAAGTTTACACCCAGCG	GCTAAATAACAGAGCCAGGAAG	3	3	1	325	0	0	-
CAM20	[5HEX]TAACAGGCAGGAATGCAGG	TCAGCCAGTGTTGGAGGTC	4	24	7	203-210	0.63	0.804	0.1191
CAM24	[5HEX]CCCACTTCAGTCTTCAGAGC	TGGAGTATTTGGGATTGGAG	5	1A	2	97-101	0.259	0.23	-0.0649
TG01_000	[6-FAM]TTGCTACCARAATGGAATGT	TTGCTACCARAATGGAATGT	2	1A	5	210-214	0.407	0.533	0.1484
TG01_148	[5HEX]TTGCAACACATTCTAATATTGC	TTTAAAGTACATCAAACAACAAAATC	6	1	2	185-187	0.111	0.107	-0.0194
TG02_078	[5HEX]TGTTAAAGCCTGTTCCATAGG	TTCCCCATAAAGTATGTACGC	7	2	2	291-292	0.407	0.509	0.102
TG03_098	[5HEX]TTTGCCTTAATTCTTACCTCATTTG	TTGCAACCTCTGTGGAAGC	8	3	2	229-230	0.037	0.037	-0.0029
TG04_004	[6-FAM]CTGGAGCAGTATTTATATTGATCTTCC	GAAGATGTGTTTCACAGCATAACTG	8	4A	1	170	0	0	-
TG04_061	[6-FAM]GACAATGGCTATGAAATAAATTAGGC	AGAAGGGCATTGAAGCACAC	9	4	4	188-193	0.259	0.24	-0.0615
TG05_046	[6-FAM]AAAACATGGCTTACAAACTGG	GCTCAGATAAGGGAGAAAACAG	4	5	1	329	0	0	-
TG06_009	[6-FAM]AAGCCTTGCTTACATTTTATGGTG	GGGGTGGTAACTGAAATAAAGTATAGG	3	6	1	119	0	0	-
TG07_022	[6-FAM]CAGAAGACTGTGTTCCTTTTGTTC	TTCTAATGTAGTCAGCTTTGGACAC	7	7	1	415	0	0	-
TG08_024	[5HEX]CCCACAAATCCTGAATTTCATATC	ACTGGCTTATAAAGTCCATGGTTG	10	8	2	122-123	0.63	0.484	-0.1397
TG11_011	[6-FAM]ACAAACTAAGTACATCTATATATCTGAAG	TAAATACAGGCAACATTGG	5	11	3	209-213	0.259	0.293	0.0411
TG12_015	[6-FAM]ACAACAGTGGCTTTACTGTGTGA	TACAGCAGCTGCAGCAAAGT	10	12	2	277-279	0.185	0.23	0.0985
TG13_017	[6-FAM]GCTTTGCATCTTGCCTTAAA	GGTAACTACAACATTCCAACTCCT	6	13	4	308-318	0.185	0.208	0.1053
TG22_001	[5HEX]TTGGATTTCAGAACATGTAGC	TCTGATGCAAGCAAACAA	9	22	2	247-249	0.296	0.257	-0.0766

Appendix 6.1. continued

	White-crowned penduline tit (*Remiz coronatus*)					Cape penduline tit (*Anthoscopus minutus*)				
Microsatellite I.D.	Allele number	Allele size range	Ho	He	Null allele frequency	Allele number	Allele size range	Ho	He	Null allele frequency
CAM06	2	282-284	0.037	0.037	-0.0029	1	286	0	0	-
CAM13	7	215-221	0.852	0.794	-0.0431	5	213-218	0.37	0.361	-0.0169
CAM17	3	202-204	0.407	0.528	0.1054	5	203-211	0.444	0.487	0.0671
CAM18	1	325	0	0	-	4	333-339	0.481	0.563	0.0718
CAM20	9	198-212	0.333	0.708	0.3683	6	203-209	0.889	0.825	-0.0476
CAM24	2	97-101	0.259	0.283	0.0345	1	101	0	0	-
TG01_000	5	210-215	0.593	0.581	-0.0144	3	189-191	0.259	0.319	0.1222
TG01_148	1	187	0	0	-	1	187	0	0	-
TG02_078	2	291-292	0.481	0.484	-0.0065	1	292	0	0	-
TG03_098	2	229-230	0.519	0.509	-0.0189	2	233-234	0.519	0.503	-0.0244
TG04_004	1	170	0	0	-	1	168	0	0	-
TG04_061	4	188-196	0.222	0.208	-0.0512	6	189-194	0.667	0.748	0.055
TG05_046	1	329	0	0	-	3	327-333	0.593	0.514	-0.0786
TG06_009	1	119	0	0	-	2	119-121	0.111	0.107	-0.0194
TG07_022	1	415	0	0	-	1	416	0	0	-
TG08_024	2	122-123	0.296	0.257	-0.0766	3	121-123	0.222	0.208	-0.0511
TG11_011	4	209-215	0.63	0.604	-0.0647	3	214-217	0.222	0.205	-0.052
TG12_015	2	277-279	0.259	0.23	-0.0649	2	278-280	0.111	0.107	-0.0194
TG13_017	3	308-312	0.407	0.452	0.0501	2	281-283	0.111	0.331	0.4892
TG22_001	2	247-249	0.037	0.037	-0.0029	2	245-247	0.259	0.44	0.2493

Appendix 6.1. continued

	Blue tit (*Cyanistes caeruleus*)					Great tit (*Parus major*)				
Microsatellite I.D.	Allele number	Allele size range	Ho	He	Null allele frequency	Allele number	Allele size range	Ho	He	Null allele frequency
CAM06	-	-	-	-	-	1	282	0	0	-
CAM13	5	212-216	0.889	0.711	-0.1498	3	223-225	0.259	0.338	0.1177
CAM17	5	202-210	0.481	0.521	0.0168	2	194-200	0.148	0.14	-0.0302
CAM18	3	340-344	0.704	0.577	-0.1127	5	338-346	0.593	0.628	0.0439
CAM20	9	200-212	0.963	0.836	-0.0818	9	193-296	0.815	0.809	-0.0177
CAM24	6	118-133	0.778	0.658	-0.1033	1	99	0	0	-
TG01_000	4	210-213	0.667	0.665	-0.03	2	215-216	0.333	0.372	0.0461
TG01_148	1	185	0	0	-	3	185-189	0.074	0.073	-0.0098
TG02_078	2	273-288	0.037	0.037	-0.0029	3	273-275	0.074	0.073	-0.0098
TG03_098	5	243-247	0.556	0.579	0.0138	3	241-244	0.444	0.425	-0.0563
TG04_004	1	166	0	0	-	1	166	0	0	-
TG04_061	4	195-198	0.519	0.523	-0.0025	5	190-198	0.407	0.406	-0.0373
TG05_046	1	336	0	0	-	1	336	0	0	-
TG06_009	1	117	0	0	-	2	119-121	0.185	0.171	-0.0415
TG07_022	3	416-418	0.37	0.498	0.1458	2	419-420	0.185	0.171	-0.0415
TG08_024	1	122	0	0	-	3	122-124	0.222	0.205	-0.052
TG11_011	6	209-219	0.667	0.693	0.0216	2	217-219	0.222	0.201	-0.0532
TG12_015	2	279-281	0.037	0.037	-0.0029	3	279-283	0.444	0.435	-0.0251
TG13_017	4	219-225	0.111	0.109	-0.0189	9	396-424	0.63	0.591	-0.046
TG22_001	4	147-248	0.074	0.143	0.2979	3	245-249	0.481	0.451	-0.0479

Appendix 6.1. continued

	Coal tit (*Periparus ater*)					Long-tailed tit (*Aegithalos caudatus*)				
Microsatellite I.D.	Allele number	Allele size range	Ho	He	Null allele frequency	Allele number	Allele size range	Ho	He	Null allele frequency
CAM06	1	282	0	0	-	-	-	-	-	-
CAM13	6	228-233	0.852	0.724	-0.0961	6	215-223	0.852	0.799	-0.0384
CAM17	3	192-196	0.481	0.488	0.0045	8	216-225	0.704	0.823	0.0601
CAM18	4	334-340	0.63	0.609	-0.0377	4	342-346	0.333	0.452	0.122
CAM20	8	196-203	0.704	0.808	0.0598	8	195-202	0.778	0.792	0.0004
CAM24	2	97-99	0.074	0.073	-0.01	2	101-111	0.037	0.037	-0.0029
TG01_000	7	223-232	0.704	0.732	0.0147	1	206	0	0	-
TG01_148	2	187-189	0.037	0.037	-0.0029	2	187-189	0.037	0.037	-0.0029
TG02_078	1	273	0	0	-	2	286-288	0.074	0.073	-0.01
TG03_098	5	237-242	0.778	0.808	0.0047	5	238-242	0.667	0.661	-0.0161
TG04_004	3	166-170	0.519	0.451	-0.0696	2	149-166	0.444	0.352	-0.124
TG04_061	9	197-206	0.704	0.767	0.0219	9	199-207	0.852	0.825	-0.0283
TG05_046	1	333	0	0	-	2	336-338	0.259	0.23	-0.0649
TG06_009	3	119-123	0.296	0.268	-0.073	1	119	0	0	-
TG07_022	2	411-417	0.111	0.171	0.2033	2	416-418	0.037	0.037	-0.0029
TG08_024	1	122	0	0	-	3	124-126	0.667	0.559	-0.0997
TG11_011	4	213-220	0.333	0.372	0.0619	8	226-264	0.741	0.72	-0.0305
TG12_015	3	279-283	0.407	0.4	-0.0012	8	285-294	0.778	0.854	0.0425
TG13_017	13	405-432	0.741	0.797	0.0239	5	253-261	0.37	0.358	-0.0184
TG22_001	1	248	0	0	-	2	244-252	0.407	0.507	0.0993

Appendix 6.1. continued

	Veinous-throated parrotbill (*Paradoxornis webbianus*)					Willow warbler (*Phylloscopus trochilus*)				
Microsatellite I.D.	Allele number	Allele size range	Ho	He	Null allele frequency	Allele number	Allele size range	Ho	He	Null allele frequency
CAM06	3	278-282	0.259	0.238	-0.0622	-	-	-	-	-
CAM13	9	220-231	0.593	0.78	0.1214	5	216-220	0.889	0.788	-0.0743
CAM17	11	218-237	0.852	0.823	-0.0306	17	202-228	0.963	0.931	-0.0266
CAM18	5	344-349	0.593	0.651	0.0336	4	338-344	0.704	0.64	-0.0562
CAM20	6	210-215	0.778	0.626	-0.1442	10	206-216	0.926	0.844	-0.0573
CAM24	5	108-118	0.63	0.604	-0.0347	7	116-133	0.667	0.714	0.0295
TG01_000	8	234-242	0.556	0.708	0.1143	10	220-246	0.926	0.841	-0.0625
TG01_148	3	187-193	0.296	0.414	0.1435	3	185-189	0.222	0.266	0.0699
TG02_078	2	290-292	0.556	0.465	-0.0983	2	287-289	0.074	0.073	-0.01
TG03_098	2	238-240	0.074	0.073	-0.01	7	229-236	0.741	0.706	-0.0333
TG04_004	3	154-168	0.667	0.465	-0.1949	5	160-170	0.481	0.614	0.1287
TG04_061	8	187-201	0.778	0.77	-0.0165	4	188-194	0.37	0.383	-0.0187
TG05_046	2	338-340	0.148	0.14	-0.0302	3	333-337	0.111	0.108	-0.0191
TG06_009	2	117-119	0.185	0.171	-0.0415	2	117-119	0.037	0.037	-0.0029
TG07_022	4	409-418	0.185	0.176	-0.04	3	414-420	0.519	0.488	-0.0355
TG08_024	3	125-127	0.407	0.44	0.0547	3	126-128	0.519	0.476	-0.0536
TG11_011	7	212-224	0.815	0.71	-0.0927	6	210-226	0.667	0.662	-0.0154
TG12_015	6	283-288	0.667	0.769	0.0653	3	280-284	0.444	0.507	0.0582
TG13_017	4	237-245	0.222	0.266	0.0705	4	240-244	0.519	0.495	-0.0081
TG22_001	9	277-286	0.741	0.79	0.0231	-	-	-	-	-

Appendix 6.1. continued

	Eurasian reed warbler (*Acrocephalus scirpaceus*)					Sedge warbler (*Acrocephalus schoenobaenus*)				
Microsatellite I.D.	Allele number	Allele size range	Ho	He	Null allele frequency	Allele number	Allele size range	Ho	He	Null allele frequency
CAM06	6	309-319	0.556	0.607	0.0634	-	-	-	-	-
CAM13	7	211-217	0.815	0.805	-0.0142	5	211-215	0.889	0.711	-0.1498
CAM17	6	201-211	0.889	0.762	-0.0884	5	202-210	0.481	0.521	0.0168
CAM18	6	335-348	0.519	0.614	0.0796	3	340-344	0.667	0.602	-0.0712
CAM20	9	193-211	0.778	0.841	0.0231	9	199-212	0.889	0.83	-0.04
CAM24	3	120-124	0.444	0.49	0.069	6	118-132	0.778	0.658	-0.1033
TG01_000	4	212-215	0.704	0.67	-0.0319	4	210-213	0.63	0.676	0.0099
TG01_148	1	187	0	0	-	1	185	0	0	-
TG02_078	3	288-290	0.111	0.108	-0.0191	1	288	0	0	-
TG03_098	7	233-239	0.889	0.782	-0.0714	3	229-231	0.519	0.465	-0.0696
TG04_004	3	162-168	0.333	0.289	-0.0865	2	164-166	0.037	0.037	-0.0029
TG04_061	7	188-197	0.852	0.774	-0.0527	6	194-199	0.593	0.53	-0.0716
TG05_046	2	333-335	0.111	0.107	-0.0194	1	336	0	0	-
TG06_009	3	117-121	0.111	0.108	-0.0191	1	117	0	0	-
TG07_022	3	408-410	0.407	0.624	0.1949	5	405-413	0.444	0.486	0.0294
TG08_024	2	123-124	0.185	0.23	0.0985	2	123-124	0.519	0.503	-0.0244
TG11_011	5	215-223	0.704	0.611	-0.0835	6	209-219	0.667	0.693	0.0216
TG12_015	4	282-288	0.444	0.381	-0.1143	3	280-284	0.37	0.322	-0.0952
TG13_017	4	217-223	0.259	0.328	0.1289	4	219-225	0.111	0.109	-0.0189
TG22_001	5	89-253	0.333	0.406	0.066	2	251-253	0.037	0.037	-0.0029

Appendix 6.2. All species used to infer the *Sylvioidea* phylogeny in the *BEAST analysis, all accession numbers for the gene sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/).

Species	English name	Family	Super-family	Cyt-B sequence	ODC sequence
Acrocephalus schoenobaenus	Sedge warbler	*Acrocephalidae*	*Sylvioidea*	AJ004243	FJ883144
Acrocepahlus scirpaceus	Eurasian reed warbler	*Acrocephalidae*	*Sylvioidea*	AJ004771	FJ883145
Cyanistes caeruleus	Blue tit	*Paridae*	*Sylvioidea*	AF347961	KF183742
Parus major	Great tit	*Paridae*	*Sylvioidea*	AY495412	EU680749
Periparus ater	Coal tit	*Paridae*	*Sylvioidea*	AF347959	KF183786
Phylloscopus trochilus	Willow warbler	*Phylloscopidae*	*Sylvioidea*	Z73492	-
Anthoscopus minutus	Cape penduline tit	*Remizidae*	*Sylvioidea*	Chapter 1	Chapter 1
Remiz coronatus	White-crowned penduline tit	*Remizidae*	*Sylvioidea*	Chapter 1	Chapter 1
Remiz pendulinus	European penduline tit	*Remizidae*	*Sylvioidea*	Chapter 1	Chapter 1
Paradoxornis webbianus	Vinous-throated parrotbill	*Timaliidae*	*Sylvioidea*	JX565699	EU680748
Hirundo rustica	Barn swallow	*Hirundinidea*	*Sylvioidea*	JX236387	EF625337
Panurus biarmicus	Bearded tit	*Panuridae*	*Sylvioidea*	JX236397	EU680747
Auriparus flaviceps	Verdin	*Remizidae*	*Sylvioidea*	Chapter 1	Chapter 1
Baeolophus wollweberi	Bridled titmouse	*Paridae*	*Sylvioidea*	AF347956	KF183793
Baeolophus bicolor	Tufted titmouse	*Paridae*	*Sylvioidea*	AF347957	KF183795
Baeolophus inornatus	Oak titmouse	*Paridae*	*Sylvioidea*	AY607681	KF183798
Poecile montanus	Willow tit	*Paridae*	*Sylvioidea*	AF347944	KF183810
Poecile hudsonicus	Boreal chickadee	*Paridae*	*Sylvioidea*	AF347949	KF183817
Poecile sclateri	Mexican chickadee	*Paridae*	*Sylvioidea*	AF347947	KF183814
Poecile carolinensis	Carolina chickadee	*Paridae*	*Sylvioidea*	AF347941	KF183816
Poecile gambeli	Mountain chickadee	*Paridae*	*Sylvioidea*	AY329470	KF183812
Poecile atricapillus	Black-capped chickadee	*Paridae*	*Sylvioidea*	AF284066	KF183811
Eremopterix verticalis	Grey-backed sparrow-lark	*Alaudidae*	*Sylvioidea*	AY165164	-
Ammomanes grayi	Gray's lark	*Alaudidae*	*Sylvioidea*	AY165168	KF060556
Chersomanes albofasciata	Spike-heeled lark	*Alaudidae*	*Sylvioidea*	AY165165	EU680716
Galerida cristata	Crested lark	*Alaudidae*	*Sylvioidea*	AY769746	-
Eremalauda starki	Stark's lark	*Alaudidae*	*Sylvioidea*	AY165162	-
Alauda arvensis	Eurasian skylark	*Alaudidae*	*Sylvioidea*	JX236372	EF625336
Eremophila alpestris	Horned lark	*Alaudidae*	*Sylvioidea*	AF290137	KF060568
Calandrella cinerea	Red-capped lark	*Alaudidae*	*Sylvioidea*	KF060420	KF060561
Pycnonotus barbatus	Common bulbul	*Pycnonotidae*	*Sylvioidea*	HM633367	HM633779
Aegithalos caudatus	Long-tailed tit	*Aegithalidae*	*Sylvioidea*	DQ792803	EU680703

Species	English name	Family	Super-family	Cyt-B sequence	ODC sequence
Cisticola exilis	Golden-headed cisticola	*Cisticolidae*	*Sylvioidea*	JX869890	-
Cisticola juncidis	Zitting cisticola	*Cisticolidae*	*Sylvioidea*	HQ608851	-
Locustella fluviatilis	River warbler	*Locustellidae*	*Sylvioidea*	AJ004764	HQ121556
Locustella luscinioides	Savi's warbler	*Locustellidae*	*Sylvioidea*	AJ004763	-
Megalurus gramineus	Little grassbird	*Locustellidae*	*Sylvioidea*	HQ333042	HQ333091
Megalurus timoriensis	Tawny grassbird	*Locustellidae*	*Sylvioidea*	JN827115	HQ706336
Sylvia atricapilla	Blackcap	*Timaliidae*	*Sylvioidea*	EF446853	EU680770
Sylvia communis	Common whitethroat	*Timaliidae*	*Sylvioidea*	AJ534538	-
Sylvia curruca	Lesser whitethroat	*Timaliidae*	*Sylvioidea*	AJ534536	KC512697
Zosterops japonicus	Japanese white-eye	*Timaliidae*	*Sylvioidea*	HQ608850	FJ358079
Zosterops lateralis	Capricorn silver-eye	*Timaliidae*	*Sylvioidea*	JN827240	-
Phylloscopus sibilatrix	Wood warbler	*Phylloscopidae*	*Sylvioidea*	Z73491	-
Phylloscopus fuscatus	Dusky warbler	*Phylloscopidae*	*Sylvioidea*	HQ608823	-
Phylloscopus collybita	Common chiffchaff	*Phylloscopidae*	*Sylvioidea*	HQ608821	FJ358084
Acrocephalus paludicola	Aquatic warbler	*Acrocephalidae*	*Sylvioidea*	AJ004291	FJ883141
Acrocephalus melanopogon	Moustached warbler	*Acrocephalidae*	*Sylvioidea*	AJ004282	FJ883138
Acrocephalus stentoreus	Clamorous reed warbler	*Acrocephalidae*	*Sylvioidea*	AJ004789	FJ883137
Acrocephalus arundinaceus	Great reed warbler	*Acrocephalidae*	*Sylvioidea*	AJ004253	FJ883128
Acrocephalus palustris	Marsh warbler	*Acrocephalidae*	*Sylvioidea*	AJ004294	FJ883142
Riparia riparia	Sand martin	*Hirundinidae*	*Sylvioidea*	AF074578	-
Pygochelidon cyanoleuca	Blue-and-white swallow	*Hirundinidae*	*Sylvioidea*	AF074586	-
Tachycineta bicolor	Tree swallow	*Hirundinidae*	*Sylvioidea*	GU460236	JX299002
Progne tapera	Brown-chested martin	*Hirundinidae*	*Sylvioidea*	AF074588	-
Progne subis	Purple martin	*Hirundinidae*	*Sylvioidea*	EU427742	-
Progne chalybea	Grey-breasted martin	*Hirundinidae*	*Sylvioidea*	AY825948	JX298999
Delichon urbicum	Northern house martin	*Hirundinidae*	*Sylvioidea*	DQ008517	EU680721
Petrochelidon nigricans	Tree martin	*Hirundinidae*	*Sylvioidea*	AY825983	-
Petrochelidon pyrrhonota	American cliff swallow	*Hirundinidae*	*Sylvioidea*	AF074591	-
Hirundo tahitica	Pacific swallow	*Hirundinidae*	*Sylvioidea*	AY825967	-
Hirundo neoxena	Welcome swallow	*Hirundinidae*	*Sylvioidea*	GU460230	-
Outgroups					
Myiarchus tyrannulus	Brown-crested flycatcher	*Tyrannidae*	-	JQ004335	DQ435489
Corvus corone	Carrion crow	*Corvidae*	*Corvoidea*	JQ864491	EU272116
Turdus merula	Eurasian Blackbird	*Turdidae*	*Muscicapoidea*	AY286396	EU154863
Ficedula hypoleuca	European pied flycatcher	*Muscicapidae*	*Muscicapoidea*	HM633303	EU680728
Oenanthe oenanthe	Northern wheatear	*Muscicapidae*	*Muscicapoidea*	GU055483	GU358885
Saxicola torquatus	African stonechat	*Muscicapidae*	*Muscicapoidea*	HM633376	JX256191

Appendix 6.3. Estimated breeding ranges and population sizes of 25 *Sylvioidea* species obtained from Birdlife international (http://www.birdlife.org.uk/datazone/home).

Species	English name	Family	Breeding area (km^2)	Estimated Pop size
Acrocephalus schoenobaenus	Sedge warbler	*Acrocephalidae*	12,000,000	31,100,000
Acrocepahlus scirpaceus	Eurasian reed warbler	*Acrocephalidae*	13,900,000	20,450,000
Aegithalos caudatus	Long-tailed tit	*Aegithalidae*	18,600,000	87,300,000
Parus major	Great tit	*Paridae*	32,600,000	686,000,000
Periparus ater	Coal tit	*Paridae*	18,700,000	210,750,000
Phylloscopus trochilus	Willow warbler	*Phylloscopidae*	15,800,000	771,500,000
Remiz pendulinus	European penduline tit	*Remizidae*	5,890,000	1,054,300
Hirundo rustica	Barn swallow	*Hirundinidea*	43,400,000	190,000,000
Alauda arvensis	Skylark	*Alaudidae*	33,000,000	602,500,000
Eremophila alpestris	Horned lark	*Alaudidae*	25,000,000	140,000,000
Galerida cristata	Crested lark	*Alaudidae*	28,500,000	56,600,000
Certhilauda chuana	Short clawed lark	*Alaudidae*	127,000	75,000
Delichon urbicum	Northern house martin	*Hirundinidae*	16,200,000	174,300,000
Riparia riparia	Sand martin	*Hirundinidae*	28,900,000	50,000,000
Poecile montanus	Willow tit	*Paridae*	21,800,000	325,500,000
Poecile palustris	Marsh tit	*Paridae*	9,040,000	45,200,000
Panurus biarmicus	Bearded tit	*Panuridae*	10,800,000	3,600,000
Paradoxornis zappeyi	Grey hooded parrotbill	*Timaliidae*	41,100	9,250
Acrocephalus arundinaceus	Great reed warbler	*Acrocephalidae*	17,400,000	21,990,000
Locustella luscinioides	Savi's warbler	*Locustellidae*	556,000	3,475,000
Phylloscopus collybita	Common chiffchaff	*Phylloscopidae*	17,000,000	452,000,000
Cettia cetti	Cetti's warbler	*Cettiidae*	4,540,000	11,435,000
Sylvia atricapilla	Blackcap	*Timaliidae*	6,860,000	137,900,000
Sylvia communis	Common whitethroat	*Timaliidae*	9,160,000	103,400,000
Sylvia curruca	Lesser whitethroat	*Timaliidae*	8,170,000	61,500,000

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