Adaptive evolution of hosts and parasites: how can species maintain high evolutionary rates and persist despite very low effective population sizes?

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Cover photo: front view of faces of female *Exoneura robusta* (top image) and *Inquilina schwarzi* (bottom image) collected from Dandenong Ranges, Victoria, Australia.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Nahid Shokri Bousjein

This thesis is dedicated to:
to the memory of my beloved parents,
my lovely husband and little daughter

Abstract

Classical evolutionary theory indicates that rates of adaptive evolution are limited by effective population size (N_e). For pairs of species that are locked in co-evolutionary arms races, these effects of N_e become even more extreme on species with the smaller N_e. Yet, there are species that have persisted over extensive evolutionary time, despite their very low effective population sizes, raising question how these species have been able to survive for a long-term despite their very low adaptability? In this thesis, I use Australian allodapine social host and parasite bees which have substantial disparity in their effective population sizes to address this question. Earlier phylogenetic studies of Australian allodapine host and parasite bees revealed that parasites species have managed to persist over long evolutionary time (since 15 million years ago), and also have tracked their hosts through multiple speciation episodes regardless of their much lower effective population sizes. Allodapine parasite species are locked in tight arms races with their hosts, and are closely related to their hosts. I organize this study into four research data chapters, focusing on host bee *Exoneura,* which is the most speciose genus of the tribe allodapini, and its obligate social parasite *Inquilina,* which displays the highest species richness.

In the first data chapter, I estimate the relative effective population sizes of *E. robusta* and its social parasite *I. schwarzi* through population demographic data collected over several years. My analyses show that the inquiline species have effective population sizes that are about an order of magnitude lower than their hosts.

In the second data chapter, I show that the population-wide sex ratio of *I. schwarzi* is close to parity. I then argue that this pattern of bias is consistent with local resource competition, where inquiline females compete to inherit their natal colony. I also discuss how extremely female-biased sex ratios of *E. robusta* species, combined with overall sex ratio parity in inquilines, may decrease the disparity in effective population sizes between these two species which are locked in an evolutionary arms race.

In the third data chapter, I examine the hypothesis suggesting that parasite lineages with much smaller N_e have been survived for a long evolutionary time because they might have been able to accelerate their rates of molecular evolution. To do so, I compare the rate of mitochondrial molecular evolution between *E. robusta* and its parasite *I. schwarzi;* and *E.*

angophorae and its parasite *I. excavate*. Our finding doesn't support the prediction that rates of molecular evolution in mitochondrial genes are higher in parasite species than their hosts.

In the fourth chapter, I assess how effective population size of *Exoneura* species have changed since late Pleistocene, particularly the last glacial maximum (LGM), using *COI* sequence data and the Bayesian Skyline analyses. Contrasting the timeline of inferred Bayesian Skyline Plots with the glacial history of Australia, suggests that populations of *Exoneura* species are surprisingly unaffected during the LGM and begin to expand with the post-LGM warming period.

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General Introduction

1. Allodapine bees

The bee tribe Allodapini belong to the family Apidae, subfamily Xylocopinae. Allodapines are mainly restricted to sub-Saharan Africa, Australia, Madagascar and southern Asia. Phylogenetic studies revealed that the origin of the Allodapini (crown age) dates back to approximately 53 million years ago (Rehan *et al*., 2012).

Bees of this tribe exhibit a wide range of behaviour, ranging from solitary to highly eusocial nesting assemblages (Tierney *et al*., 2002, 2008; Schwarz *et al*., 2003, 2007). Previous studies have indicated that social nesting is an ancestral trait for the tribe and therefore solitary or strictly subsocial species represent a loss of sociality (Schwarz *et al*., 2003, 2006).

Allodapine bees nest in the pithy centres of dead plant stems (Michener 1970b; Schwarz *et al*., 1998) and nearly all species form small colony sizes, usually with fewer than four females per nest (Schwarz *et al*., 2011). Most species of allodapines, other than *Exoneurella tridentata*, lack morphologically distinct queen and worker castes and egg laying is often attributed to more than one female (Schwarz *et al*., 1998, Smith & Schwarz, 2006a).

The allodapines are unique among bees in that brood are fed progressively by adults in an undivided communal tunnel without cell partitions in dead plant stems and reeds (Figure 1) (Michener, 1974, 2007). Given the structure of the nest and method of brood rearing, the brood are therefore in constant contact with other larvae, adult females and potential predators and parasites. However, to reduce brood predation and parasitisation in the event of maternal death, alloparental cares in this tribe seems to be well defined (Bull & Schwarz, 1996; Hogendoorn & Schwarz, 2001, Tierney *et al*., 2008) and has been suggested as an ancestral trait for the allodapines (Schwarz *et al*., 2011).

There are currently 15 genera and over 300 described species of allodapine bees (Michener, 2007), but many more species are still undescribed (e.g. Chenoweth & Schwarz, 2011).

1.1. Australian allodapine bees

The Australian allodapine bees divide into two main clades:

(i) the genus *Braunsapis*, which is mainly distributed in tropical and subtropical regions of Australia, although a number of species are found in arid and semi-arid regions (Reyes, 1993). *Braunsapis* species are also distributed through Africa, Madagascar, and southern Asia (Michener, 1975).

(ii) The exoneurine clade which is found only in Australia (Bull *et al*., 2003; Schwarz *et al*., 2003) and comprises four genera consisting of *Exoneura* Smith, *Exoneurella*, *Brevineura*, and *Inquilina* Michener. Of these, *Exoneura* is the most speciose genus and *Inquilina* is a genus of obligate social parasites (of *Exoneura* hosts) and displays the most species rich group of parasites within the tribe Allodapini (Michener, 2007).

The genera *Exoneura*, *Brevineura* and *Inquilina* are mainly restricted to temperate zones of Australia while *Exoneurella* is generally distributed in arid/semi-arid regions of the continent (Michener, 1965, 1971a, 1975; Bull *et al*., 2003; Schwarz *et al*., 2003; Chenoweth & Schwarz, 2011). In addition, the types of habitats these genera occupy generally differ. *Exoneura* and *Inquilina* are most common in cool, wet forests. *Exoneurella* is found in dry woodland and inland xeric habitats, and *Brevineura* is most commonly found in coastal to subcoastal heath and woodlands (Schwarz *et al*., 1998).

1.1.1. Australian allodapine social parasites

Inquilina is a socially parasitic genus that occurs only in Australia. Initially it had been suggested that *Inquilina* arose from within extant *Exoneura* lineages (Michener, 1983), but subsequent studies supported a sister group relationship between the two genera (Lowe & Crozier, 1997; Schwarz *et al*., 2003; Smith *et al*., 2007).

Inquilina spend most of their life span within their host's (*Exoneura*) nest, except for a very brief period of dispersal. *Inquilina* species exhibit reduced mouthparts and pollen-collecting scopal hairs and this is thought to be an adaptation to a life history of reduced foraging and rearing of brood in a host colony (Figure 2) (Michener, 1965, 1971a, 1975).
Allodapine inquilines are host specific and have life cycles that are phenologically closely tied to their hosts (Michener, 1965; Smith & Schwarz, 2006 a,b; 2009). Once an inquiline

invades a host nest, it effectively replaces the host queen as the principle egg layer, however, the queen is not killed and generally continues to live alongside the parasite in the colony (Michener, 2007). *Inquilina* commences egg-laying after its host (Smith & Schwarz, 2009), but its brood develop in synchrony with the host brood and reach adult eclosion at about the same time (Michener, 1965; Smith & Schwarz, 2009).

Despite the low level of morphological variation among *Exoneura* host species, *Inquilina* species display significant inter-specific morphological variation in adult morphology. Smith and Schwarz (2009) suggested that this variation might be due to: (i) the strong selection pressures on morphological traits that are related to host exploitation, (ii) the smaller population sizes of social parasites compared to the host species, which could lead to higher rates of molecular evolution (e.g. Bromham & Leys, 2005) and a consequent drift in morphological traits that may have no adaptive significance.

Recent collection of parasites based on samples of host colonies suggested seven species of *Inquilina* in Australia. However, because inquilines are rarely collected on flowers and their rates of host-nest colonisation are low, it is likely that their true level of species diversity is yet to be detected (Smith & Schwarz, 2009).

1.1.2. Historical biogeography

Earlier molecular phylogenetic studies indicate that allodapine bees originated in Africa (Bull *et al*., 2003; Schwarz *et al*., 2003, 2006) but arrived into Australia via two temporally different migrations. The first migration gave rise to the exoneurine genera endemic to southern Australia. This clade migrated from Africa to Australia via Antarctica about 50-30 Mya and diversified about 34 Mya into four genera (*Exoneura*, *Exoneurella*, *Brevineura*, and *Inquilina)* in Australia (Schwarz *et al*., 2006).

The second migration involved the genus *Braunsapis* which first dispersed into southern Asia and then arrived into Australia via Indian Ocean Rim dispersal, around late Miocene 12-7 Mya (Schwarz *et al*., 2004; Fuller *et al*., 2005).

1.1.3. Phylogeny

Previous studies have been extensively examined the phylogeny of allodapines, including non-host, host and parasite species (Chenoweth & Schwarz, 2011; Smith *et al*., 2013). The most recent and informative phylogenetic tree is provided in figure 3, where the phylogeny of 84 allodapine species, including 19 socially parasitic taxa and their hosts are presented. The inferred phylogenetic tree indicates at least nine independent origins of parasitism (red dots in the tree) and that, social parasites show close phylogenetic relationships to their host species in all cases.

More specifically, the resultant tree indicated that the divergence age of the genus *Inquilina* from their sister host clade, *Exoneura*, dates back to approximately 15 Mya (Smith *et al*., 2007, 2013). The long evolutionary persistence of *Inquilina* along with its multiple speciation events provides an opportunity to examine how species with small effective population sizes can manage to survive when locked into evolutionary arms races with hosts that have much larger effective population sizes.

1.1.4. Sex allocation

Allodapine bees exhibit ubiquitous female-biased sex allocation regardless of their broad variation in social and life history traits (Schwarz *et al*., 1998). For instance, previous studies on *Exoneura richardsoni* (Cronin & Schwarz, 1997), *E. bicolor* (Schwarz, 1988) *E. nigrescence* (Figure 4) (Bull & Schwarz, 2001) and *Braunsapis protuberans* (Joyce & Schwarz, 2007) found that sex ratios are all strongly female biased.

The preponderance of female-biased sex ratios in this group is thought to be due to local resource enhancement (LRE, Schwarz, 1988). LRE occurs when there are strong benefits in producing daughters first, where such daughters act in alloparental roles in the event of brood orphaning (Bull & Schwarz, 2001; Aenmey *et al*., 2006; Thompson & Schwarz, 2006).

Additional studies on allodapine bees reveal that reproductive females have the ability to adjust sex ratios according to colony size (Thompson & Schwarz 2006), with maximum female-bias in the smallest colonies and decreasing bias in larger colonies (Schwarz *et al*., 2007). It seems likely that the ability to adjust sex ratios according to colony size is an

ancestral trait for the Allodapini, given that this feature was observed in different genera of allodapine bees (*Macrogalea* Malawi sp. (Thompson & Schwarz, 2006) and *E. nigrescence* (Bull & Schwarz, 2001).

2. Effective population size and the rate of adaptive evolution

Rates of adaptive evolution are critical for understanding how species can meet diverse challenges to their survival. Slow-evolving species are much less likely to adapt to rapidly changing threats, so it is important to understand what factors limit, and enhance, evolutionary rates. In a series of highly influential papers in evolutionary genetics, Kimura and Ohta showed that rates of adaptive evolution scale linearly with effective population size (N_e) (Kimura & Ohta, 1971, 1974a, 1974b). Recent genomic studies have supported this theoretical prediction (Gossmann *et al*., 2012), indicating that low effective population size really can put a brake on rates of adaptive evolution. Whilst effective population size is a key parameter in evolutionary biology, it is often misunderstood. It is not a measure of population size per se, but instead a measure of the number of individuals contributing gametes to the next generation and how equally they contribute (Wright, 1931). Small values of N_e will lower the number of favourable mutations entering a gene pool, and at the same time increase the likelihood that deleterious mutations spread due to increased genetic drift (Weber & Diggins, 1990; Woolfit, 2009). When effective population size becomes very small, rates of adaptive evolution can become critically low and, combined with the effects of inbreeding and genetic drift, can greatly increase the risk of extinction. These risks are widely appreciated, and the management of endangered species frequently involves attempts to increase the N_e of managed populations to minimize these problems.

Despite these problems of small N_e, it is clear that a large number of species have naturally low effective population sizes, and in many cases these low N_e values have persisted over long evolutionary time. This raises the question of how such species were able to persist for a long evoluatinary time despite very low effective population sizes. It is possible that some of these species occupy niches that change little over time, so that capacity for rapid evolutionary change is not imperative.

However, in some cases species with low N_e are locked into continuing evolutionary conflict with their enemies, and if these enemies have much larger N_e, we would expect the low N_e species to eventually lose out in these battles and become extinct.

2.1. Allodapine social host and parasite model system

Allodapine bees have long been known for harbouring a high diversity of socially parasitic species with multiple origins (Michener, 1975). allodapine host and social parasite model has 3 key attributes that make them ideal to examine how species with small effective population size were able to persist for a long evoluationary period. These keys are as follows: (i) Social parasites have much smaller N_e than their hosts; (ii) Social parasites and their hosts live in extremely similar environments and have the same generation times; and (iii) There are multiple origins of parasitism, followed by multiple subsequent cospeciation events, so that phylogenetic contrasts have statistical power. I now outline each of these features.

2.1.1. Effective population size of *Inquilina*

Previous demography-based studies indicated effective population size (N_e) for inquilines is approximately 5% that of hosts (Smith & Schwarz 2006b, 2008, 2009; Smith *et al*., 2007, 2013). Consequently, the shifts from social living to social parasitism entail reductions in N_e of about 95% or more. Given the linear association between N_e and rates of adaptive evolution according to the classical evolutionary theory (Kimura & Ohta, 1971), it is predicted that social parasites will have much lower potential rates of adaptive evolution than their hosts. Moreover, since *Inquilina* seems to be locked in tight co-evolutionary arms races with its host, the effects of small N_e could strongly disadvantage inquiline species. However, phylogenetic studies have shown that inquilines have managed to not only survive over long periods of evolutionary time, but they have tracked their hosts through multiple speciation episodes (Smith *et al*., 2013) (Figure 3). Long-term evolutionary persistence of *Inquilina* (in some cases extending back to approximately 20 millions years ago) and the relatively large morphological variation of this genus (compared to small variation in hosts), provide a major problem for classic models of adaptation based on effective population sizes.
Subsequently, there is a potentially important paradox surrounding social inquilines: if inquilines have smaller effective population sizes than their hosts, how are they able to survive long-term evolutionary races against their hosts when the hosts should be capable of faster evolutionary rates.

2.1.2. Social parasites and hosts share the same environment and generation times.

Allodapine social parasites spend their entire life-cycle in their host's nests, where they are fed by hosts who are unable to recognize them as heterospecifics once invasion has been successful (Michener, 1965, 1975; Schwarz *et al*., 2007, Smith & Schwarz, 2006, 2008). The strong integration between parasite and host life cycles (egg-laying and brood rearing patterns of parasites are closely integrated with that of the host (e.g. Michener, 1965; Smith & Schwarz, 2006a,b) means that allodapine hosts and social parasites have similar generation times and live in the same nest environment, negating any effect of generation time and environment on the on rates of adaptive evolution (e.g. Thomas *et al*., 2010b).

2.1.3. Multiple origins of social parasitism followed by subsequent co-speciation events.

The phylogeny of allodapines, including non-host, host and parasite species has been extensively examined (Chenoweth & Schwarz, 2011). The most comprehensive phylogeny covering host and parasite pairs (Smith *et al*., 2013) indicates 9 independent origins of parasitism available for comparisons between host and social parasite strategies, but subsequent speciation events increases the actual number of phylogenetic contrasts that can be made to >20. Because host/social parasite lineage divergences are well supported, along with subsequent cospeciation events, and divergence dates are also well know, we are able to carry out phylogenetic contrasts within a well-supported and well-dated evolutionary framework. Intriguingly, Smith (2008) found that for Australian social parasites in the genus *Inquilina*, pairwise genetic distances for mitochondrial genes were often much smaller than for the nuclear gene EF-1α. Furthermore d_N/d_S rates of EF-1α are highly variable among *Inquilina* species, much more so than for mitochondrial genes, suggesting very uneven rates of adaptive evolution for at least one nuclear marker. Lastly, when describing five new Australian social allodapine parasites, Smith (2008) and Smith & Schwarz (2009) noted that *Inquilina* species were very strongly differentiated in a wide variety of morphological traits, whereas their host species showed very little differentiation. This is unexpected given the much smaller *N*e of parasites compared to their hosts. However, the d_N/d_S ratios and highly variable morphologies of parasite species is consistent with rates of nuclear change.

3. Aims of project

This thesis consists of four data chapters, using two genera of the Australian exoneurine clade: *Exoneura* and their obligate parasites *Inquilina*. The general aim of this project was to understand how inquilines are able to survive long-term evolutionary races against their hosts when the hosts have much greater effective population size and consequently much faster rates of adaptive evolution.

The specific aims were to:

- Estimate the relative effective population size (N_e) of pairs of Australian allodapine bee host species and their inquilines through population demographic data (Chapter 1).

- Explore the sex-allocation pattern in parasitic *Inquilina* and their host *Exoneura* species to understand whether sex allocation biases might modify evolutionary arm races in terms of relative effective populations sizes (Chapter 2).

- Compare rates of mitochondrial molecular evolution between pairs of Australian allodapine bee host species and their inquilines to assess earlier hypotheses that rates of molecular evolution should be higher in socially parasitic species with smaller effective population sizes (Chapter 3).

- Examine how the effective population size of *Exoneura* and their *Inquilina* social parasites have changed since the late Pleistocene, particularly since the last glacial maximum (LGM) (Chapter 4).

I conclude this thesis with a general discussion which presents a synthesis of the research and broader implications of this study and discusses the limitations and requirements for future research.

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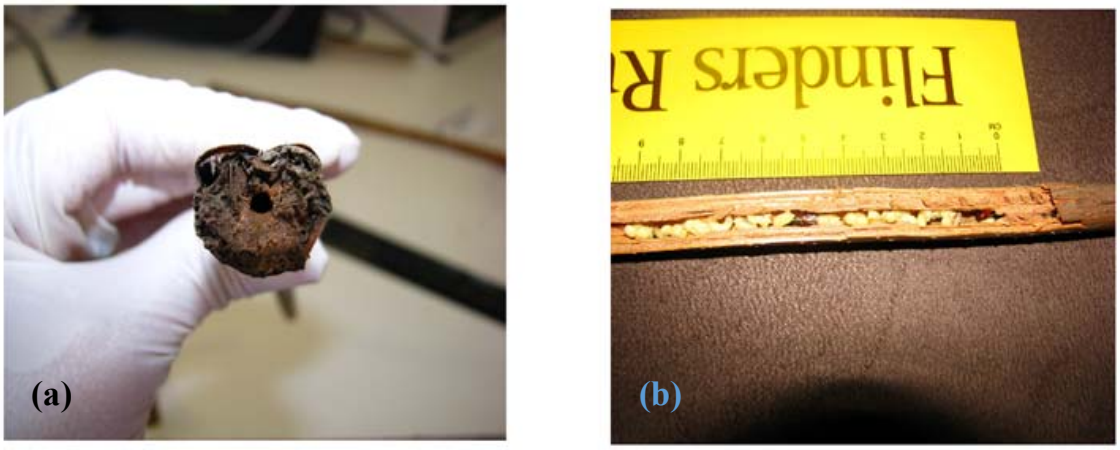

Figure 1. (a) View of the basal end of a dead tree fern frond, *Cyathea australis,* showing the entrance of an *Exoneura* nest, collected from Dandenong Ranges, Victoria, Australia; (b) Longitudinal cut of nest comprising adult *Exoneura* females and pupae.

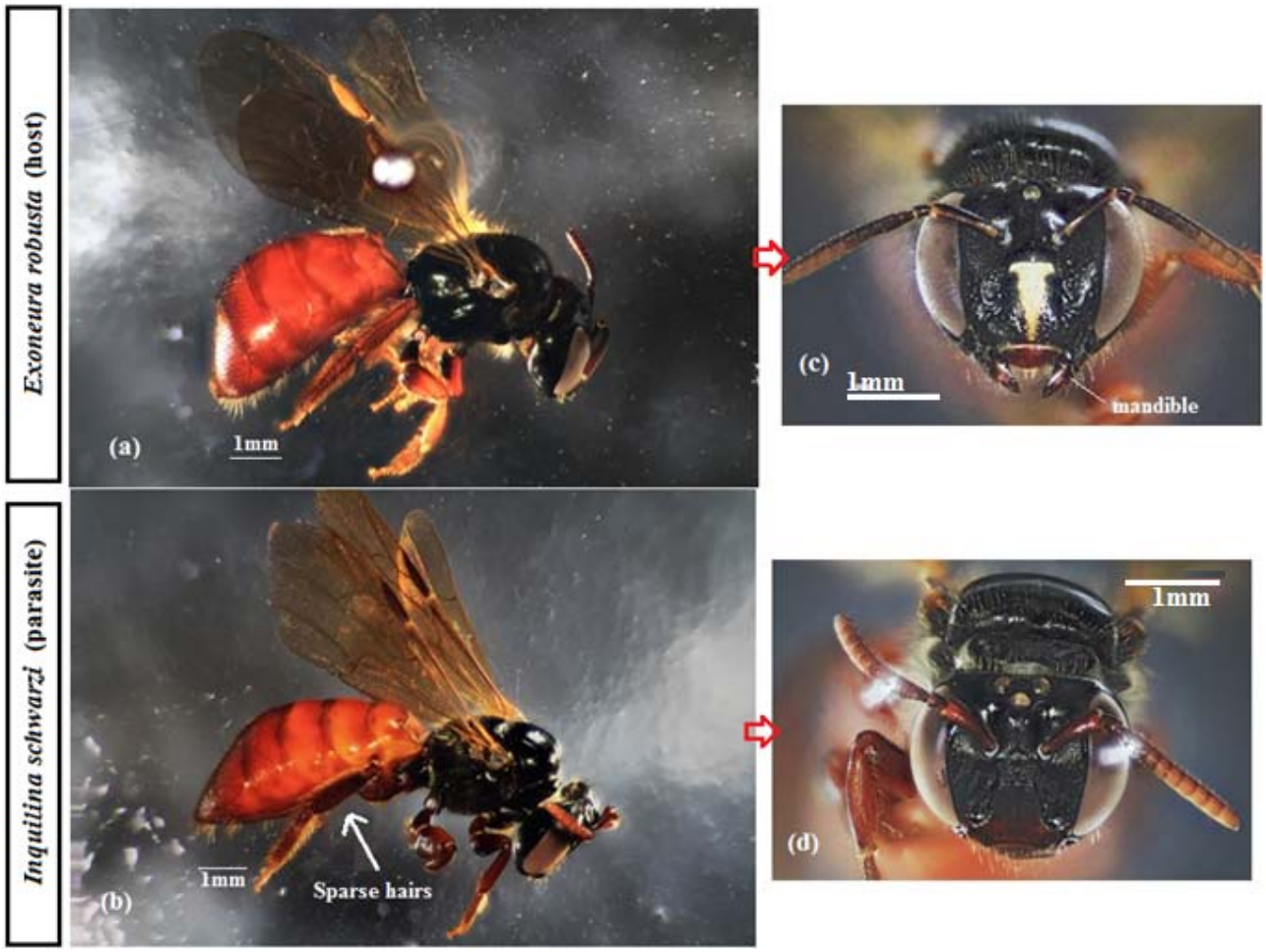

Figure 2. (a) & (b) female specimens of *E. robusta* and its social parasite *I. schwarzi* respectively. White arrow displayes sparse scopal hairs of parasaite species; (c) and (d) front view of faces of *E. robusta* and *I. schwarzi* respectively, showing lack of mandible in parasite species. Bees were collected from Dandenong Ranges,Victoria, Australia.

Figure 3. Molecular phylogeny of allodapine social parasites (red branches) and their hosts (green branches). Red dots indicate multiple origins of social parasitism. Red brackets connect parasite species with their hosts (Adapted from Smith *et al*., 2013, Biological Journal of the Linnean Society).

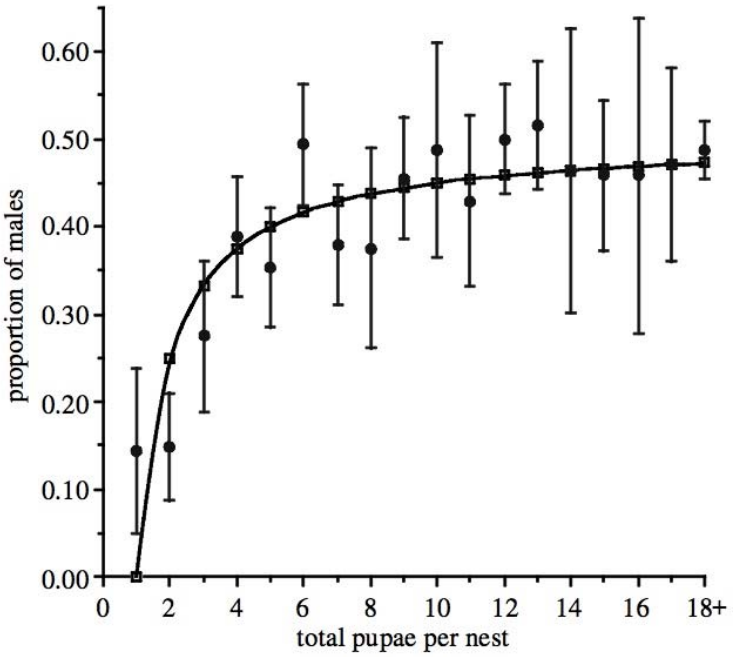

Figure 4. Numerical sex ratio of *Exoneura nigrescens* (with 95% confidence interval) plotted against the total number of pupae per nest (filled circles) and the numerical sex ratio investment predicted by insurance via protogyny model for a single female insurance (open squares) (adapted from Bull & Schwarz, 2001, Journal of the Royal Society).

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Chapter I:

Small effective population sizes of bee social parasites compared to their hosts raise important questions for evolutionary arms races

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Name of principal author (candidate): Nahid Shokri Bousjein

Contribution to the paper: Sorting samples, carried out analyzing of the data and wrote the manuscript (80%).

Signature:  **Date:** 2.7.2017

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Contribution to the Paper: Contributed to review the paper critically and was corresponding author during submission process (5%).

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Contribution to the Paper: Designer of research, assisted in fieldwork, supervised the direction of the study, gave advice on analyses and critically reviewed the manuscript (15%).

Signature:  **Date: 2.7.2017**

1. Abstract

Social parasitism in insects has raised major questions in evolutionary biology, firstly in terms of adaptations that parasites use to circumvent host defenses and, secondly, in terms of whether social parasites have arisen via allopatric or sympatric speciation. Here we raise a third and major evolutionary issue: *a priori* considerations suggest that social parasites have much smaller effective population sizes (N_e) than their hosts, and should therefore have much slower rates of evolution than their hosts. Arms races should therefore be weighted in favour of host species, raising the question of how social parasites have been able to persist over long evolutionary time.

Very few studies, however, have actually estimated the relative sizes of N_e for social insects and their social parasites, and therefore the dimensions of unequal host-parasite evolutionary rates are unknown. Here we use extensive samples of allodapine bee host species and their inquilines from two localities over multiple years to gauge their relative N_es. We show that inquiline species have N_es that are about an order of magnitude lower than their hosts, so explaining the evolutionary persistence of social parasitism poses a major puzzle for evolutionary biology. We propose several hypotheses that may be able to address this puzzle and discuss how they could be evaluated.

Key Words: arms races, bees, coevolution, effective population size, inquilines, social parasites.

2. Introduction

Host-parasite co-evolutionary arms races have been implicated in many widespread phenomena, such as maintenance of sexual reproduction (e.g. Hamilton, 1980; Ladle, 1992; Lively, 2010) and the evolution of female mate-choice based on male adornments (Hamilton & Zuk, 1982). In both of these situations, the larger effective population sizes (N_e) of endo and ecto-parasites have been important considerations. This is because larger N_e confers faster evolutionary rates, pushing hosts into strategies that maximize genetic responses to their parasitic enemies. There is, however, one situation where parasites have smaller N_e than their hosts, viz. obligate social parasites which are unable to breed outside their host colonies, a situation referred to as inquilinism. Inquilinism has evolved multiple times in insects, particularly in ants (Savolainen & Vepsäläinen, 2003) and in allodapine bees (Smith *et al.,* 2007, 2013), but it is also well known in bumble bees (Michener, 2007) and paper wasps (Cervo, 2006). Inquilinism in these groups has attracted attention because its origins have controversially been used to infer sympatric speciation (e.g. Buschinger, 1990; Bourke & Franks, 1991; Savolainen & Vepsäläinen, 2003; but see Smith *et al.,* 2013).

Here we raise a potentially important paradox surrounding social inquilines: *if inquilines have smaller effective population sizes than their hosts, how are they able to survive long-term evolutionary races against their hosts when the hosts should be capable of faster evolutionary rates?* Vulnerability to extinction has already been noted for socially parasitic bumblebees because of small population sizes and host specificity (Suhonen, Rannikko & Sovari, 2015), but their vulnerability to arms races with their hosts has not been addressed. The imperative for high evolutionary rates in arms races has been widely appreciated (e.g. Haraguchi & Sasaki, 1996; Pal *et al.,* 2007; Paterson *et al.,* 2010), and classical evolutionary theory (e.g. Kimura & Ohta, 1971) as well as later considerations (reviews in Woolfit, 2009; Lanfear, Kokko & Eyre-Walker, 2014) predict that both mutation rates and N_e are critical for determining rates of adaptive evolution because they influence the number of potentially advantageous mutations that can enter a population. Small values of N_e will lower the number of favourable mutations entering a gene pool, and at the same time increase the likelihood that deleterious mutations spread due to increased genetic drift (Weber & Diggins, 1990; Woolfit, 2009). When effective population size becomes very small, rates of adaptive

evolution can become critically low and, combined with the effects of inbreeding and genetic drift, can greatly increase the risk of extinction.

The arguments above assume that inquilines have smaller N_e than their hosts, but we are aware of only one study that has explicitly looked at these relative N_es (Erler & Lattorff, 2010). The dearth of such studies is understandable because inquilines are rarely collected outside their host colonies, so that estimating infestation rates *per se* is difficult and requires very large sample sizes for host colonies. However there are further problems for unpicking the issues associated with estimating relative N_es of hosts and parasites in Hymenoptera. One important problem concerns the estimation of effective population size in haplodiploids with complementary sex determination (Hedrick & Parker, 1997), especially when population sizes become very small, such that the effect of sterile diploid males arising from inbreeding limits the number of males that contribute genes to the next generation (Zayed, 2004; Zayed & Packer, 2005).

Allodapine bees are ideal for examining infestation rates because inquilinism has evolved repeatedly within the tribe, at least more than 13 times (Smith *et al.*, 2013) and also because they are stem nesters with small colony sizes (Schwarz, Richards & Danforth, 2007), so that large numbers of host colonies can be obtained and processed readily.

2.1. *Exoneura* and *Inquilina* life cycles

Exoneura Smith species nest in simple unlined and unbranched tunnels in soft pithy stems, and brood are reared progressively and not separated from each other by cells (Figure 1). Because all brood within a nest are in an undivided and communal nesting lumen, and the brood are in physical contact with each other, it is difficult for females in multifemale colonies to restrict maternal care to just their own offspring, so that parental care can easily spill over into alloparental care (Schwarz, Richards & Danforth, 2007). In temperate regions of Australia *Exoneura* is univoltine and during brood rearing the maximum number of adult females per nest does not exceed eight, and modal colony sizes are usually 2-3 females per nest. Foundresses disperse from their natal nests in late spring, and new colonies can be founded by up to six females who are usually related to each other (Schwarz 1986, 1987). Dissection data reveal no apparent reproductive hierarchies in newly founded colonies,

although they suggest the presence of some effectively sterile females in colonies that re-use their natal nest (Schwarz, 1986; Silberbauer & Schwarz, 1995; Schwarz, Lowe & Lefevere, 1996). Whilst the possibility of multiple mating has not been explicitly explored for *Exoneura* species, estimates of intra-colony relatedness among adult nestmates and female brood are relatively high ($r \approx 0.4 – 0.7$; Schwarz, 1987; Schwarz, Lowe & Lefevere, 1996; Langer, Hogendoorn & Keller, 2004; Langer *et al.,* 2006; Harradine, Gardner & Schwarz, 2012) and, given the existence of multiple egg-laying females in most colonies, are consistent with females being only singly mated.

Social parasitism has evolved at least 13 times in the Allodapini, probably because of the opportunities that communal progressive rearing provide for inter- and intra-specific cheating (Smith *et al*., 2013). Species of the Australian parasite genus, *Inquilina* Michener, are completely dependent on their hosts for brood-rearing; they have reduced mouthparts and pollen-collecting scopae and are rarely caught on flowers (Michener, 1965). These inquilines are host-specific (Smith & Schwarz, 2009) and have life cycles that phenologically closely tied to their hosts (Michener, 1965; Smith & Schwarz, 2006a,b). *Inquilina* commences egg-laying after its host and it is able to kill host brood (Smith & Schwarz, 2009), but the inquiline brood develops in synchrony with any remaining host brood and reaches adult eclosion at about the same time (Michener, 1965; Smith & Schwarz, 2009; results below). *Inquilina* has not been observed to kill or eject reproductive host females (Michener, 1965; Smith & Schwarz, 2009).

2.2. Calculating relative effective population sizes

Whilst effective population size, N_e, is a key concept in evolutionary biology, it is remarkably difficult to measure. While there are genetic methods involving allelic diversity at microsatellite loci or d_N/d_S ratios for protein coding genes, these methods can be very problematic if there have been historical changes in population size, such as bottlenecks, or selection on coding genes (e.g. Gregory & Witt, 2008; Whitney & Garland, 2010). In our study we do not attempt to estimate absolute N_e for hosts and their inquilines, but instead their relative sizes. We do this by estimating the likely ratio of reproductive females in host/parasite pairs, but we take into account the complicating effects of reproductive skew (uneven sharing of reproduction within cooperatively breeding animal groups) in host

colonies, relatedness among host nestmates, and sex ratio biases.

Here we collated data from large-scale nest collections, spanning >10 years, of three Australian allodapine bee species, each of which is attacked by its own inquiline allodapine species. Colony samples had been obtained for other studies on sociality and sex allocation in the host species, but records of colony contents taken at the times of collection allow us to use these data to explore rates of infestation and relative N_e of hosts and their inquilines. We predict that the N_e of inquilines will be much less than that of their hosts and we outline potential mechanisms for the inquiline's survival over evolutionary time.

3. Materials and Methods

3.1. Sources of data

Our data were taken from collections of entire colonies conducted over a series of studies on allodapine social behavior between 1989 and 2014 in two widely separated localities, the Dandenong Ranges and Cobboboonee State Forest, both in Victoria, Australia (Figure 2). Collection of nests was not influenced by the presence of inquilines since *Inquilina* has no effect on physical nest characteristics (Michener, 1965).

Sampling sites, collection dates and sample sizes are given in Table 1 along with *Inquilina* infestation rates (see Results, below). Our samples covered three host species: (i) *Exoneura robusta* Cockerell1922, which was only sampled from the Dandenong Ranges; (ii) *Exoneura nigrescens* Friese1899, which was only sampled from Cobboboonee Forest; and (iii) *Exoneura angophorae* Cockerell 1912, which was sampled from both regions. All three species are univoltine (Schwarz, 1986; Bull & Schwarz, 2001; Schwarz, Lowe & Lefevere, 1996). These species are infested by three inquiline species, namely *Inquilina schwarzi* Michener 1983, an undescribed species of *Inquilina*, and *I. excavata* (Cockerell 1922) respectively. The phylogenetic relationships between these three host species and the two described *Inquilina* species were described by Smith *et al*., 2013.

3.2. Collection methods

All samples were taken in the early morning, early evening, or during rainy weather when all nest occupants are present. Nests were stored in insulated boxes on ice for transporting to the lab where they were opened and all colony contents recorded. The Dandenong Ranges samples were collected from the region between Toolangi State Forest, Fern Tree Gully, and Gembrook in the Dandenong Ranges, east and north east of Melbourne. This region is wet montane forest with a tall eucalypt overstory (Figure 2). Nests occurred in the dead fronds of the tree fern *Cyathea australis*. The Cobboboonee samples were collected from Cobboboonee State Forest and the adjacent Glenelg National Park in western Victoria. This region comprises open eucalypt woodland interspersed with heathland, and nests were collected from the dead flower scapes of the grasstree genus *Xanthorrhoea* (Figure 2).

Nest collections were conducted from late spring (November) to late summer (February), corresponding to the major brood rearing period for allodapines in Victoria (Schwarz, 1986; Schwarz, Lowe & Lefevere, 1996; Silberbauer & Schwarz, 1995). During this period inquilines have already dispersed from their natal nests to take up residence in host colonies (Smith & Schwarz, 2006b, 2008), so our collections represent a period in host/inquiline life cycles when dispersal has been completed and brood rearing is underway.

Data recorded from each nest included the number of adult females of each host species, the number of adult inquilines, the total number of brood in each colony (counting callows as brood), and nest age in terms of whether or not the nest had been newly founded (new) or if it was being re-used after a previous year of brood rearing (old). Old nests can be identified by the presence of dark stains on the nest walls from earlier deposition of nectar and larval faeces (Schwarz, 1986, 1987). This distinction in nest age is important because colony sizes in new nests are smaller than in old nests (Schwarz, 1986, 1987, 1994; Schwarz, Lowe & Lefevere, 1996).

3.3. Co-presence of inquiline adults and brood in mature host colonies

When estimating relative effective population sizes of *Exoneura* and *Inquilina* it is important to know whether *Inquilina* females are nest-loyal and remain with their brood for life. This is

because if parasitic females switch nests during brood rearing, depositing eggs in each, then counts of adult female inquilines during brood rearing might underestimate the actual number of parasite brood being produced.

Determining whether inquilines switch nests would be indicated by the presence of inquiline brood in nests that lack an adult inquiline. However, distinguishing between *Exoneura* and *Inquilina* brood is difficult because their larval and pupal morphology is very similar (Michener & Syed, 1962). Instead we used one sample from Cobboboonee Forest, where we reared all post-feeding brood to the adult stage. All post-feeding larvae (prepupae) and pupae were transferred to tissue culture wells and maintained at ambient room temperature until they eclosed as adults. For this we obtained a total of 116 nests of *E. nigrescens* containing prepupae and pupae, collected between 30 December 1989 and 13 January 1990. Post-feeding brood in these nests were transferred to tissue culture wells and allowed to develop through to adult eclosion, where the identity of *Inquilina* was evident from adult morphology.

3.4. Virulence of *Inquilina*

Arms races between hosts and parasites can be impacted by the extent to which parasites reduce their host's reproductive output, which we will refer to as virulence. In this sense, higher virulence levels will exert higher selection on hosts to prevent infestation. However, there might also be selection on parasites to lower virulence if this means that parasite offspring are able to inherit a still-functioning host colony (Schmid-Hempel, 1998; Kirchner & Roy, 2002; Galvani, 2003).

We examined virulence from two collections from the Dandenong Ranges in late summer (25-26 February 2013 and 27 February 2014) when nearly all brood had reached pupal stage or were callow adults. This is a colony phase prior to dispersal of either host or parasite brood, and pupae were reared through to adult eclosion in the lab to determine species.

3.5. Statistical analyses

Because our data were derived from multiple collections over many years, pooling data within species is not straightforward because of possible year effects. Therefore, before

combining host samples within localities, we tested where infestation rates differed across sampled years for each host species using Chi-square analyses. However, in some cases we conducted the Fisher's exact test as infestation rates were so low (they did not reach the minimum expected counts of 5 or more for each cell in a 2x2 table).

We continued our analyses by asking whether rates of inquiline infestation of host colonies differed across host/locality combinations when all samples were combined, and we then explored whether infestation rates varied with host-colony age (new or old) and with colony size.

3.6. Calculating relative effective population sizes (N_e)

Although N_e is a key concept in evolutionary genetics, it can be extremely difficult to estimate. For haplodiploids like bees, it will be affected by variation in the number of offspring per individual, as well as sex ratios (Hedrick & Parker, 1997), and for social insects these will be determined by factors such as reproductive skew, colony productivity, and sex ratios. In allodapine bees at least, these factors can interact in complex ways (Schwarz, Richards & Danforth, 2007). For example, skew can be influenced by relatedness among nestmates (Langer, Hogendoorn & Keller, 2004; Harradine, Gardner & Schwarz, 2012), whilst skew is also associated with colony productivity (Schwarz, 1986), and both of these co-vary with sex allocation (Schwarz, 1994).

For our calculations we considered how previous studies of intra-colony relatedness, skew, colony size and sex ratios may affect N_e in the host species *Exoneura robusta*, which is the most thoroughly studied allodapine to date, and the species for which we also have the most extensive data on *Inquilina* infestation. When estimating key parameters affecting N_e, we took the most conservative approaches that tend to underestimate true N_e, and then considered how more realistic approaches could affect N_e.

4. Results

Inquilina infestation rates for our samples are summarized in Table 1. Chi-square tests indicated no significant year effects for re-used nests of *Exoneura nigrescens* (N = 626, P =

0.092), *Exoneura angophorae* (N = 280, P = 0.25), but a marginally significant effect for *E. robusta* (N = 363, Fisher's exact test, P = 0.043). The latter significant effect is largely due to a lower than expected number of infested nests in 1993 (0 observed infested nest versus an expected count of 0.1, from a total two nests), 2002 (13 observed infested nests versus an expected count of 17.3, from a total of 299 nests) and a higher than expected frequency in 2013 (8 versus 3.7 from a total of 64 nests). However infestation rates in these three years were very low (0%, 3.6% and 2.2% respectively), suggesting that overall infestation rates are likely to be small.

We obtained 21 nests of *Exoneura angophorae* from Cobboboonee Forest, none of which contained *Inquilina* species, whereas we obtained 280 nests from the Dandenong Ranges of which 15.7% contained *Inquilina excavata*. Although the *Inquilina*-infested proportion of nests was only marginally significantly different between these two samples (X^2= 0.113, P = 0.049), the Cobboboonee sample was small and the power of our test is therefore low. Consequently, we did not include the Cobboboonee samples in further analyses.

We then compared overall rates of *Inquilina* infestation for the *E. angophorae, E. robusta* and *E. nigrescens* samples using Chi-square analysis. This indicated a significant difference in infestation rates (X^2 = 47.681, P < 0.001, Table 1). The results in Table 1 suggest that the major difference in infestation rates involves the higher rate in *E. angophorae*, and when this species was removed from analysis there was no difference between *E. robusta* and *E. nigrescens* (X^2 = 3.269, P = 0.071).

We then examined whether infestation rates differed between newly founded and re-used nests in each of the three host species using logistic regression where presence/absence of *Inquilina* was the dependent variable, and nest age and host colony size (number of adult host females) were the independent variables. For these analyses, nests where age status was unknown were not included. Our data suggest that inquilines were more frequently found in old compared to new nests in all species, so that 14.1%, 3.7% and 2.6% of old nests were infested by inquilines in *E. angophorae, E. robusta and E. nigrescens* respectively, while those of new nests were 2.0%, 1.3% and 0.0%. Moreover, logistic regression analysis indicated that colony age was a statistically significant predictor of inquiline infestation in *E. angophorae* (P < 0.001) and *E. robusta* (P = 0.002) but not for *E. nigrescens* (P = 0.994,

Table 2). Colony size did not predict inquiline infestation in any of the species when colony age was included as a categorical independent variable ($P > 0.05$ for all three tests, Table 2).

4.1. Brood rearing results

Of the 116 *E. nigrescens* nests (from Cobboboonee, December 1989 and January 1990) where post-feeding brood were reared to maturity, twelve (10.3%) contained an *Inquilina* adult from the parental generation. Only four of these (33.3%) also contained *Inquilina* brood, ranging from 1 to 11 prepupae/pupae per nest. Of the 104 nests without an *Inquilina* adult, only two (1.9%) contained *Inquilina* post-feeding brood (comprising three and four brood respectively). Consequently, *Inquilina* brood can be reared to maturity by hosts in the absence of an *Inquilina* adult, but the proportion of such nests in our sample is very small (1.9%). When combined, these 116 nests contained 594 host and 25 *Inquilina* post-feeding brood reared to adult stage, and of these 25 *Inquilina*, 12 were female. Consequently, 4.04% of all post feeding brood in these nests were inquilines, and 1.94% were female inquilines.

4.2. Virulence results

Our 2013 and 2014 February (late summer) samples retrieved a total of 36 nests of *Exoneura robusta* containing an *Inquilina* adult and eight such nests of *E. angophorae*. Brood in 33 of the infested *E. robusta* nests and seven of the infested *E. angophorae* nests only comprised pupae or callows. Of the remaining nests with some larvae, the maximum number of larvae per nest was two, and these could have been host or parasite brood. Consequently, our estimates of the number of *Inquilina* brood per nest, based on post-feeding brood, are likely to closely represent actual virulence levels. The proportion of *Inquilina* among pupae and callows from *E. robusta* nests is summarized for different brood numbers as a box plot in Figure 3. A total of 50% of *E. robusta* nests with 10 or fewer brood did not contain any *Inquilina* brood, and the overall proportion of parasite brood, combining all infested nests, was 20.5%. Of the eight parasitized *E. angophorae* nests, only four contained an *Inquilina* pupa and in each case this was only a single female. However total brood numbers in the *E. angophorae* nests were very low and the total proportion of pupae/callows, pooled across all eight nests that were parasites was 21.9%.

4.3. Estimating relative N_e for *Exoneura robusta* and its parasite *Inquilina schwarzi*

Of the 300 *Exoneura robusta* nest in our sample where nest age was recorded, 204 (68%) were newly founded and 96 (32%) were re-used. However, our data were not always from exhaustive sampling in a region. In an exhaustive collection of *Exoneura robusta* nests from a study site in the Dandenong Ranges shortly after foundress dispersal, Schwarz (1986) found that of a total of 97 nests, 73 (~ 75%) were newly founded nests and 24 (~ 25%) were re-used nests, and these contained an average of 2.6 and 4.4 females respectively. In a genetic study of the same population that used microsatellites to estimate reproductive skew, Langer *et al*., (2006) obtained an overall estimate of skew that was very small, but different from zero (B = 0.06 ± 0.03) and that skew estimates did not differ between newly founded and re-used nests. However, they pointed out that the low value of skew in re-used nests could be false, given small sample sizes for that nest category. These results broadly match those from dissection data (Schwarz, 1986) which found that all foundresses in newly founded nests had large ovaries, and that most re-used nests contained multiple reproductive females, though some also contained effectively sterile females. Because there is only ever one *Inquilina* female in brood rearing colonies of *E. robusta*, we can use the above parameters to calculate the relative number of reproductive *Inquilina* females to host females. Using an extremely conservative approach we could assume no skew in newly founded nests and complete skew in re-used nests (i.e. only one reproductive female per nest). Using the proportion of newly founded to re-used nests reported by Schwarz (1986), and assuming *Inquilina* infestation rates of 1.3% and 3.7% respectively (above), and also assuming that infested nests produce no host brood at all, we can then estimate the proportion of *Inquilina* females per nest as (0.75 * 0.013) + (0.25 * 0.037) = 0.019 females per nest. If we then assume that hosts never reproduce in infested nests we can calculate the mean number of reproductive host females per nest as {(0.75-0.013)*2.6 + (0.25-0.037)*1} = 2.129. This suggests a value of approximately 1 *Inquilina* female per 100 reproductive host females.

Some important caveats must be placed on the calculations above. Firstly, dissection data (Schwarz, 1986) and estimates of skew (Langer *et al*., 2006) indicate the presence of more than one reproductive host female in re-used nests, such that the above calculations will underestimate the relative of N_e of hosts to inquilines. Secondly, these calculations assume

no host brood is produced in infested nests, whereas our data indicate low virulence, and this assumption will again underestimate host N_e. Thirdly, relatedness between nestmates will tend to decrease N_e for the host species, and we now consider this.

Using microsatellite data from the Dandenong Ranges population of *Exoneura robusta* Langer *et al*., (2006) obtained an overall intra-colony relatedness estimate for adult females of r = 0.39, and separate estimates for newly founded and re-used nests did not significantly differ. Using larger samples, but from the same Dandenong Ranges locality, Harradine, Gardner & Schwarz (2012) found a very similar relatedness estimate (r = 0.40), but found significant variation across colonies. It is not straightforward to use these estimates of relatedness to adjust N_e because of variation in intra-colony relatedness and differences in colony size. However, we could again take a highly conservative approach and assume only one reproductive host female in each re-used nest and also assume that reproductive contributions from multiple females in newly founded are only equivalent to the genetic contribution of a single female, then we would estimate that for each *Inquilina* female there are roughly 50 reproductive host females. This is very clearly an underestimate of relative N_e because it assumes that there is only one reproductive female in re-used nests, and that the multiple reproductive females in newly founded nests are effectively genetic clones. Nevertheless, it indicates a huge disparity in N_e of hosts and inquilines.

Lastly, we need to consider the effect of sex ratio bias on estimates of N_e. Bees are haplodipoid, and in haplodiploids sex ratio bias distorts N_e differently from diplodipoids because males donate their entire genome to each of their daughters, whilst sons arise from unfertilized eggs. N_e as a function of sex ratio in haplodiploids is given by the equation $N_e = 9\,N_f N_m/(2N_f + 4N_m)$ where N_f and N_m refer to the number of females and males respectively (Wright, 1933; Hedrick and Parker, 1997). We graph this function in Figure 4 where we use an arrow to indicate the reported population-wide sex ratio for *Exoneura robusta*, r ≈ 0.15, based on a sample of 225 nests containing 1857 pupae (Schwarz, 1988). Using sex ratio bias in *E. robusta* to adjust estimates of relative N_e is not trivial because sex ratios vary with colony size and colony age (Schwarz, 1988) and both of these also affect colony productivity, which will influence the genetic contribution of female nestmates to future generations. Nevertheless, Figure 3 indicates that female biased sex ratios have the potential to greatly reduce N_e, and reported sex ratios for *E. robusta* suggest that N_e could be decreased by nearly

50% compared to an unbiased sex ratio. However, this would not be sufficient to bring the disparity in N_e between *Exoneura robusta* and *Inquilina schwarzi*, calculated above, below an order of magnitude, especially given the extremely conservative estimates of relative N_e that we have provided.

5. Discussion

Interpretation of our data is complex because empirical estimation of effective population size, *per se*, is not straightforward (e.g. Woolfit, 2009), and made even more difficult by the effects of haplodiploidy (Hedrick & Parker, 1997), relatedness among host nestmates, reproductive skew in host colonies, and sex ratio bias. However, we have taken an extremely conservative approach that is likely to greatly underestimate the relative N_e of hosts, compared to their inquilines, and this still yields a massive difference in relative N_e, suggesting that host species are likely to have a much greater capacity for evolutionary change in any arms races.

We found that overall rates of inquiline infestation are not statistically different between *E. robusta* and *E. nigrescens,* with rates of ~5.8% and ~3.4% respectively, but differ from *E. angophorae* where the rate is ~15.7%. However, infestation rates will underestimate relative N_e of hosts compared to their inquilines because most host colonies contain more than one reproductive female, and because many inquiline females fail to reproduce. Whilst female biased sex ratios in the host species will tend to decrease N_e, this has only a marginal effect, compared to the large discrepancy in the number of reproductive hosts and their inquilines.

Our findings of low levels of infestation by social parasites match some studies on other socially parasitic species (e.g. Schönrogge *et al.,* 2002; Thomas, Schönrogge & Elmes, 2005; Biani & Wcislo, 2007). For instance, infestation rates of ant inquilines have been estimated as ranging from 1 to 15% (Davies, Bourke & de L Brooke, 1989). For the polistine social parasites *Polistes sulcifer* and *P. atrimandibularis*, parasitization rates of four *Polistes* hosts ranged from 0-30% (Fanelli, Cervo & Turilazzi, 2001), though the latter value was based on only seven host colonies, For host species where > 40 colonies were sampled, parasitization rates varied from 4.6-17.5%. Low population sizes of socially parasitic bumblebees has also been noted as an extinction risk (Suhonen, Rannikko & Sovari, 2015), although the rate of

parasitism in the European bumblebee *Bombus terrestris* by the social parasite *B.* (*Psithyrus*) *vestalis* has been estimated at approximately 42% (Erler & Lattorf, 2010). At the same time, Carvell *et al.,* (2008) found that experimental colonies of *B. terrestris* suffered invasion rates 62-97% by *B. vestalis* and those rates varied with floral resource availability. Goulson (2003) also noted that the frequency of bumblebee nests infested by *Psithyrus* is highly variable across localities and years, but reported infestation rates by *Psithyrus* do appear to be much higher than for allodapine bees, *Polistes* wasps and ant inquilines. Although our allodapine dataset suggest insignificant infestation rates across sampling years in *E. angophorae* and *E. nigrescens*, and just marginal significance in *E. robusta*, we cannot rule out that the effect of year might be impacted by the irregular samplings carried out over years.

5.1. Implications for evolutionary races between hosts and parasites

Theoretical arguments linking rates of adaptive evolution to effective population size are well developed (e.g. reviews by Woolfit, 2009, and Lanfear, Kokko & Eyre-Walker, 2014), so the persistence of enemies that are locked into evolutionary arms races, but which have very unequal N_es, raises a critical evolutionary puzzle: how can a species with lower potential for evolutionary change survive competition with its enemy, when the enemy should have higher rates of adaptive evolution? We now propose several mechanisms that might allow this to occur:

(i) Accelerated evolutionary rates in inquilines. It is possible that inquiline lineages have been able to increase rates of adaptive evolution above those that are based purely on N_e. For example, they may have evolved mechanisms that increase recombination rates (Hill & Robertson, 1966; Felsenstein, 1974) and/or increased mutation rates (Biémont & Vieira, 2006; Biémont, 2010; Pritham, 2009). However, small N_e combined with heightened mutation rates should also increase the rate at which deleterious mutations become fixed, and this should impose long-term genetic load on inquilines (Lynch & Conery, 2003; Lynch, 2007a,b).

(ii) Trade-offs between host cooperation and defence against parasites. Cooperative nesting provides strong benefits for many allodapine species (Schwarz, Richards & Danforth, 2007) and can involve nesting between unrelated conspecifics (e.g. Schwarz & O'Keefe, 1991;

Melna & Schwarz, 1994; Zammit, Hogendoorn & Schwarz, 2008; Harradine, Gardner & Schwarz, 2012). If discrimination mechanisms that increase rejection of inquilines from host colonies also lead to greater rejection of potentially beneficial conspecific cooperators, there could be a trade-off between rejecting potentially beneficial cooperators and allowing inquilines to gain entry. It is possible that this trade-off could limit the ability of hosts to evolve inquiline exclusion strategies.

(iii) Rates of parasitization are too low to generate strong selective responses in hosts. Inquilines are completely dependent on their hosts for reproduction, so that selection on them to overcome host defences will be extreme. However, if inquiline population sizes are low and infestation rates also low, then selection pressures on host species to prevent attack may be low. Such a situation could lead to fluctuating selection pressure on hosts, where there is strong selection pressure when inquilines are common, but low pressure when inquilines are rare (Erler *et al*., 2014). The evolutionary dynamics here may be non-trivial and may also interact with selection on host virulence, which we discuss later.

(iv) Inquilinism is not able to survive long-term evolutionary races. Lastly, it is possible that because of much smaller N_e values, inquilines are indeed unable to survive in long-term evolutionary arms races with their hosts. In the case of *Inquilina*, this might seem unlikely, given that the crown age of this genus is between 15-20 Mya (Smith *et al.,* 2013). However, one possible escape from such long-term races might arise if inquiline species are able to occasionally switch from a long-term host, where host defenses have been gradually improving, to a new host that has not had recent evolutionary 'experience' with that inquiline lineage. In such cases it may take substantial time for a new host to evolve defenses against any such 'novel' parasite species, and that once such defenses gradually become effective, the parasite may be able to switch hosts again. This possibility could be assessed by determining whether host switching is frequent in inquiline lineages. We note such escaping from host defences need not require that new hosts are free of other inquiline species at the time of switching, but it would require that adaptations to any existing inquiline associations do not provide effective defense against the new, switching, inquiline species. In fact, if such host switching does occur, we might expect that the new 'switching' species would have substantial advantages over an older parasite species, because of its novelty to the new host, and would outcompete the older inquiline species. Consequently, this hypothesis predicts

both host switching by inquilines and increased extinction rates for inquilines with long-term associations with a particular host lineage.

The arguments above consider only infestation rates per se, but virulence of inquilines is also an important consideration. The low rates of virulence that we found surprised us. These rates could reflect an ability of host species to lower the efficacy of parasites once they have entered a nest, but they could also reflect selection for lowered virulence of parasites. Lowered virulence can be expected under various circumstances (e.g. Schmid-Hempel, 1998; Galvani, 2003), but two appear to be especially pertinent to our system. Firstly, if parasites are host-specific and different host species are in competition, then parasites with higher virulence may overly disadvantage their host species in that competition and therefore lower their own future prospects for obtaining new hosts (Kirchner & Roy, 2002). Secondly, if there is vertical transmission of parasites, then high virulence will lower the likelihood of inquiline offspring being able to 'inherit' a host offspring (in our case, colonies surviving into later years of brood rearing). Degradation of hosts for future exploitation can lead to selection for lower virulence (Galvani, 2003). Bees in the genus *Exoneura* can re-use nests over many generations (Schwarz, Richards & Danforth, 2007), so parasite virulence in any one year would involve a trade-off between vertical and horizontal transmission. If gaining entry to a new host nest is very costly, but remaining in a natal host colony has few costs, we would expect selection for low virulence. Assessing this hypothesis is tractable in at least two ways: (i) if inquilines are able to assess the suitability of a host nest for future re-use by hosts, they should increase virulence as the host nest approaches its 'use-by' date; (ii) if different inquiline species have hosts whose nesting substrates differ in longevity, higher rates of virulence should occur where substrates are more ephemeral.

6. Conclusion

The importance of unequal effective population sizes between hosts and parasites has been recognized for many coevolutionary systems, but has been almost entirely neglected for inquiline parasites where N_e can be much smaller than for the host. This situation is the reverse of the Hamilton-Zuk hypothesis where parasites have much larger N_es than hosts. Our data indicate that such 'reverse' situations do, in fact, arise and they entail important evolutionary puzzles for how parasites with low N_e manage to persist over evolutionary time.

Future studies will need to consider these puzzles, with an emphasis on rates of evolution where both capacities for adaptive evolution, and selection pressure for evolutionary change, differ between hosts and social parasites. It seems likely that for hosts and their social parasites, co-evolutionary arms races are likely to be lop-sided competitions in more ways than one.

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Table 1. Sample sizes, collection dates and *Inquilina* infestation rates for three *Exoneura* species obtained over two regions.

Host species	Collection dates	Non-infested nests	Infested nests with *Inquilina*
Exoneura angophorae (Dandenong Ranges)	1992,1993, 2002, 2013	236 (84.3%)	44 (15.7%)
Exoneura angophorae (Cobboboonee Forest)	1989	21 (100%)	0 (0%)
Exoneura nigrescens (Cobboboonee Forest)	1989, 1990	605 (96.6%)	21 (3.4%)
Exoneura robusta (Dandenong Ranges)	1993, 2002, 2013	344 (94.2%)	21 (5.8%)

Table 2. Logistic regression of *Inquilina* infestation (presence/absence) onto colony age (as a binary variable of 'newly founded' and 're-used') and colony size (as an interval variable).

	Colony age			Colony size		
Host species	Sig.	B	Wald	Sig.	B	Wald
E. angophorae	.000	2.632	27.355	.892	.021	.018
E. nigrescens	.994	19.288	.000	.269	-.321	1.223
E. robusta	.002	1.911	9.854	.716	-.51	.133

Figure 1. Two-females nest of *Exoneura robusta*, showing adults, eggs and larvae in the rear half of a nest tunnel in a frond of the tree fern *Cyathea australis*. Nest tunnels are unbranched and undivided by cells, and brood are reared progressively by adults.

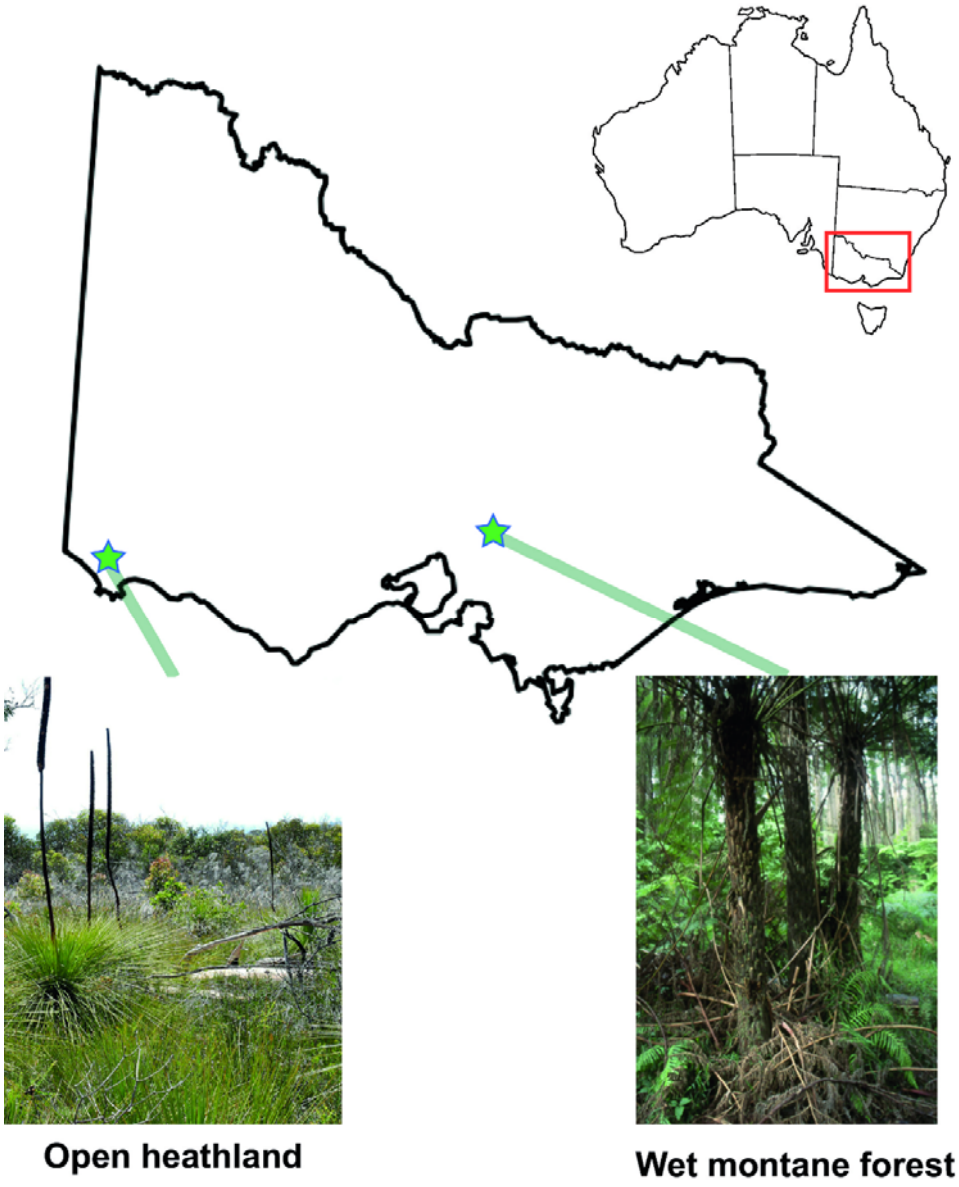

Figure 2. Collection localities of *Exoneura* and *Inquilina* species in this study.

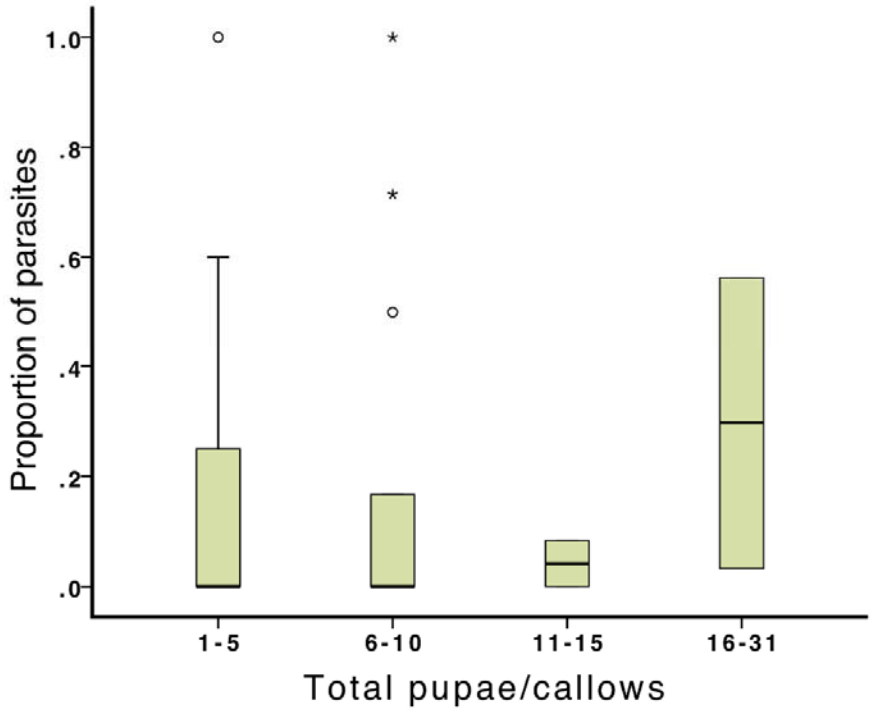

Figure 3. Inter-quartile boxplots of the proportion of inquilines among mature brood (pupae and callows) as a function of brood number for 33 nests of *Exoneura robusta* that had been infested by *Inquilina schwarzi*. Colonies were collected in late summer 2013 and 2014 from the Dandenong Ranges. Hollow circles represent outliers and asterisks represent extremes.

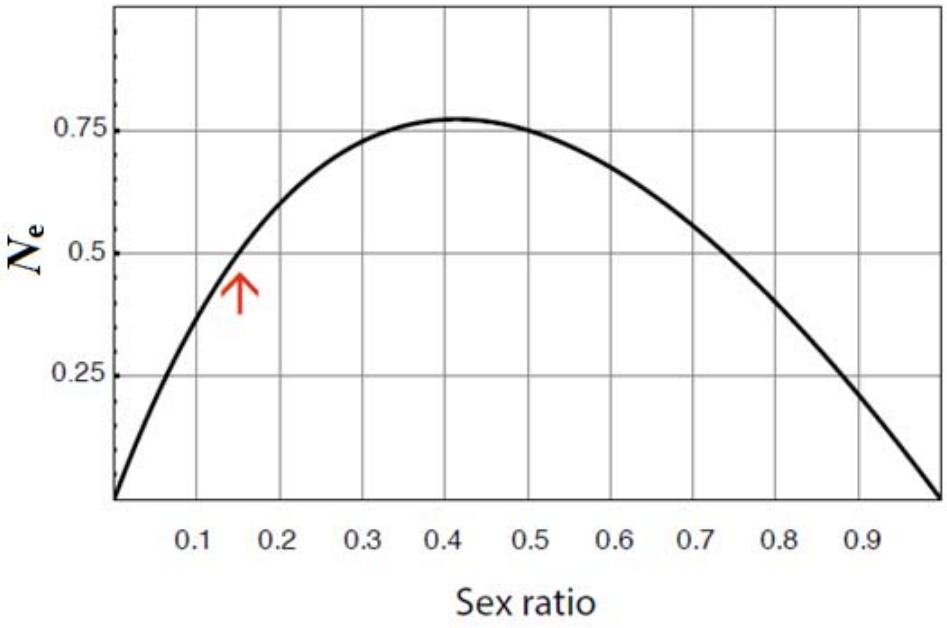

Figure 4. Graph of effective population size (N_e) as a function of sex ratios for haplodiploids, $N_e = 9\ N_f N_m/(2N_f + 4N_m)$, where N_f is the number of females and N_m is the number of males. The red arrow indicates the population-wide sex ratio of *Exoneura robusta*, r ≈ 0.15.

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Chapter II:

Sex Ratios in a Socially Parasitic Bee and implications for host-parasite interactions

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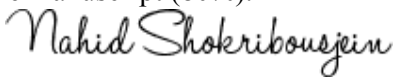
Contribution to the paper: Carried out filed work and lab experiment, analyzing of the data and writing the manuscript (80%).

Signature:  **Date:** 2.7.2017

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Contribution to the paper: Assisted in lab experiments(0.5%).

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Contribution to the Paper: Designer of research, assisted in fieldwork, supervised the direction of the study, gave advice on analyses and critically reviewed the manuscript (15%). **Signature:**  **Date: 2.7.2017** **Date:**

1. Abstract

Obligate social parasites of Hymenoptera, known as inquilines, have received enormous attention due to the elaborate adaptations they exhibit for exploiting their hosts, and because they have frequently been used to infer sympatric speciation. Their population biology can be difficult to infer as they are both rare and difficult to extract from host nests. Sex allocation has been studied for very few inquilines of social Hymenoptera. Here we report sex ratio patterns in the allodapine bee *Inquilina schwarzi,* which is an obligate social parasite of another allodapine, *Exoneura robusta*. We show that the sex ratio of this inquiline varies with its brood size, it is female-biased in the smallest brood, but becomes more even in larger brood, where the population-wide sex ratio is close to parity. We argue that this pattern of bias is consistent with local resource competition, where inquiline females compete to inherit their natal colony. We also argue that extremely female-biased sex ratios of the host species, combined with overall sex ratio parity in the parasite, may help ameliorate disparity in effective population sizes between these two species, which are locked in an evolutionary arms race.

Key Words: Bees, effective population size, inquilines, local resource competition, sex ratio, social parasites.

2. Introduction

Allodapine bees (family Apidae, tribe Allodapini) provide a model taxon for examining sex allocation bias. Nearly all species where sex allocation has been examined exhibit female biased allocation, with the proportion of males among maturing brood being as low as, or lower than 15% in several genera (Schwarz et al. 2007). This bias has largely been attributed to local resource enhancement (LRE, Schwarz 1988) and the production of 'insurance daughters' who play an alloparental role in the event of maternal death prior to brood maturation (Bull and Schwarz 2001; Aenmey et al. 2006; Thompson and Schwarz 2006). Both models assume a non-linear increase in fitness through investment in daughters, until an optimal number of daughters has been produced within a brood (West 2009).

Most detailed studies of allodapine sex allocation have indicated that the degree of bias depends strongly on colony size (Schwarz et al. 2007). For example, a tropical allodapine bee, *Macrogalea* Malawi sp., has a very clear shift away from female-biased sex ratios once colonies contain more than six adult females, indicating the ability of females to assess the number of their nestmates (Thompson and Schwarz 2006). In another allodapine, *Exoneura nigrescens*, sex allocation varies quite precisely with the number of brood in a nest, indicating a remarkable ability of reproductive females to anticipate their final brood numbers (Bull and Schwarz 2001). The genus *Macrogalea* is a sister clade to all other allodapines and *Exoneura* Smith is one of the most distal clades (Chenoweth and Schwarz 2011), suggesting that the ability to adjust sex ratios according to colony size is an ancestral trait for the Allodapini. The crown age of the allodapines is in the early Eocene (Rehan et al. 2012), so we might expect a very long period of selection on an ancestral ability of allodapines to adjust sex allocation to proximate conditions in ways that maximize fitness.

Many allodapine bees are attacked by other allodapine species that have evolved a socially parasitic strategy. Social parasitism has evolved at least 13 times in the Allodapini, more than all other bees and wasps combined (Smith et al. 2013). Most of these origins have resulted in obligate parasitism, referred to as 'inquilinism' (Smith et al. 2013). In these inquilines, parasitic species enter host colonies and replace some of the host brood with their own, which are then reared by the host species (Michener 1965; Smith and Schwarz 2006b).

Sex allocation strategies in hymenopteran inquilines have rarely been explored because rates of infestation are very low (Shokri Bousjein et al. 2016) and determining inquiline sex allocation patterns requires the ability to sample sufficient parasitized colonies to characterize inquiline allocation strategies. In a study on two bumble bee social parasites (genus *Bombus*, subgenus *Psithyrus*), Fisher (1992) argued that female biased sex allocation may be due to local mate competition (LMC). LMC arises when close male relatives compete with each other for access to females and could arise if males show strong philopatry, for example by mating within or close to their natal nest (Alexander and Sherman 1977; Werren 1980). In one inquiline parasitic ant *Plagiolepis xene*, a female biased sex ratio has also been attributed to local mate competition arising from sib-mating within nests (Aron et al. 1999).

Inquilina schwarzi Michener 1983, is an Australian allodapine inquiline that infests nests of *Exoneura robusta* Cockerell 1922 (Smith and Schwarz 2006b, 2009). Inquiline females delay oviposition until host egg-laying is near completion, and is potentially a strategy to allow the inquiline's eggs to acquire odour from the host's eggs to make them less detectable by the hosts (Smith and Schwarz 2006b). Once inquiline egg production has commenced, some, but not all, host eggs are destroyed by the inquiline (Smith and Schwarz 2006b). In infested colonies *Inquilina* Michener brood development and maturation occur in synchrony with that of the host brood (Michener 1965) where they eclose as adults at the same time (Shokri Bousjein et al. 2016). Importantly, in most *Inquilina*-infested host nests, not all host brood are killed by the inquiline. This may be a strategy that allows one of the inquiline's daughters to 'inherit' a host colony (Shokri Bousjein et al. 2016), avoiding the costs of dispersal difficulties of infiltrating a new host colony.

Here we explore sex ratios of the inquiline *I. schwarzi* based on samples obtained during brood maturation over a three year period and explore what kinds of selective mechanisms might shape sex allocation patterns.

3. Methods

3.1. Nest collection

Colonies of *E. robusta* were collected from the Dandenong Ranges, Victoria, Australia, between Kee Wee Rup North and Gembrook. This area comprises tall wet eucalyptus forest, and colonies were found in dead fronds of the tree fern *Cyathea australis*. Bee nests were collected whole in intact dead fronds of *C. australis* during early mornings and early evenings of late February in 2013, 2014 and 2015, when all colony occupants were present in the nest. Fronds were transferred to insulated boxes on ice and transported to Flinders University where they were kept at 4˚C until opening.

3.2. Estimating sex ratios

Our samples were taken at a time when host and inquiline brood were in the process of eclosing into adults, and therefore colonies comprised a mixture of parental generation females, newly eclosed brood, and late-stage immatures. Calculating *Inquilina* sex ratios at this time is not straightforward since it was not possible to confidentally decide whether any particular adult inquiline female was from the parental generation or a recently eclosed brood member. Wing wear as a function of foraging activity can be used to assign females to generational cohorts in the host species *E. robusta* (Schwarz 1986) but, being social parasites, *Inquilina* do not forage (Michener 1965; Smith and Schwarz 2006b) and wing wear may not correspond to age. However, a recent study (Shokri Bousjein et al. 2016) indicated that approximately 98% of nests with maturing inquiline brood also had the mother of those brood still present. Therefore, in our study inquiline brood sex ratios would be better estimated by treating one of the adult inquiline females in each nest as a mother rather than a brood.

Given the above considerations, we estimated inquiline sex ratios in two ways: (i) by treating each inquiline adult or immature as a member of the brood; and (ii) by treating one adult inquiline female as a mother and calculating sex ratios from the remaining inquiline individuals. We subsequently refer to these sex ratios as ‘raw’ and ‘adjusted’ sex ratios respectively. Raw sex ratios are highly likely to over-estimate the proportion of inquiline females in a brood, because including a mother will falsely inflate estimates of female

allocation, especially where brood numbers are very small. On the other hand, 'adjusted' sex ratios may slightly underestimate female biased allocation if an inquiline mother was no longer present in a host nest.

4. Results

A total of 53 *E. robusta* colonies containing *I. schwarzi* adults or brood were collected from February 2013 (N = 6 colonies), February 2014 (N = 30) and February 2015 (N = 17).

Before analyzing sex ratios we examined whether the total number of inquilines, hosts and adjusted and non-adjusted *Inquilina* sex ratio differed across our three samples (2013 – 2015) using non-parametric Kruskal Wallis tests, as sex ratio data is 0 -1 truncated. These results indicated no significant yearly effects for any of the variables ($X^2_2 = 5.660$, P = 0.059; $X^2_2 = 1.133$, P = 0.567; $X^2_2 = 0.396$, P = 0.820; $X^2_2 = 2.022$, P = 0.364, respectively), so the yearly samples were pooled for subsequent analyses.

We then explored whether raw and adjusted *Inquilina* sex ratios were influenced by the number of hosts and inquilines, based on adults and post-feeding brood reared to adulthood, using generalized linear modeling (GLM) where normal distribution of errors was also assumed in this analysis. GLM was used because QQ plots (not shown here) indicated non-normality for both the dependent variable (inquiline sex ratio) and the independent variables (number of hosts and number of inquilines). When we examined the adjusted *Inquilina* sex ratio data, our GLM analysis indicated a highly significant effect of *Inquilina* numbers (β = 0.094, 95% likelihood ratio $X^2_1 = 7.262$, P = 0.007, 95% profile likelihood CI = 0.03 – 0.159) but no effect of the total number of *E. robusta* adults (β = 0.002, 95% likelihood ratio $X^2_1 = 0.039$, P = 0.884, 95% profile likelihood CI = -0.018 – 0.022). When we examined raw sex ratio data we found a highly significant effect of the number of *Inquilina* (β = 0.072, 95% likelihood ratio $X^2_1 = 23.714$, P < 0.001, 95% profile likelihood CI = 0.046 – 0.099) but no effect of the total number of *E. robusta* adults (β = -0.003, 95% likelihood ratio $X^2_1 = 0.0.329$, P = 0.566, 95% profile likelihood CI = -0.012 – 0.007).

The adjusted sex ratio of *I. schwarzi* as a function of the number of inquilines present in a nest is summarized as a boxplot in Figure 1. This shows that when there is only one inquiline brood in a nest, the inquiline sex ratio is strongly female biased, and as brood number increases the sex ratio becomes less biased and maybe even male biased, though our samples for larger *Inquilina* brood numbers are very limited. A non-parametric correlation indicates that this pattern is significant (Spearman's $\rho = 0.675$, N = 15, $P = 0.006$).

Lastly, we estimated population-wide inquiline sex ratios in two ways: (i) firstly we calculated the raw and adjusted sex ratio for each colony and then obtained a mean across these estimates and the 95% confidence interval for these means was obtained via bootstrapping with 1000 pseudoreplicates; (ii) secondly we pooled the total number of inquiline brood across all nests from all collections and used these to calculate a point estimate of sex ratio, where each inquiline individual was treated as an equally weighted data point. Mean raw sex ratio was estimated as r = 0.223 (95% CI = 0.094 – 0.385) and mean adjusted sex ratio was 0.280 (95% CI = 0.125 – 0.491). The population-wide sex ratio for the raw data (where every female is included) was r = 0.22 and for the adjusted data (where one *Inquilina* in each nest is regarded as a mother and not used to calculate sex ratios) it was r = 0.47. It is not possible to calculate confidence intervals for this latter estimate.

5. Discussion

Our data indicate that sex ratios in *I. schwarzi* vary with inquiline brood number, regardless of whether one of the *I. schwarzi* adult females is treated as the mother or whether all adults and pupae are treated as brood. Furthermore, sex ratios in *I. schwarzi* are strongly female biased for the smallest brood numbers and become less biased, or even male-biased, in larger brood. However, the total number of males and females recovered across the three years of our study is close to parity when one adult female was regarded as the mother of the remaining adults and pupae, suggesting that at a population level sex allocation is unbiased. Our findings of even sex allocation of *I. schwarzi* match some previous studies on other socially parasitic species. For instance, sex-investment ratios were found to be quite even in *Epimyrma ravouxi*, *Harpagoxenus sublaevis*, *Leptothorax duloticus*, and *Formica subnuda* which all are slave making ants (Bourke et al. 1988; Bourke 1989; Bourke and Franks 1995; Savolainen and Deslippe 1996).

In slave-making ants, brood are reared by the host workers, while slave-maker workers can less easily enforce their own interests by manipulating resource allocation to male or female larvae. Therfore, it seems that the slave-maker queen rather than the slave-maker workers is in control of sex ratios (Ettorre and Heinze 2001).

We now discuss possible evolutionary drivers for the observed patterns in *I. schwarzi*.

Fisher (1992) argued that female biased sex allocation in two socially parasitic bumble bees in the subgenus *Psithyrus* may be due to local mate competition (LMC). However, Bourke (1997) argued that this was unlikely because LMC predicts increasing female bias with larger brood numbers, whereas no effect of brood number sex ratios was found in those data. LMC arises when close male relatives compete with each other for access to females. In that case, competition between the males will result in some failing to mate, and the production of those males would therefore be a waste of the mother's resources, so mothers would preferentially adjust the sex ratio to be female-biased. For *I. schwarzi* LMC could occur if male inquilines show high levels of philopatry and if local populations are sufficiently small to make competition between close relatives likely. We are unable to exclude this possibility for *I. schwarzi*; however, LMC leads to declining rates of mean fitness returns through male investment and therefore predicts a decrease in male investment as brood number increases (West 2009), opposite to what we find. Therefore, our data are incompatible with LMC.

Local resource enhancement (LRE) arises when close female relatives cooperate in a way that leads to increasing per capita fitness returns through females as a function of investment in daughters. Although LRE predicts greater female bias in small brood (Schwarz 1988), as we find for *I. schwarzi*, it is difficult to see how inquiline females would be able to cooperate in a way that increases their mean fitness. Although *I. schwarzi* females can remain in their natal nests during autumn and winter, this period does not involve any oviposition or brood rearing, and females disperse in spring so that there is only one inquiline female per host nest during brood rearing in spring and summer. We therefore believe that LRE is unlikely to operate in *I. schwarzi*.

Lastly, Local resource competition (LRC; Clark 1978) arises when females compete for limited resources, such that increasing investment in daughters leads to declining returns in

mean daughter fitness. LRC predicts female bias in the smallest brood numbers, with increasing investment in males as brood numbers become larger, which is consistent with our data on *I. schwarzi*. LRC could arise if inquiline sisters compete to inherit their natal host nests and where successful inheritors have greater mean fitness than females who are forced to disperse and invade new hosts. We believe that this scenario is highly plausible for two reasons. Firstly, any odour cues of inquiline brood raised within a host colony would be familiar to their co-developing host brood, therefore if any ability to recognize non-nestmates is based on familiarity of semiochemicals, such inquilines should be less easily detected as enemies. Secondly, the very low rate of inquiline infestation, 5.8% of all host *E. robusta* nests (Shokri Bousjein et al. 2016), suggests that the likelihood of being able to successfully invade a new host colony is very low. Consequently, it is plausible that one female in an inquiline brood could 'inherit' her host colony, but that subsequent inquiline sisters would have lower fitnesses because of the need to successfully invade new host colonies.

Our results are important for several reasons. Firstly, they suggest that the ancestral ability to adjust sex ratios according to benefits of cooperation (LRE) in allodapines can be co-opted to adjust sex ratios in line with the costs of competition (LRC). Secondly, our GLM results suggest that *I. schwarzi* is able to adjust sex ratios according to its anticipated brood number, rather than just its host colony size.

Lastly, our data have some implications for understanding evolutionary arms races between *Exoneura* and *Inquilina*, which we now discuss. Because *I. schwarzi* is an obligate social parasite of *E. robusta* we expect that selection will operate on *E. robusta* to evolve strategies for minimizing rates of parasitism, but will also operate on *I. schwarzi* to overcome host defences, leading to an evolutionary 'arms race'. However, rates of adaptive evolution are curtailed by effective population size, N_e (e.g. Kimura & Ohta 1971; Woolfit 2009; Lanfear et al. 2014). Shokri Bousjein et al. (2016) have shown that infestation rates of *I. schwarzi* are approximately 5.8% of potential host colonies, and that relative N_e may be as much as two orders of magnitude greater for the host than its inquiline species. However, for haplodiploids, such as ants, bees and wasps, N_e is a function of sex ratios, viz. $N_e \sim 9 N_f N_m/(2N_f + 4N_m)$ where N_f and N_m refer to the number of females and males respectively (Wright 1933; Hedrick & Parker 1997). *Exoneura robusta* has extremely female biased sex allocation at a population-wide level, $r \approx 0.15$, due to local resource enhancement (Schwarz

1988). However, our data indicate that *I. schwarzi* has a population-wide sex ratio close to parity, and this may serve to decrease the disparity in N_e that is otherwise suggested purely by infestation rates, which we now discuss.

Figure 2 shows the relationship between N_e and sex ratio for haplodiploids with panmixis and where arrows indicate the population-wide sex ratios estimated for both *E. robusta* (Schwarz 1988) and *I. schwarzi* (data presented here). This figure suggests that the relative disparity in N_e between hosts and parasites is much less than predicted by infestation rates alone, due to the extremely female biased allocation in *E. robusta*. In fact, the relative N_e of *I. schwarzi* is approximately 50% higher than if it had the same population-wide sex ratio as its host.

Sex ratio patterns in inquilines have largely been ignored in previous studies, despite the attention given to origins of inquilinism and mechanisms that inquilines use to exploit their hosts. Ability to adjust sex ratio from a multitude of inter and intra specific parameters suggests that we often may underestimate the level of complication within social insects and their parasites. These systems require attention as the evolution of adjusted sex ratios and the associated evolutionary arms race repercussions will allow better predictions and models of host/parasite interactions on an ancestral and global scale.

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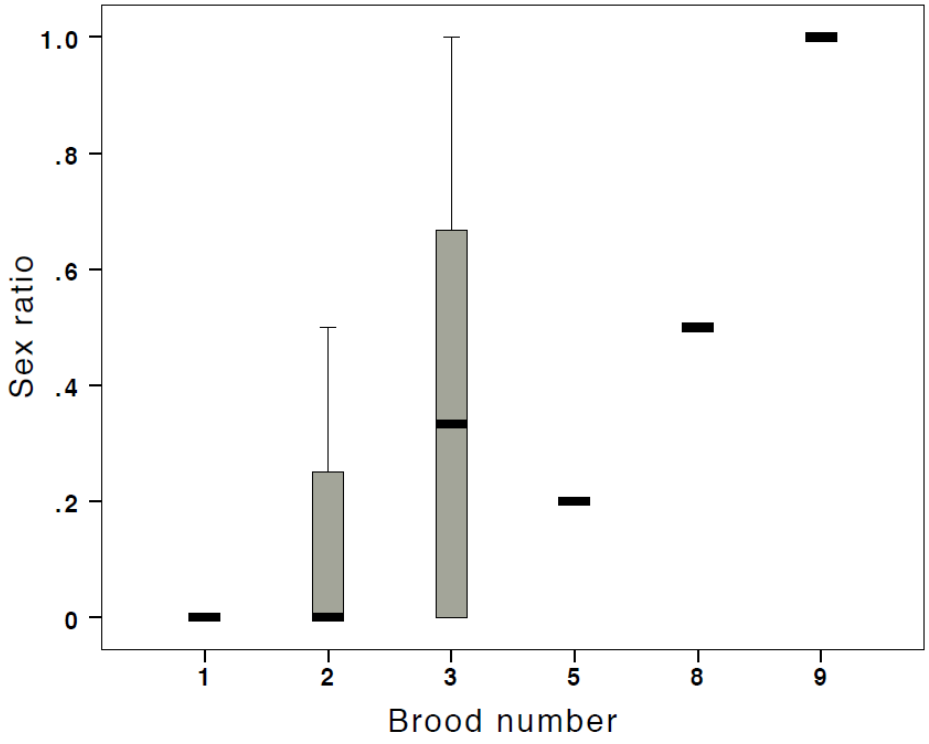

Figure 1. Box plot with interquartile ranges of the sex ratio of *Inquilina schwarzi* as a function of inquiline brood number. Sex ratios and brood numbers were calculated by treating one adult female in each nest as mother of the remaining parasites.

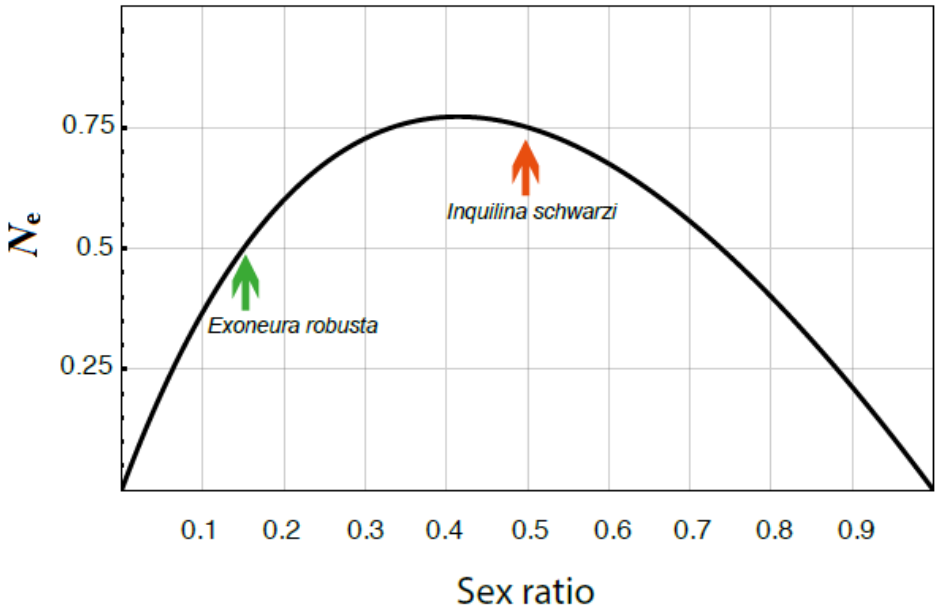

Figure 2. Graph of effective population size, N_e, as a function of sex ratio for panmictic hapodiploids. The population-wide sex ratio of the host *Exoneura robusta* is indicated by a green arrow, and the sex ratio of its inquiline *Inquilina schwarzi* by a red arrow.

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Chapter III:

Similar rates of molecular evolution between social parasitic bees and their host species despite substantial disparity in their effective population size

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1. Abstract

Social parasites are predicted to have different evolutionary rates than their social hosts, because of their much smaller effective population sizes (N_e) and their adaptation to a very different life-cycle, which is largely completed within host colonies. Very small N_e should increase the likelihood of slightly deleterious mutations spreading to fixation because of genetic drift, and there should also be strong selection on genes that are associated with parasitic strategies, which could include changes in metabolic rates. Accordingly, socially parasitic species have been predicted to have faster rates of molecular evolution than their hosts because of greatly reduced effective population size. In this study, we use two pairs of Australian allodapine host-parasite bee species, where parasite species have effective population sizes which are about an order of magnitude smaller than their host. To compare rates of molecular evolution between host and parasite species, 11 mitochondrial (mt) protein coding genes were recovered using Next Generation Sequencing (NGS) methods and nonsynonymous to synonymous substitution rates ratios (d_N/d_S) were estimated. We found that both host and parasite mtDNA genes were subjected to purifying selection, but with no evidence that efficiency of purifying selection is less in the parasite species. Our analyses also did not support predictions that rates of molecular evolution in mitochondrial genes are higher in parasite species than their hosts. We proposed some possibilities that might explain why this study does not support the predicted differences in rates of molecular evolution between hosts and their social parasites.

Key words: inquiline, mitochondrial genes, non-synonymous substitutions, relaxed selection, slightly deleterious mutations, synonymous substitutions.

2. Introduction

Effective population size (N_e) is a key parameter in evolutionary biology, and is predicted to affect the rates of molecular evolution of species (e.g. Woolfit & Bromham, 2003; Woolfit, 2009). In populations with reduced N_e, selection becomes less efficient at removing deleterious mutations because genetic drift becomes substantial and can overwhelm purifying selection operating on slightly deleterious mutations, and simultaneously, lessen the efficacy of positive selection for slightly advantageous mutations (Ohta, 1973; Weber & Diggins, 1990; Woolfit, 2009). These effects can be further amplified if there is inbreeding, which lowers N_e even more (Charlesworth & Charlesworth, 1987).

It is expected that most nonsynonymous substitutions fall into a 'nearly neutral' (slightly deleterious/advantageous mutations) category in such lineages, because strongly deleterious mutations are removed by selection and highly favourable mutations are rare (Ohta, 1973; 1992). Due to the effect of small N_e in such species, the nonsynonymous substitution rate (d_N) for the slightly deleterious mutations increases because of enhanced genetic drift while d_N for the slightly advantageous mutations decreases due to the lower efficiency of positive selection. Nevertheless, given that slightly detrimental mutations comprise substantial proportion of mutations in such lineages (Eyre-Walker *et al.*, 2002; Woolfit, 2009), previous studies suggested that the ratio of non-synonymous to synonymous substitution rates (referred to as ω) is greater in lineages with small N_e (Ohta, 1992).

The ratio ω can also provide information on the mode and strength of selection acting on a protein coding genes (Yang & Bielawski, 2000; Nielsen, 2005), where $\omega = 1$ signifies neutral evolution, $\omega < 1$ indicates purifying selection and $\omega > 1$ indicates positive selection. Empirically measured d_N/d_S ratios can therefore provide insights into the history of selection and population sizes of particular species and genes (Wagner, 2002).

2.1. Practical consequences of varying N_e

The issues above regarding ω and N_e can potentially become very problematic for species with small N_e that are locked into co-evolutionary arms races with other species that have

much larger effective population sizes, and consequently much greater rates of adaptive evolution (Kimura & Ohta, 1971). This situation could arise in parasite-host species dyads if parasites have much smaller N_e than their hosts, where we expect hosts to have higher rates of adaptive evolution and parasites to accumulate deleterious mutations more rapidly, as suggested for some species of socially parasitic inquiline bees (Shokri Bousjein *et al*., 2016).

Several studies have compared rates of molecular evolution in organisms assumed to have different effective population sizes, but they have not produced consistent results. Spradling *et al.,* (2001) compared the rate of cytochrome *b* evolution in 21 rodent species and found an inverse relationship between N_e and the rate of molecular evolution. Johnson and Seger (2001) showed an increase in evolutionary rates of island avian species, which are supposed to have smaller effective population sizes, compared to those occurring on the mainland. Furthermore, Woolfit and Bromham (2003) revealed that long-term reduction in N_e of endosymbiotic microorganisms compared to their free-living relatives caused an increase in the nonsynonymous substitution rates of the 16S rRNA gene. Bromham and Leys (2005) conducted a comparative analyses on social parasites of bees, wasps and ants, using many types of DNA sequences and found that social parasites tend to have faster rates of nonsynonymous substitution than their social hosts, which they attributed to the effect of lower N_e of parasites on rate of molecular evolution.

In contrast, Erler *et al*., (2014) found similar rates of evolution among almost all defence-related genes (antimicrobial peptide genes) when comparing between host and socially parasitic bumblebees. A recent study conducted by Helbing and Lattorff (2016) revealed that three antiviral siRNA genes, evolved faster in host bumblebees compared to their respective parasitic species. Furthermore, Fouks and Lattorff (2016) discovered contrasting evolutionary rates for three "social effect" genes including foraging, salivary gland secretion 3 and vitellogenin between social *Bombus* and their parasitic species.

To further examine this issue, we used allodapine bee host species and their obligate social parasite bee species, otherwise known as inquilines. Inquilinism is an advanced form of social parasitism where the parasitic species spends its entire life cycle within the nest of the host species and are therefore permanent parasites.

Allodapine inquilines have strongly reduced mouth parts and pollen-collecting scopae. Given these morphological variations, they are completely dependent on their host's society for brood rearing (Michener, 1965, 1970b, 1971a, 1975, 1983). They are locked in tight co-evolutionary arms races with their host because they are obligate parasites and host specific (Smith & Schwarz, 2009; Smith *et al*., 2013). Allodapine host and parasite genera are sibling clades (Smith *et al*., 2013) and their life history traits (e.g. body size, generation times) are similar (Michener, 1965; Smith & Schwarz, 2006 a,b).

Our earlier study (Shokri Bousjein *et al*., 2016) revealed that allodapine inquilines have N_e that are about an order of magnitude lower than their host. Despite this, phylogenetic analyses of allodapine parasites showed that they have been able to persist for long periods of evolutionary time (from 15 million years ago) and are presumed to have followed their hosts through multiple speciation events (Smith *et al*., 2013).

In this study, we test whether small N_e of allodapine parasite species from the genus *Inquilina* Michener give rise to faster rates of molecular evolution (d_N/d_S) compared to their *Exoneura* Smith host species; as theoretically predicted (Woolfit & Bromham, 2003; Woolfit, 2009). We target mitochondrial genes to examine this hypothesis because: firstly, inquiline species may be expected to have very different metabolic rates based on their radically different life history (e.g. adaptation associated with parasitism could induce different levels of energy requirements in parasite species). Therefore, mitochondrial genes might be expected to indicate differences in molecular evolutionary rates due to their involvement in energy metabolism. Secondly, the rates of molecular evolution is higher in mitochondrial genes than nuclear genes due to the mitochondrial haploid genome, its maternal inheritance and smaller N_e (Rand, 1994; Ballard & Whitlock, 2004). Thus, they may be expected to better reflect recent differences in rates and patterns of evolution between closely related host-parasites species.

We also expect *a priori* that purifying selection is less effective on mitochondrial genes of parasite species compared to their hosts because of enhanced effects of genetic drift on species with small N_e as predicted (Ohta, 1973; Weber & Diggins, 1990; Woolfit, 2009).

3. Material and methods

3.1. Sampling methods

Our study focused on *Inquilina schwarzi* Michener 1983 and *Inquilina excavata* Cockerell 1922, which infest colonies of the semisocial allodapine bees *Exoneura robusta* Cockerell 1922 and *E. angophorae* Cockerell 1912 respectively (Smith & Schwarz, 2009). Sampling was undertaken in December 2013, from the Gembrook region in the Dandenong Ranges of Victoria, Australia. Nests containing host and parasite species were collected from dead and fallen fronds of the tree fern, *Cyathea australis*, early in the morning when bees were not active (Figure 1). All collected nests were immediately stored in insulated boxes on ice and transported back to Flinders University for nest dissection.

3.2. RNA preparation and high throughput sequencing

Specimens were then snap frozen on dry ice and the head and metasoma of each species were dissected on dry ice in the laboratory and immediately preserved in RNAlater® (Weber *et al*., 2010) to prevent RNA degradation. In order to replicate sampling procedures, head and metasomal tissues were analysed separately, and we pooled tissues from 2-3 individuals per species. Total RNA from pooled tissues was extracted using RNeasy® PlusMicro Kit (Qiagen). SMARTer™ cDNA Synthesis Kit and Advantage®2 PCR Kit (Clontech Labratories, Inc) were used to synthesize double-stranded complementary DNA and PCR-amplify products. PCR cycle optimization was assessed on gel and products were purified using Ultraclean® PCR Clean up kit (MO BIO Labratories, Inc). DNA library preparation and sequencing were outsourced to Australian Genome Research Facility (AGRF) for sequencing on an Illumina platform, which generated 100 bp paired-end reads.

3.3. Quality control and trimming

Raw sequence data was quality controlled using FASTQC (Babraham Institute) and trimmed using CUTADAPT (Martin, 2011) to remove Nextra adapters, SMARTER PCR

Primers, reads containing suspected poly-Adenine and poly-Thymine tails, low quality reads (phred scores < 30) and sequences shorter than 25 bp.

3.4. Transcriptome assembly

The Trinity platform was used for *de novo* assembly of transcript sequences using a default k-mer of 25 (Hass *et al*., 2013). The quality of assembled contigs was then assessed by mapping them back to raw reads using the BOWTIE 2 aligner and the quantity of proper-paired reads was calculated. Read coverage statistics were also retrieved using BEDTOOLS (v.2.22.0).

3.5. Orthology inferences

We used two approaches to determine and verify orthologous genes: 1) Sequence similarity inference 2) phylogenetic reconstruction (Tekaia *et al*., 2016).

3.5.1. Sequence similarity inference

We matched assembled transcripts using the BLASTX algorithm against seven NCBI reference species (mitochondrial protein coding genes), with a 10^{-6} E-value cut-off. Reference species used in this study included the European honeybee *Apis mellifera* (Hymenoptera: Apidae); Red dwarf honey bee *Apis florae* (Hymenoptera: Apidae); Guaraipo bee *Melipona bicolor* (Hymenoptera: Apidae); Urussu bee *Melipona scutellaris* (Hymenoptera: Apidae); Bumble bee *Bombus ignitus* (Hymenoptera: Apidae); Plasterer bee *Colletes gigas* (Hymenoptera: Colletidae); Chalk yellow face bee *Hylaeus dilatatus* (Hymenoptera: Colletidae).We then ran two reciprocal best similarity hit methods (tBLASTn and BLASTn), with a 10^{-6} E-value threshold to verify putative orthologs. We considered stringent criteria to call reciprocal best hits as orthologs if we found: alignment length ≥50%; protein identity ≥30% and nucleotide similarity ≥ 50% (Tommaso *et al*., 2011). However, a few best hit contigs didn't match to any of the reference species mtDNA genes due to their short length. We therefore performed BLAST2BLASTN alignment between best-hit contigs that were perfectly matched to reference species mtDNA genes (subject sequence) and un-matched best-hit contigs (query sequence).

3.5.2. Phylogenetic reconstructions

For each species, a consensus sequence was generated from best hit contigs of head and metasoma using pairwise alignment, Geneious algorithm and all other parameters set to default. Consensus sequences and reference species orthologous genes were imported into Geneious v.9.1.4 (Kearse *et al*., 2012) and multiple sequence alignments were created using Translation alignment and MAFFT/Geneious algorithms. All reading frames also were inspected in Geneious.

All nucleotide alignments were then analysed using a Bayesian inference method implemented in MrBayes v.3.2 (Huelsenbeck & Ronquist, 2001). For MCMC analyses, we partitioned nucleotide sequences by codon positions and applied a GTR + I + Γ nucleotide substitution model (least restrictive model was chosen to avoid potential errors related to incorrect a priori model selection) for each codon partition. Analyses were run two times, with each run comprising 10 million generations, using three heated chains and one cold chain and with variable rate permitted, and sampling every 1000th iteration. Likelihood plots and standard deviation of split frequencies (below 0.01) were used to verify stationarity distribution and length of run. Parameter trace files of each run were also examined in TRACER v.1.6 (Rambaut et al., 2014) and the first 25% of trees were discarded as burn-in. FigTree v1.3.1 (Rambaut et al., 2010) was then used to visualise the Bayesian Inference (BI) phylogenetic tree and *Hylaeus dilatatus* and *Colletes gigas* were used as outgroups to root the tree.

3.6. Pair-wise genetic distance of mtDNA genes between host and parasite species

Uncorrected pair-wise genetic distances (*P*) for amino acid, nucleotide sequences, synonymous and non-synonymous substitution for each host-parasite/host-host/parasite-parasite pairs were calculated using MEGA6 (Tamura *et al*., 2013). A bootstrap method with 500 replicates, including both transition and transversion for substitution mutation, pairwise deletion of ambiguous data and Nei-Gojobori method (Proportion) (Nei & Gojobori, 1986) was applied to estimate genetic divergence.

3.7. Detection of positive selection acting on host and parasite mtDNA

As argued in the introduction, it is assumed that both purifying and positive selection are less efficient in inquilines due to their much smaller N_e than their hosts. Here we used an Adaptive Branch-Site Random Effects Likelihood method (aBSREL) implemented in HyPhy v.2.2.4 (Kosakovsky Pond *et al*., 2005) to identify whether positive selection has a role in the evolution of the mtDNA genes of host and parasites ($\omega > 1$). This method is a developed class of "branch-site" models which quantifies selective pressures on sequences, by the ω ratio where it varies among both codon sites and individual branches in the phylogeny (Smith *et al*., 2015).

3.8. Evolutionary tests

Hyphy v.2.2.4 (Kosakovsky Pond *et al*., 2005) was utilised to compare rates of molecular evolution of mtDNA genes between each host and its associated parasite using the inferred Bayesian tree and likelihood function created by Hyphy.
Evolutionary comparisons were carried out at two levels:

1) We first estimated branch-by-branch variation in rates when an average of d_N/d_S over all codon positions in the gene sequence was considered for comparison (Kosakovsky Pond *et al*., 2005). These analyses were carried out by fitting the global model as a null hypothesis (H_0), with the local model as the alternative hypothesis (H_A). Likelihood ratio tests (LRT) were then used to explore evidence of branch-by-branch rate heterogeneity if H_0 was rejected.
2) We then compared the rate of molecular evolution when codons within a gene were assumed to have different substitution rates. A hypothesis testing framework RELAX (Wertheim *et al*., 2015) was used to compare rates of molecular evolution between host and parasite species and also to test for relaxation or intensification of selection, quantified by the ω ratio. RELAX partitions branches as disjoint sets and calculates a selection intensity parameter (k), and uses LRT to examine whether selection intensity on test and reference branches is identical or different.

4. Results

4.1. Transcriptome assembly statistics

In total, Illumina sequencing generated between 27.3 and 37.7 million paired-end reads, of which 16.6 - 29.2 million remained after quality trimming. Total assembled transcripts varied from 28372 - 82102 per species, and contig N50 ranged from 552 - 1526bp. Overall, 30.4 - 54.3 million reads in the assembled files were aligned to the post-trimming transcripts and 52.83 - 77.62% of reads were determined as proper pairs. Detailed summary statistics on reads and assembly can be found in Table 1.

4.2. Mitochondrial gene content of hosts and parasites transcripts

BLASTX searches of the assembled contigs against reference species recovered 10 - 11 protein coding mtDNA genes of hosts and parasites species. These genes comprised COI, CO2, CO3, Cyt-b, ND-1, ND-3, ND-4, ND-4L, ND-5, ATP6 and ATP8. Of these, ND-3 was the only one not recovered in *I. schwarzi*. In total, Illumina sequencing of hosts and parasites transcripts covered 77- 85% of the reference species mitochondrial protein-coding genes. Both reciprocal best hit methods showed that majority of suggested contigs derived from BLASTX search met the defined criteria (alignment length ≥50%; protein identity ≥30% and nucleotide similarity ≥50%). ND-4L of both host species and *I. schwarzi* was the only gene which failed to meet those standards. We therefore undertook BLAST2BLASTN alignment using best-hit contigs of *I. excavata* to verify orthology of ND-4L best hit contigs from each species. Information concerning the length and depth coverage of identified mtDNA genes of focal host and parasite species is shown in Tables 2 and 3 respectively.

4.3. Phylogenetic tree inference

Two different approaches were used to reconstruct Bayesian gene trees. Phylogenetic trees for each individual mtDNA genes were reconstructed using both host/ parasite focal species and the reference species. Gene trees based on individual genes were sometimes not well-supported (data not shown here) which is not unexpected given the short sequences for some genes, and we therefore constructed a phylogenetic tree by concatenating all mtDNA genes.

The inferred Bayesian tree (with posterior probability node support) corroborated BLAST methods results and was presented in figure 2. It revealed that all predicted mtDNA genes of hosts and parasites species formed fully resolved monophyletic clade with maximal posterior probability (PP) support (PP=1.0).

4.4. Genetic distance of d_N/d_S

P-distance comparisons revealed that in all host and parasite mtDNA genes except ATP8, the proportion of the synonymous substitution distance was higher than nonsynonymous changes ($d_S > d_N$), which is strong evidence of purifying selection operating on mtDNA genes (Table 4). Furthermore, at all comparison levels, genetic divergence between parasite species were higher than hosts one, for almost all mtDNA genes, except for ATP8 (Table 4).

4.5. Test of positive selection

The adaptive branch-site random effects method showed no statistically significant evidence of positive selection ($d_N/d_S > 1$) on any host and parasite mitochondrial genes (Table 5).

4.6. Comparison of evolutionary rates between host and parasite

For HYPHY analyses, we excluded all reference species from data set and used only aligned mitochondrial genes of the focal taxa.

4.6.1. Estimating branch-by-branch variation in rates

Likelihood ratio tests for each gene resulted in support for global rates, which posits the same ω for all host and parasite branches (Table 6). Moreover, estimation of tree-wide global rates for all mitochondrial genes (across the entire alignment and tree) revealed that ω-values were less than 1, which implies the occurrence of purifying selection on the mtDNA genes of both host and parasite species (Table 6).

Based on inferred equal ω among host and parasite branches, we improved our comparisons by allowing variation in rates across all codon positions of each gene.

4.6.2. Estimating codon-by-codon variation in rates

We tried four different treatments on host and parasite branches for RELAX analyses as follows. Under the first treatment, the combined parasite clade was compared against the combined host clade. For the second treatment, the rate of evolution was compared between the common ancestral lineages for each of the host and parasite clades. Each parasite was also compared with its own host for the third and fourth treatments. Since effective population size (N_e) differences among species seems to act at genome-wide level (Mitterboeck & Adamowicz, 2013), we concatenated all mitochondrial genes into a single alignment and analysed them together. However, we found no evidence that efficiency of purifying selection is less in the parasite species compared to host ones. Our analyses also did not support predictions that rates of molecular evolution in mitochondrial genes are higher in parasite species than their hosts (Table 7).

5. Discussion

The *P*-distance and global d_N/d_S ratio analyses indicated that all host and parasite mtDNA genes have undergone purifying selection, which is the major mode of selection acting on mitochondrial genes (Castellana *et al*., 2011; Soares *et al*., 2013; Stewart *et al*., 2008). However, using RELAX, we found no evidence that efficiency of purifying selection is reduced in parasite lineages due to possible increased genetic drift associated with much smaller N_e. The RELAX outcome also did not suggest that the rates of molecular evolution of mtDNA genes is higher in parasite species than their hosts, in any of the treatments.

Our finding here is inconsistent with some previous studies, which found inverse relationships between N_e and rates of molecular evolution (Wu & Li, 1985; DeSalle & Templeton, 1988; Johnson & Seger, 2001; Spradling *et al*,. 2001; Weinreich, 2001; Woolfit & Bromham, 2003, Bromham & Leys, 2005). This discrepancy might be partly due to some deficiencies in previous studies, but also to different approaches used for comparisons. For instance, Wu and Li (1985) showed that globin genes evolve at higher rates in rodents with

smaller N_e compared to primate lineages. However, each analysis in their study was based on a single comparison, which might have limited the breadth of their conclusions. In addition, Spradling *et al*. (2001) found differences in rates of Cyt-b among rodent species with different N_es, but for the rate comparison, they didn't use phylogenetically independent comparison methods (e.g. as developed by Felsenstein, 1985) which led to inflated type I and type II errors (Harvey & Rambaut 1998). Johnson and Seger (2001) also confirmed an opposite relationship between N_e and rates of evolution using island and mainland bird species, however, they used small and taxonomically restricted dataset for their comparison. Later, Wright *et al.* (2009) used a much larger and more varied dataset and found no significant difference between island and mainland species.

Although Woolfit and Bromham (2003) found increased rates of evolution in endosymbiotic bacteria and fungi with small effective population sizes, their conclusions were based only on a single gene, 16S rRNA. Furthermore, Bromham and Leys (2005) used hosts and parasites of bumble bees, allodapine bees and ants for their comparisons and found consistently higher rates of molecular evolution in parasite lineages than hosts. However, they used concatenated mtDNA, nuclear and ribosomal genes for almost all their comparisons, which may conflate quite different evolutionary processes (e.g. recombinant nuclear genes with non-recombinant mt genes). In the case of the allodapine bees in their study, they did not explicitly compare inquiline species with their corresponding hosts, while in our study, we explicitly compared rates between each host and its specific parasite.

Nevertheless, our finding in this study raises a critical question which is: why the comparisons failed to support the predicted differences in efficiency of positive (using aBSREL method) and purifying selection (using RELAX method) between hosts and their social parasites? We now put forward several possibilities that might explain this outcome.

One possible interpretation is that N_e of inquilines is not reduced enough to affect the rates of molecular evolution despite their comparatively lower N_e compared to the host (Shokri Bousjein *et al.*, 2016). For example, whilst *Inquilina* has lower N_e than its hosts, it is possible that its effective population size is still large enough to allow effective purifying selection. In addition, whilst it has been found that N_e of allodapine inquilines is about an order of magnitude lower than their hosts based on incidences of parasitization (Shokri

Bousjein *et al*., 2016), N_e could also be influenced by variation in the number of offspring per individual, sex ratio (Hedrick & Parker, 1997; Shokri Bousjein *et al*., 2017), reproductive skew, colony productivity, reproductive success among individuals. These factors are connected to each other in a complex way in allodapine bees (Schwarz *et al*., 2007). For instance, reproductive skew can be affected by relatedness among nest mates (Langer *et al*., 2004; Harradine *et al*., 2012), however, skew is also linked with colony productivity (Schwarz, 1986), and both of these co-vary with sex allocation (Schwarz, 1994). Because of these complicating factors, a precise estimation of N_e is problematic. Nevertheless, recent genetic methods such as using d_N/d_S ratio is currently trying to obtain an accurate measure of effective population size, however these methods is impractical when there have been historical changes in population size or selection on coding genes (Gregory & Witt, 2008; Whitney & Garland, 2010).

An alternative issue concerns how great the effect of a change in N_e should be on the rates of molecular evolution. Although it is clear that N_e may have considerable effects on the rates of evolution, estimation of the magnitude of that effect is not simple (Bromham & Leys, 2005; Woolfit, 2009). Previous studies (e.g. Bachtrog, 2008; Woolfit, 2009) suggested that the distribution of fitness effects of both slightly deleterious and advantageous mutations must be considered when the degree of the effect of a change in N_e on rates of evolution is determined. Woolfit (2009) argued that if two lineages have different N_e (one with the larger, N_eL, and another with the smaller, N_eS), the proportion of slightly deleterious mutations, that have a selective coefficient between $1/N_e$L and $1/N_e$S, determines the difference in evolutionary rates between two lineages. Earlier studies obtained inconsistent results on the size of that proportion. Ohta (1977) suggested that the effect of a change in population size on the rate of evolution is expected to be quite large because a substantial proportion of mutations fall in the range from $1/N_e$L to $1/N_e$S. By contrast, Kimura (1979) suggested fewer mutations will be in this range, therefore the difference in evolutionary rates between lineages with different N_e will also be low. However, Silander *et al.*, (2007) argued that the distribution of selective coefficients of slightly deleterious mutations is dynamic between taxa and even within a species. Initially, slightly deleterious mutations was often taken into account to determine the difference in rates of evolution because they are more common in small population, while slightly advantageous mutations are assumed to be rare in such lineages (Woolfit, 2009). However,

Charlesworth & Eyre-Walker (2007) found that slightly advantageous mutations which are under positive selection, are also relatively common in small populations. Therefore, it was suggested to consider the distribution of fitness effects of both types of mutations while comparing rates of molecular evolution (Woolfit, 2009).
Another possibility, which might be worth considering here, is that other aspects than N_e might have a substantial influence on rates of evolution. Previous studies found that fecundity is strongly positively correlated with rates of evolution (Bomham & Leys, 2005, Welch & Bromham, 2005). One possible explanation for this pattern is that the number of genome copies per generation scales linearly with fecundity, thereby generating more opportunities for DNA copy error/mutations in species with higher fecundity. This effect might be evident in a higher number of synonymous mutations and consequently higher rates of evolution in those species (Bomham & Leys, 2005). Thus, the impact of this feature on rate of evolution is completely opposite to that of the N_e effect, with higher rates for host species (which have high fecundities) relative to inquilines (which have low fecundities) (Shokri Bousjein *et al.*, 2016).

In conclusion, our analyses suggest that relatively small N_e of parasite lineages has seemingly had no strong impact on rates of molecular evolution of their protein coding mtDNA genes compared to their hosts. This does not match the patterns found by Bromham and Leys (2005) who included *Exoneura* and *Inquilina* in their study and it also conflicts with some broader theoretical considerations. Our results indicate a need to consider a variety of theoretical bases for comparative rates of evolution for host/parasite relationships, and these may include the evolution of high-stake arms races into more benign relationships such as symbiosis or mildly deleterious associations. At present, the evolution of more benign species interactions has not been explored in terms of rates of molecular evolution and we argue that our data calls for such an examination in future studies.

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Table 1. Transcriptome read and assembly summary.

Taxa	Relation	body segment	QA paired-end reads (read length)	Total sequences after trimming	*De novo* assembled transcripts (contig N50)	Total aligned reads (% proper pairs)
E. robusta	Host	Head	29,320,666 (100bp)	20985442	82102 (968bp)	37226484 (52.83%)
		Metasoma	27,303,651 (100bp)	16655057	28372 (552bp)	30498594 (55.68%)
I. schwarzi	Parasite	Head	30,839,546 (100bp)	22396119	65944 (992bp)	40886113 (60.78%)
		Metasoma	32,631,666 (100bp)	23734470	77700 (1189bp)	43935616 (51.03%)
E. angophorae	Host	Head	35,318,489 (100bp)	25741159	66426 (1080bp)	47616902 (77.62%)
		Metasoma	34,386,652 (100bp)	24592950	76988 (1135bp)	44565173 (61.88%)
I. excavata	parasite	Head	32.160,656 (100bp)	24967610	77109 (1526bp)	46464257 (73.44%)
		Metasoma	37,715,844 (100bp)	29259350	69007 (1301bp)	54356494 (65.36%)

Table 2. Characteristics of the mitochondrial genes of focal species. The mtDNA best hit contigs derived from BLASTx which met determined criteria of tBLASTn and BLASTn (alignment length ≥50%; protein identity ≥30% and nucleotide similarity ≥ 50%) are highlighted. ND-4L best hit contigs of both host and one inquiline species didn't meet those criteria and obtained from BLAST2BLASTN alignment.

Size (bp) / Species	ATP6	ATP8	CO1	CO2	CO3	Cyt-b	ND-1	ND-3	ND-4	ND-4L	ND-5
E. robusta	**684**	**156**	**1538**	**624**	**762**	**1135**	**891**	**354**	**1286**	**115**	**1167**
E. angophorae	**684**	**156**	**1537**	**648**	**736**	**1090**	**909**	**246**	**1280**	**100**	**919**
I. excavata	**684**	**156**	**1538**	**675**	**769**	**1137**	**572**	**267**	**1284**	**102**	**1166**
I. schwarzi	**684**	**156**	**1538**	**675**	**691**	**1134**	**912**	**Not found**	**1287**	**111**	**1086**

Table 3. mtDNA sequence coverage depth. The first row indicates mean sequence coverage depth per nucleotide base (± s.e.) for transcripts of mtDNA orthologous genes. The maximum depth for a single nucleotide base within each transcript is presented in square brackets and the sample size (*n*) represents the full length of the unedited transcript (number of bases). Data not available for three mtDNA genes of *I. schwarzi.*

Species \ mtDNA genes	ATP6	ATP8	CO1	CO2	CO3	Cyt-b	ND-1	ND-3	ND-4	ND-4L	ND-5
E. robusta	40845.29± 51903.94	40845.29± 51903.94	45725.88± 24890.49	40845.29± 51903.94	34627.03± 46309.80	10981.58± 7903.98	1114.69± 797.81	2692.59 ± 5118.60	1357.3± 1418.59	1231.52± 1393.71	1047.35± 1386.47
	[245927]	[245927]	[101606]	[245927]	[245927]	[32668]	[2835]	[22143]	[5719]	[5719]	[5719]
	n = 3754	n = 3754	n = 2852	n = 3754	n = 5186	n =2356	n =1182	n =1191	n = 2841	n =3169	n =3579
E. angophorae	16900.99± 18738.06 [83137] n = 2339	16900.99± 18738.06 [83137] n = 2339	36389.30± 23593.51 [109632] n = 2848	16900.99± 18738.06 [83137] n = 2339	15557.903± 17675.71 [83137] n = 4671	7241.53± 6729.82 [27454] n = 2386	253.354± 142.80 [678] n = 2037	7.539± 5.88 [19] n = 623	650.97± 614.25 [2285] n = 2743	650.97± 614.25 [2285] n = 2743	105.91± 92.33 [384] n = 1852
I. schwarzi	20558.93±39 881.38	20558.93±3 9881.38	43874.29±3 3564.59	22297.61±36 449.83	18678.74±36 015.61	-----	-----	-----	2114.44±2 197.66	2114.44±2 197.66	509.50±712 313
	[200586]	[200586]	[157338]	[200586]	[200586]	-----	-----	-----	[8443]	[8443]	[2893]
	n = 3077	n = 3077	n = 3067	n = 3845	n = 3862	-----	-----	-----	n = 1732	n = 1732	n = 1522
I. excavata	7912.18±120 95.31 [65897] n =2706	7912.18±12 095.31 [65897] n =2706	21023.45±1 7168.40 [73903] n = 3223	11378.52± 18462.48 [98885] n = 3017	7587.12±106 80.48 [65897] n = 3659	2848.26±2 791.28 [11284] n = 3090	1465.23±1 813.33 [6472] n = 2153	33.18±2 1.09 [77] n = 613	695.48±72 0.44 [2735] n = 3052	695.487±7 20.44 [2735] n = 3052	283.20±337. 22 [1526] n = 2107

Table 4. Pair-wise genetic distance of mitochondrial genes. Uncorrected *P*-distances calculated using nucleotide (nuc) and amino acid (aa) alignments (± s.e.).

mtDNA genes	Species	*P*-distance-nuc	*P*-distance-aa	*P*-distance of Synonymous Substitution	*P*-distance of Nonsynonymous Substitution
ATP8	*E.robusta-E.angophorae*	0.044±0.015	0.117±0.046	0.018±0.019	0.051±0.020
	I.schwarzi-I.excavata	0.038±0.015	0.098±0.043	0.018±0.021	0.043±0.019
	E.robusta-I.schwarzi	0.044±0.017	0.117±0.045	0.018±0.020	0.051±0.019
	E.angophorae-I.excavata	0.070±0.020	0.176±0.051	0.018±0.018	0.083±0.026
ATP6	*E.robusta-E.angophorae*	0.020±0.005	0.044±0.013	0.0300±0.015	0.018±0.005
	I.schwarzi-I.excavata	0.039±0.007	0.066±0.016	0.084±0.024	0.029±0.007
	E.robusta-I.schwarzi	0.071±0.009	0.132±0.022	0.129±0.030	0.058±0.010
	E.angophorae-I.excavata	0.055±0.008	0.097±0.019	0.084±0.023	0.049±0.011
CO1	*E.robusta-E.angophorae*	0.020±0.004	0.005±0.003	0.086±0.015	0.002±0.001
	I.schwarzi-I.excavata	0.042±0.005	0.023±0.007	0.161±0.020	0.011±0.003
	E.robusta-I.schwarzi	0.050±0.005	0.033±0.007	0.178±0.021	0.016±0.004
	E.angophorae-I.excavata	0.052±0.005	0.043±0.008	0.171±0.019	0.020±0.004
CO2	*E.robusta-E.angophorae*	0.022±0.006	0.028±0.012	0.068±0.024	0.011±0.005
	I.schwarzi-I.excavata	0.040±0.007	0.076±0.017	0.077±0.022	0.031±0.007
	E.robusta-I.schwarzi	0.056±0.008	0.082±0.018	0.127±0.031	0.039±0.009
	E.angophorae-I.excavata	0.044±0.008	0.074±0.018	0.096±0.026	0.032±0.008
CO3	*E.robusta-E.angophorae*	0.033±0.007	0.045±0.013	0.102±0.025	0.018±0.006
	I.schwarzi-I.excavata	0.032±0.007	0.066±0.016	0.056±0.020	0.027±0.007
	E.robusta-I.schwarzi	0.060±0.009	0.113±0.020	0.095±0.024	0.052±0.010
	E.angophorae-I.excavata	0.077±0.10	0.117±0.021	0.161±0.031	0.057±0.010

Table 4. Continued.

mtDNA genes	Species	*P*-distance-nuc	*P*-distance-aa	*P*-distance of Synonymous Substitution	*P*-distance of Nonsynonymous Substitution
Cyt-b	*E.robusta-E.angophorae*	0.030±0.005	0.033±0.009	0.091±0.019	0.015±0.004
	I.schwarzi-I.excavata	0.033±0.005	0.040±0.009	0.098±0.019	0.016±0.004
	E.robusta-I.schwarzi	0.049±0.006	0.069±0.013	0.125±0.022	0.031±0.006
	E.angophorae-I.excavata	0.055±0.006	0.072±0.013	0.142±0.022	0.033±0.006
ND-1	*E.robusta-E.angophorae*	0.019±0.004	0.027±0.009	0.053±0.017	0.011±0.004
	I.schwarzi-I.excavata	0.033±0.008	0.032±0.013	0.115±0.030	0.013±0.005
	E.robusta-I.schwarzi	0.045±0.006	0.054±0.013	0.144±0.027	0.022±0.005
	E.angophorae-I.excavata	0.042±0.008	0.048±0.016	0.142±0.033	0.020±0.006
ND-3	*E.robusta-E.angophorae*	0.008±0.006	0.024±0.018	0.000±0.000	0.010±0.007
	E.angophorae-I.excavata	0.025±0.012	0.037±0.026	0.063±0.043	0.015±0.011
ND-4	*E.robusta-E.angophorae*	0.025±0.005	0.035±0.009	0.066±0.016	0.016±0.004
	I.schwarzi-I.excavata	0.033±0.005	0.049±0.010	0.077±0.017	0.023±0.005
	E.robusta-I.schwarzi	0.059±0.006	0.114±0.015	0.091±0.019	0.052±0.007
	E.angophorae-I.excavata	0.059±0.007	0.111±0.014	0.094±0.019	0.051±0.007
ND-4L	*E.robusta-E.angophorae*	0.010±0.010	0.000±0.000	0.060±0.058	0.000±0.000
	I.schwarzi-I.excavata	0.039±0.018	0.030±0.029	0.176±0.097	0.012±0.012
	E.robusta-I.schwarzi	0.057±0.022	0.030±0.031	0.117±0.077	0.012±0.012
	E.angophorae-I.excavata	0.030±0.017	0.032±0.034	0.125±0.086	0.012±0.013
ND-5	*E.robusta-E.angophorae*	0.017±0.004	0.013±0.006	0.063±0.017	0.005±0.003
	I.schwarzi-I.excavata	0.044±0.006	0.055±0.012	0.109±0.020	0.026±0.005
	E.robusta-I.schwarzi	0.063±0.008	0.080±0.014	0.141±0.021	0.042±0.008
	E.angophorae-I.excavata	0.050±0.007	0.042±0.011	0.152±0.026	0.023±0.006

Table 5. Adaptive Branch Site REL summary. Significant positive diversifying selection on each branch was tested using the likelihood ratio test by comparing a null model (MG94) in which no positive selection rate class is allowed on each branch and full model which allows site-to-sire rate variation. Accordingly, none of the mtDNA genes were found to be under significant positive selection (indicated by values in column five). The small-sample Akaike Information Criterion (AICc, Sugiura, 1978) was used to compare the goodness of fit of two models. Values in column two indicate that CO1, CO2, ND-4 and ND-5 support full model, allowing multiple ω rate classes (indicated also by asterisks in column one).Values in column three and four indicate proportion of branches and trees of each gene for a particular value of ω.

mtDNA genes	Trees rateclasses	%of branches	% of tree length	Proportion of branches under significant positive selection	AICc MG94	AICc model Full model
ATP6	1	%100	%100	0	2059.58	2059.58
ATP8	1	%100	%100	0	493.26	493.26
CO1*	1	%83	%36	0	4530.30	4520.11
	2	%17	8.8%	0		
CO2*	1	%83	%59	0	2051.92	2046.55
	2	%17	%13	0		
CO3	1	%100	%100	0	2380.42	2380.42
Cyt-b	1	%100	%100	0	3411.70	3411.70
ND-1	1	%100	%100	0	2416.55	2416.55
ND-3	1	%100	%100	0	882.31	882.31
ND-4*	1	67%	73%	0	3690.51	3688.41
	2	33%	24%	0		
ND-4L	1	%100	%100	0	357.61	357.61
ND-5*	1	83%	81%	0	3365.34	3360.69
	2	17%	16%	0		

Table 6. Tree wide global rate of selection versus local rates. Tree wide global rate posits ω does not vary from branch to branch in the tree and provides a crude measure of the overall strength of selection acting on each mtDNA gene. Local model allows a separate ω in every branch of the tree. The likelihood ratio test was used to compare global model (null hypothesis) and local model (alternative hypothesis) on each mtDNA gene and resulted in a very strong ($p \geq 0.05$ in all cases) support in favor of the global rate. DF= Degrees of freedom.

mtDNA genes	Tree wide Global rate of selection	Tree wide Global rate versus local rates of selection		
		2*Likelihood Ratio(LR)	DF	P-value
ATP6	0.196	2.590	5	0.762
ATP8	0.533	1.529	5	0.909
CO1	0.0346	9.669	5	0.085
CO2	0.127	2.500	5	0.776
CO3	0.123	6.544	5	0.256
Cyt-b	0.0786	3.971	5	0.553
ND-1	0.0744	0.580	5	0.988
ND-3	0.256	1.756	3	0.624
ND-4	0.110	8.039	5	0.154
ND-4L	0.044	4.238	5	0.515
ND-5	0.087	8.457	5	0.132

Table 7. Test for Relaxed selection of mtDNA genes in parasite species compared with hosts. RELAX test was used to examine whether selection is relaxed or intensified on a subset of test branches compared with a subset of reference branches in a predefined tree. The relaxation coefficient, k, is used to estimate selection intensity. In the null model, the selection intensity is constrained to 1 for all branches, whereas in the alternative model, k is allowed to differ between reference and test groups. Acceptance or rejection of the alternative model is tested using a likelihood-ratio test, but Akaike Information Criterion was also included as measures of fit of the null model and the alternative model. Four different treatments were examined on host and parasite branches for RELAX analyses which are as follows. Treatment 1: the combined parasite clade was selected as a test branch and the combined host clade was treated as a reference branch. Treatment 2: common ancestral lineage of parasite was tested against common ancestral lineage of host. Treatment 3: *I. schwarzi* as a test branch and *E. robusta* as a reference branch. Treatment 4: *I. excavata* as a test branch and *E. angophorae* as a reference branch. All treatments were also tested on concatenated mitochondrial genes. Two treatments were examined on ND-3 gene as it didn't find in *I. schwarzi*. No significant differences in purifying selection efficiency and rates of molecular evolution were found between host and parasite species in any treatments. ω reported here is calculated under the accepted null model.

mtDNA genes	Treatments	Relaxation coefficient (k)	P Value	LR	ω test / reference branches	AICcNull model	AICc Alternative model
ATP6	1	1.55	0.252	1.31	0.162	2057.99	2058.80
	2	1.04	0.959	0.00	0.0001	2074.67	2076.83
	3	1.57	0.328	0.95	0.153	2073.02	2074.22
	4	1.00	0.978	0.00	0.000	2072.55	2074.72
ATP8	1	1.00	0.976	0.00	0.00	489.39	492.02
	2	1.13	0.976	0.00	0.00	514.22	517.13
	3	1.00	0.979	0.00	0.00	514.22	517.14
	4	1.00	0.977	0.00	0.00	513.47	516.39

Table 7. Continued.

mtDNA genes	Treatments	Relaxation coefficient (k)	P Value	LR	ω test / reference branches	AICcNull model	AICc Alternative model
CO1	1	0.78	0.122	2.38	0.0193	4521.09	4520.78
	2	49.75	0.486	0.48	0.0329	4531.17	4532.76
	3	1.07	0.744	0.11	0.0105	4532.07	4534.04
	4	0.67	0.107	2.58	0.0215	4534.02	4533.51
CO2	1	0.75	0.307	1.04	0.0893	2047.53	2048.61
	2	1.00	0.992	0.00	0.0413	2060.51	2062.68
	3	0.57	0.240	1.38	0.0931	2065.60	2066.39
	4	0.96	0.885	0.02	0.0918	2061.16	2063.30
CO3	1	0.56	0.050	3.81	0.111	2385.22	2383.52
	2	3.85	0.528	0.40	0.0001	2396.03	2397.78
	3	1.01	0.979	0.00	0.249	2397.65	2399.79
	4	0.61	0.251	1.32	0.079	2398.96	2399.79
Cytb	1	1.57	0.339	0.91	0.008	3411.31	3412.47
	2	0.99	0.970	0.00	0.073	3424.98	3427.07
	3	1.00	0.986	0.00	0.069	3426.27	3428.37
	4	1.07	0.803	0.06	0.052	3426.31	3428.35

Table 7. Continued.

mtDNA genes	Treatments	Relaxation coefficient (k)	P Value	LR	ω test / reference branches	AICc Null	AICc Alternative
ND-1	1	1.18	0.519	0.42	0.071	2415.20	2416.87
	2	9.94	0.987	0.00	0.063	2433.39	2435.51
	3	0.97	0.926	0.01	0.066	2433.40	2435.51
	4	1.49	0.473	0.51	0.087	2433.91	2435.51
ND-3	1	1.00	0.979	0.00	0.00	893.77	896.18
	4	1.00	0.971	0.00	0.00	898.53	900.97
ND-4	1	0.50	0.116	2.47	0.020	3685.31	3684.91
	2	1.13	0.978	0.00	0.070	3692.33	3694.41
	3	0.23	0.192	1.70	0.065	3695.26	3695.65
	4	1.02	0.976	0.00	0.00	3693.16	3695.24
ND-4L	1	1.46	0.598	0.28	0.070	358.96	361.57
	2	1.13	0.999	0.00	1.00	382.55	385.88
	3	0.26	0.317	1.00	0.0667	383.55	385.89
	4	1.00	0.989	0.00	0.00	382.55	385.88
ND-5	1	0.04	0.136	2.22	0.00	3361.79	3361.64
	2	1.13	0.983	0.00	0.00	3372.16	3374.25
	3	0.03	0.106	2.61	0.031	3374.85	3374.34
	4	1.16	0.662	0.19	0.034	3372.40	3374.30

Table 7. Continued.

mtDNA genes	Treatments	Relaxation coefficient (k)	P Value	LR	ω test / reference branches	AICc Null	AICc Alternative
Concatenated genes	1	1.89	0.0944	2.79	0.014	25441.575	25440.788
	2	4.680	0.329	0.952	0.00	25442.154	25443.213
	3	0.902	0.436	0.605	0.069	25443.003	25444.409
	4	1.050	0.845	0.038	0.0162	25444.114	25446.088

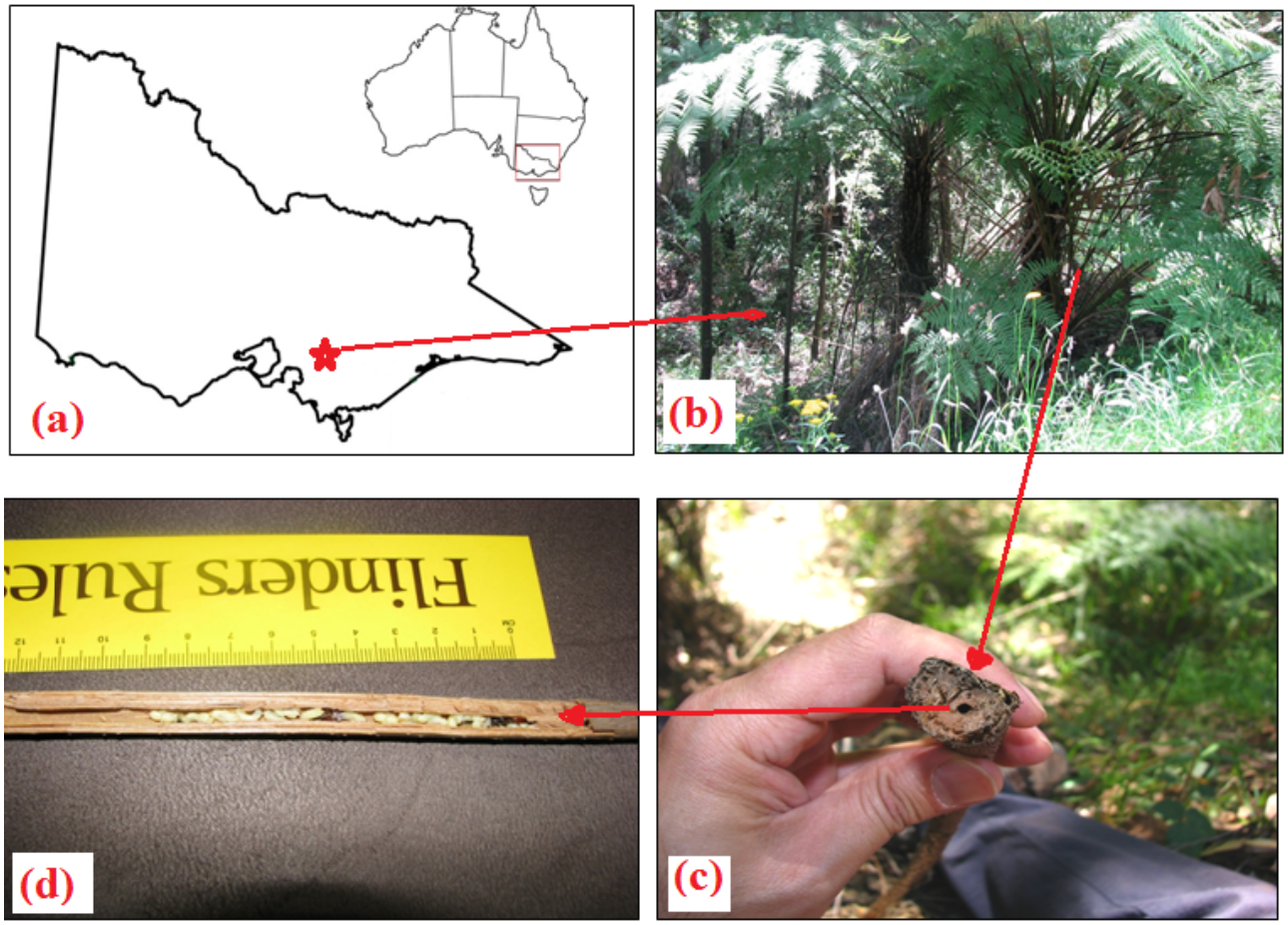

Figure 1. (a) Colourful star indicates collection locality of *Exoneura* and *Inquilina* in this study; (b) fronds of the tree fern *Cyathea australis* containing *Exoneura* nests; (c) view of the basal end of a dead tree fern frond, *Cyathea australis*, showing the entrance of an *Exoneura* nest; (d) longitudinal cut of nest comprising adult females and pupae.

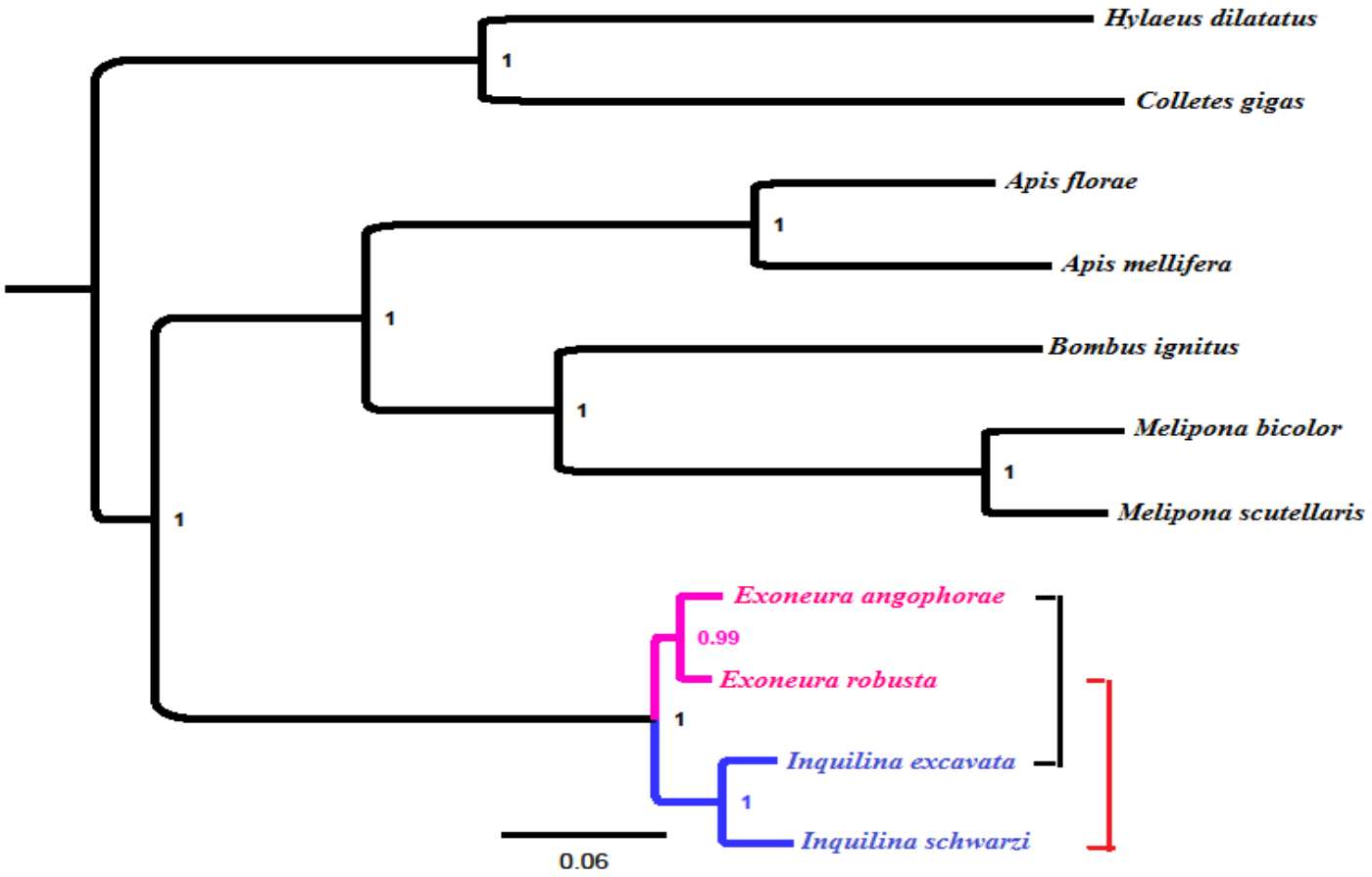

Figure 2. Mitochondrial genes phylogeny. A Bayesian tree (resulted from MrBayes analyses) derived from 11 concatenated protein coding mtDNA genes, with posterior probability node support. Parasites and hosts are indicated by coloured branches: pink for hosts and blue for parasites. The brackets to the right of the terminal branches link each host to its associated parasite.

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Chapter IV:

Demographic histories of Australian exoneurine bees from the late Pleistocene, with emphasis on changes since the last glacial maximum

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1. Abstract

Despite severe cooling and extreme aridity in Australia during the Pleistocene, and particularly since the last glacial maximum (LGM), the demographic history of Australian temperate-adapted bees has remained unknown over this epoch. The Allodapini comprise an important tribe of temperate bees ranging across montane and subcoastal regions in eastern and south Western Australia. Here, we construct population histories of this faunal group by applying Bayesian skyline plot analyses to the barcoding region of the mitochondrial gene COI, sequenced from two exoneurine genera, *Exoneura* and its social parasite *Inquilina*. Our analyses suggest that three *Exoneura* species appear to show gradually increasing population sizes since the LGM, but no sharp decline at the LGM. We argue that an ability to track changing climates both altitudinally and latitudinally may have allowed *Exoneura* species to avoid major changes in population size, though a gradual increase in population sizes since the LGM may reflect gradually declining aridity in Australia since then. We suggest that connectivity in climate related habitats may have stabilized past population sizes for some important pollinators. It will become important to understand how different pollinator groups will respond to future climate changes, and how their resilience might depend on their current climate niches.

Key words: Bayesian skyline plot, climate change, DNA barcoding, exoneurine bees, haplotype network, last glacial maximum.

2. Introduction

2.1. Background

Investigating the responses of pollinators to past climate fluctuations is critical for understanding how their changing population sizes may have affected pollination in the past, and how they may respond to future climate changes (Kremen *et al*., 2007). Concern about this issue has grown due to recent global warming and likely future scenarios (Solomon *et al*., 2007). Earlier studies found that such changes in climate could in fact induce plant-pollinator phenological asynchrony (Fitter & Fitter, 2002; Memmott *et al*., 2007; Miller-Rushing & Primack, 2008; Rafferty & Ives, 2011).

Though a growing number of studies have addressed the influence of past climate change on populations of pollinators, some key studies have relied on museum material that only extends into the past over century scales (eg. Cameron *et al*., 2011; Burkle *et al*., 2013), whilst the major Quaternary climate cycles occur over many thousands of years and encompass much larger climate fluctuations.

Bayesian coalescent analyses of molecular data allow us to infer changes in population sizes and diversification patterns of species, potentially beyond the last 100kya (Drummond *et al*., 2005) and therefore allow us to explore the effects of past climates well beyond the reach of museum collections. These approaches have been used previously to reveal how past climate change has affected population sizes of both fishes (Ruzzante *et al*., 2008) and mammals (Palkopoulou *et al*., 2013). Moreover, recent studies on bee populations using the same approaches have also discovered past population dynamics of these faunal groups relative to climate fluctuations. For instance, studies by Groom *et al*., (2013; 2014a) found that population sizes of tropical halictine bees (Halictidae) in three tropical pacific archipelagos declined markedly during the last glacial maximum (LGM), occurred at 21000 ± 2000 years ago and expanded as global climate returned to an inter-glacial warming period.

Another study by Lopez-Uribe *et al*., (2014) suggested that Neotropical orchid bees have been impacted by glacial cycles due to their limited physiological tolerance. A more recent study by Dew *et al*., (2016) showed that population size of the Australian ceratinine bee

Ceratina australensis (Apidae) expanded simultaneously with a major period of post-glacial warming. Furthermore, Shell and Rehan (2016) found a rapid population expansion of North American *Ceratina* species (Apidea: Ceratinini) when the LGM came to end.

In this study we focus on Australian cool and montane temperate-adapted exoneurine bees which are regarded as an important group of native pollinators in the continent. We target this clade because, firstly, no previous studies have explored how past climates have impacted Australian bees that are adapted to wet temperate environments. Secondly, a recent study (Dew *et al*., 2017, in-prep) on xeric-adapted Australian allodapines in the genus *Exoneurella* suggested that social bees might be less vulnerable to climate change than more solitary nesting species because social living may allow more flexible responses to fluctuating resources; this may be important for understanding *Exoneura* Smith species given their marked levels of sociality (Schwarz *et al*., 2007). Lastly, other studies found that differences in life history (e.g. fecundity and dispersal ability) and ecological traits (e.g. temperature tolerance) among different taxa can alter their responses to historical events such as climate change (Turner *et al*., 1996; Bermingham & Martin, 1998; Hodges *et al*., 2007).

2.2. Exoneurine bees and development of aridity in Australia during the Pleistocene

Exoneurines bees (comprising the genera *Exoneura*, *Exoneurella*, *Brevineura* and *Inquilina* Michener) comprise the oldest clade of the Australian allodapines (tribe Allodapini) and, apart from the genus *Exoneurella*, are almost entirely restricted to the temperate areas of Australia (Chenoweth & Schwarz 2011). *Inquilina* is an obligate social parasite of *Exoneura,* and *Exoneurella* is generally distributed in arid/semi-arid regions of the continent (Michener, 1965, 1970, 1971a, 1975*;* Bull *et al*., 2003; Schwarz *et al*., 2003; Chenoweth & Schwarz, 2011).

Phylogenetic studies indicated that exoneurine bees diverged from an African plus Malagasy clade in the mid Eocene about 42 million years ago (Mya) and likely dispersed from Africa via Antarctica in Oligocene, about 30 Mya (Schwarz *et al*., 2006; Chenoweth & Schwarz, 2011).

At approximately 6-10 Mya (mid-Miocene), accelerated aridification had commenced in Australia and consequently radiation of temperate plant groups declined (Byrne *et al*., 2008). Surprisingly, this period of aridification in Australia is associated with rapid speciation of temperate exonurine lineages while there is no evidence of increased diversification in xeric-adapted *Exoneurella* species. However, four extant *Exoneurella* species expanded their ranges during the period of aridification (Chenoweth & Schwarz, 2011).

During the Pliocene, Australian climates returned temporarily to warm and wet conditions. The beginning of the Pleistocene (two and half million to four hundred thousand years) was the initiation of major oscillations between glacial and interglacial climates for Australia (Byrne *et al*., 2008). During glacial intervals, the tropical region of Australia became drier (Fujoika *et al*., 2005) and the arid zone of the continent was expanded (Ayliffe *et al*., 1998). Extreme aridity in Australia coincided with LGM period corresponding with the end of the Pleistocene at 18000 – 23000 years ago (William, 2000 & 2001).

Nevertheless, given the progressive aridity in Australia during the Pleistocene, particularly during the LGM, the effects of the repeated glacial advances and retreats on exoneurine bees remain unknown. In order to examine such possible effects, the barcoding region of mitochondrial cytochrome oxidase *c* subunit I (COI) gene (Gompert *et al*,. 2008; Naderi *et al*,. 2008; Rajabi-Maham *et al*,. 2008) was recovered from 239 specimens of temperate exoneurines, collected from Queensland, South Australia, Victoria, and Western Australia. We first identified the number of species using phylogenetic analyses and species delimitation methods and then constructed demographic histories of each species through Bayesian skyline approaches and, finally, associated the timelines of plots with the glacial history of Australia to discover whether Pleistocene climate fluctuation, particularly the LGM, impacted exoneurines.

3. Material and Methods

3.1. Sampling localities of exoneurine bees

Exoneurine specimens were collected from five study locations comprising: 1) the Mt. Donna Buang and Gembrook regions from Dandenong Ranges (DR), Victoria; 2) the Daylesford region (DF), Victoria; 3) the Warwick region in south eastern Queensland (QL); 4) the Mont

Lofty ranges in South Australia (SA) and 5) the south western region of Western Australia (WA). All sampling regions were in temperate zones (Figure 1). Details of sampling including collection localities and dates of sampling, barcoded specimens, the number of discovered haplotypes, and identified genera from each site are provided in Table 1. Nests containing bee species were collected from dead fronds/stems of tree fern *Cyathea australis*, *Chrysanthemoides* sp. (Asteraceae), *Gahnia sieberana* (Cyperaceae), *Ferula communis* (Apiaceae), *Rosa* spp. (Rosaceae), *Rubus* spp. (Rosaceae), and *Senecio* spp. (Asteraceae). Samplings were carried out early in the morning when bees are assumed to be present in the nests. Nest entrances were blocked with adhesive tape immediately upon collection and transported back to Flinders University on ice, where they were stored at 4°C until processing. Nests were opened longitudinally with a knife and bees were morphologically identified at genus level following published descriptions (Michener, 1965, 2007; Smith & Schwarz, 2009). Then, one adult from each nest was chosen randomly and kept in 99% ethanol for DNA sequencing.

3.2. DNA extraction, PCR amplification and sequencing techniques

In total 239 specimens were used for DNA extraction and sequencing. One leg was removed from each specimen, rinsed in 10 mM Tris to remove the alcohol before the extraction process and was incubated overnight in insect lysis solution with proteinase K. Total genomic DNA was extracted using a CCDB (Canadian Centre for DNA Barcoding) Glass Fibre Plate DNA extraction protocol (Ivanova *et al*., 2006), except that the volume of the Binding Buffer and Protein Wash Buffer (PWB) were doubled and DNA washing times with PWB and Wash Buffer (WB) were increased to 5 and 20 minutes respectively. Vacuum manifold was applied for the washing step with the suggested buffers, however, to collect final DNA elute, centrifugation was used for 5 minutes at 3000 rpm. The DNA extract was kept in TLE (10mM TRIS, 0.1mM EDTA pH8) instead of Deionized, distilled water (ddH2O).

Mitochondrial cytochrome *c* oxidase subunit 1 (COI) was PCR-amplified using forward primer M414 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) and reverse primer 423 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′) (Folmer *et al.,* 1994) and finally, a 655 bp of the barcoding region of COI was sequenced for all taxa. All PCRs were carried out on Kyratec Supercycler thermal cyclers (SC300) using 25 µl reaction volumes comprising of

15.4 nuclease-free water, 0.1 µl of Immolase PCR buffer, 1 µl of each primer (5 µM concentration), 5µl of MRT Buffer and 2.5 µl of DNA.

PCR amplification involved an initial denaturation at 95°C for 10 min and 35 subsequent cycles of 94 °C for 45 s, 48 °C for 45 s, 72 °C for 1 min and a final extension of 72°C for 6 min, followed by a 2 min hold time at 25°C. Amplified PCR products were visualised on 1.5% agarose gels and purified using a PCR multiscreen filter plate (Millipore). Purified PCR products were sequenced in both directions using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified using a SEQ multiscreen filter plate (Millipore) and analysed on an ABI 3700 DNA capillary sequencer.

3.3. Molecular Phylogenetic analyses

A consensus sequence was generated from forward and reverse sequences of each specimen using Geneious Pro version 9.1.4 (Kearse *et al*., 2012). The resultant consensus sequences were then screened for *Wolbachia* contamination using a standard nucleotide BLAST program following Smith *et al*., (2012). Since coalescent analyses is sensitive to data quality, specimens with ambiguous nucleotide sites in both forward and reverse sequences were not included in the analyses (Ho & Shapiro, 2011). Multiple sequence alignments were carried out using MAFFT algorithms following default parameter setting, allowing determine sequence directions automatically and adjusting direction more accurately. All reading frames were also inspected in Geneious. Sequence lengths were trimmed to have identical lengths for all taxa, therefore, 239 sequences with 654 bp in length were left for phylogenetic reconstruction.

To conduct phylogenetic analyses, MrModeltest version 2.3 (Posada & Crandall, 1998) was utilized to estimate the best nucleotide substitution model for COI data set using an Akaike Information Criterion (AICc, Sugiura, 1978) framework. A GTR + I (Rodríguez *et al*., 1990; Yang, 1996) was found to be the most appropriate nucleotide model for all COI codon positions.

All nucleotide alignments were then analysed using a Bayesian inference (BI) method implemented in Beast v1.8.2 (Drummond & Rambaut, 2007). *Brevineura* clade (with 11 similar haplotypes, all sampled from South Australia) was used as outgroup with a mean root

height of 27.7 Ma (parameter treeModel.rootHeight set to normal distribution; mean = 27.7 and s.d. = 2.5) following estimated divergence age of Australian temperate exoneurines bees by Chenoweth and Schwarz (2011). MCMC analyses were run for 100 million generations, sampling every 1000th generation, and using unlinked substitution models for each codon position, linked clock models and trees, GTR+I for the substitution model, an uncorrelated lognormal relaxed molecular clock, with estimated substitution rate and Yule process speciation model. Two parameters including ucld.mean and ucld.stdev of lognormal clock rate priors followed an exponential distribution, mean = 0.33, and offset = 0.

The resulting log files were inspected in TRACER v.1.6 (Rambaut *et al*., 2014) to decide if posterior estimates of divergence times and substitution model parameters were stabilized given the Effective Sample Sizes (ESS). TreeAnotater v.1.8.1 (Drummond *et al*., 2012), used to construct maximum clade credibility (MCC) tree with a burn-in of 1000 trees. FigTree v1.3.1 (Rambaut *et al*., 2010) was then used to visualise the MCC tree.

Identical haplotypes were then detected in the resulting tree and removed from the final alignment. The MCMC analysis was run once more with 29 haplotypes following the same settings described above (Figure 2).

3.4. Species delimitation

We continued our analyses with species delimitation analyses to determine species boundaries in the resultant phylogenetic tree. However, for this analysis, Bayesian inference (BI) phylogenetic tree is needed as input. To do so, duplicate sequences were removed and Bayesian inference method using 29 sequences was implemented in MrBayes v.3.2. The *Brevineura* clade was again used as outgroup to root the tree. For MCMC analyses, nucleotide sequences were partitioned by codon positions and a GTR + I substitution model was applied for each partition. Analyses were run two times, with each run comprising 10 million generations, with three heated chains and one cold chain, with variable rate permitted and sampling every 1000th generation. Likelihood plots and standard deviation of split frequencies were assessed to verify stationarity of the model and length of run. Parameter trace files of each run were also examined in Tracer v.1.6 (Rambaut *et al*., 2014) and the first 25% of trees were discarded as burn-in. A 50% majority rule BI consensus tree was

constructed from the remaining trees and posterior probabilities were used to assess the robustness of nodes.

Species delimitation analyses were run using two methods: (i) a Poisson Tree Process (PTP) and (ii) bPTP (a Bayesian implementation of PTP) to the resultant COI phylogenetic tree. The PTP-master package was utilised for these analyses and the number of putative species were estimated in the phylogeny (Zhang *et al*., 2013) (Figure 3).

3.5. Haplotype networks

Network 5.0.0.0 (Fluxus Engineering 2016) was used to construct COI haplotype networks using the median joining network algorithm, with epsilon set to 10 and allowing external rooting of the networks using *Brevineura* clade. However, to export graphical haplotype genealogies, Haplotype Viewer (Salzburger *et al*., 2011) was used to generate Minimum Spanning Trees (MST). MST represents each unique haplotype, the number of individuals within each haplotype, the number of substitution between haplotypes and geographical structure of the haplotypes (Figure 4).

3.6. Pattern of historical demography

To obtain temporal patterns of historical changes in population size of the ingroup clades, Bayesian Skyline Plots (BSP) were generated for each clade. To do so, distinct clades in the MCC tree were detected and best fit nucleotide substitution models were then obtained for each clade separately using MrModelTest version 2.3 (Posada & Crandall, 1998). All individuals representing each clade were included in BSP, regardless of haplotype duplication as recommended by Grant (2015). The outgroup was not included in BSP analyses in order to constrain resolution solely on ingroups.

MCMC analyses using Beast v1.8.2 (Drummond & Rambaut, 2007) were run for 50 or 100 million generations (based on reached convergence for separate clades), sampling every 1000th generations, allowing for linked clock model and tree, GTR or HKY (assessed appropriate nucleotide substitution model for seperate clades) as substitution models, strict molecular clock with fixed rate at one and using Bayesian Skyline tree prior for BSP construction. Setting the rate parameter under a strict molecular clock with a rate of 1.0, instead of an uncorrelated relaxed clock, allows the x-axis in resultant BSP plots to be scaled

based on mean mutation rate per site per generation (Ho & Shapiro, 2011) which can be converted to calendar years using a mutation rate per generation and the number of generations per year. Because BSP analyses assume that sequences evolve neutrally (Ho & Shapiro, 2011), to construct BSP plots, we limited MCMC analysis to include only on 3rd codon positions which do not influence amino acid coding. Ultimately, the subsequent log files were inspected to assess whether ESS values were acceptable and BSP plots were then generated for each clade using TRACER v1.6 (Rambaut *et al*., 2014).

3.7. Estimate of molecular dating

Since there are no known exoneurine bee fossils (Schwarz *et al*., 2006) to estimate species divergence time, an approximate mitochondrial mutation rate was used instead. We therefore, followed a method of Groom *et al*. (2014a) which used a mitochondrial (mt) mutation rate of *Drosophila melanogaster* (6.2×10^{-8} per site per generation Haag-Liautard *et al*., 2008) to estimate molecular dating of tropical halictine bees. However, due to the effect of base composition on substitution rate (Montooth & Rand, 2008), AT frequency was estimated for each clade using PAUP v4.0b10 (Swoffort, 2002) to explore this possibly confounding factor. We obtained 78-79% AT frequency for our focal clades which is close to *Drosophila* mt genome AT bias (82%). Consequently, we believe that the *Drosophila* mt mutation rate 6.2×10^{-8} per site per generation is most likely applicable to estimate molecular dating of either our focal bees. We then scaled BSP plots based on calendar years through dividing yielded mutation rates per site per generation on x-axis by 6.2×10^{-8} per site per generation and the number of generation which is one per year for *Exoneura* and *Inquilina* (Schwarz, 1986; 1987).

4. Results

4.1. Phylogenetic topology

The maximum credibility tree from our BEAST analysis is given in figure 2, and the consensus Bayesian Inference phylogenetic (BI) tree from MrBayes analysis is presented in figure 3. In total, 29 unique haplotypes were recovered across five sampling locations.

Our phylogenetic analyses revealed six well supported clades (PP ≥ 0.95), referred to hereafter as Clades A to F. Clade A included specimens from the genus *Brevineura* which was used as outgroup in both Beast and MrBayes analyses. The ingroup contained a basal split between two genera comprising *Exoneura* and *Inquilina*. Within *Exoneura*, four major clades were recovered which we referred them to as Clades B - E. Clade F comprised species from the genus *Inquilina* and it included three haplotypes (Figure 2).

In the inferred phylogeny, high support was found for the monophyly of each genus (PP = 0.99/1) which is consistent with previous phylogenetic studies of allodapines. Clades D and E were recovered as sister clades (PP = 1) and clade D + E was a sister clade to Clade C (PP = 1).

When we assumed a mitochondrial mutation rate for 3rd codon positions of 6.2 x 10^{-8} per site per generation, and one generation per year, the BEAST analysis suggested a divergence between the *Brevineura* clade (Clade A) from *Exoneura* + *Inquilina* clades (Clades B - F) at about 27 Mya, and divergence between *Exoneura* and *Inquillina* at about 22 Mya. The crown ages for the included *Exoneura* clades was estimated at about 14 Mya which is very close to the estimated crown age of the included inquilines (approximately 14 Mya) (Figure 2). Key nodes ages and 95% credibility intervals inferred from Beast analyses are given in Table 2.

4.2. Species delimitation

PTP models for species delimitation applied to the COI data resulted in an estimated eight putative species including one species for the Clade A, four species for Clades B - E and three putative species for the Clade F. The bPTP model for the same gene led to an estimated 11 species in which the haplotypes of Clade E were inferred as four distinct species. The bPTP analyses on the rest of clades generated the same results as the PTP model (Figure 3).

4.3. Geographical patterns

Both BEAST maximum credibility tree (Figure 2) and the inferred haplotype network (Figure 4) suggested geographical structuring of haplotypes within Clades B, D, E and F. This is because: Clade B with only three haplotypes, was recovered mostly from Daylesford, but also one from the Dandenong Ranges. Specimens of Clade D, comprising six haplotypes,

were generally found from South Australia and the Dandenong Ranges, but also a single haplotype from the Daylesford. Moreover, seven haplotypes of Clade E were mostly recovered from Dandenong Ranges, but with some haplotypes from WA, Queensland and South Australia. Lastly, the Clade F was collected from the Dandenong Ranges with only one haplotype recovered from Daylesford.

However, there was no haplotype diversity detected for Clade A and all specimens of Clade C were from Western Australia.

4.4. Historical demography

We found only one haplotype for the 11 specimens of Clade A, and only three haplotypes for Clade B, which were insufficient haplotypes diversity to infer historical demography. Furthermore, we found only four haplotypes for the putatively three species of Clade F, which is again too small to infer historical demography. We therefore restricted BSP analyses to the Clades C, D and E which all belonged to *Exoneura* (Figure 5).

For these plots, we used two different scales for the x-axis, one is based on a mutation rate of 1.0 per site per generation and the other on a mutation rate of 6.2×10^{-8} per site per generation with one generation per year. The former scale allows the resulting branch lengths to be interpreted in units of mean substitutions per site, which can be converted to years given a mutation rate of 6.2×10^{-8} per site per generation and one generation per year.

Figure 5 needs to be interpreted very cautiously, for at least two important reasons. Firstly, we do not know how well the mitochondrial mutation rate for *Drosophila* reflects mutation rates for *Exoneura*, and secondly, it is known that BSP signatures of past demographic change can be largely obscured by recent changes in demographic change (Grant, 2012; 2015). For the latter issue, it is likely that the very flat lines preceding increases in population size are artefactual. However, all of Clades C, D and E show evidence of increasing population size beginning after about 1×10^{-3} mutations/site/generation, and under the assumed *Drosophila* mutant rate, and with one generation per year, this corresponds a period after the last glacial maximum. Clade D shows some evidence of an earlier start to population size increase at about 1.5×10^{-3} mutations/site/generation, which would roughly equate to the LGM or shortly before.

5. Discussion

5.1. Divergence between *Exoneura* and *Inquilina*

The maximum credibility chronogram tree (Figure 2) indicates that *Inquilina* clade diverged from *Exoneura* approximately 22 Mya. Our estimated divergence time here is consistent with that of Chenoweth and Schwarz, (2011) (16.5 ± 3.5 Mya) and Smith *et al*., (2013) (23 ± 11 Mya) when they set the age of the root node joining the Xylocopinae and the corbiculates tribes to 120 and 107 Mya respectively. It is worth noting that these two latter studies both included the majority of allodapine genera and used different methods (multiple nuclear genes and a calibrated lognormal relaxed clock), which gives us confidence that our estimate of the divergence between these two groups is relatively old.

5.2. Species delimitation

The delimitation PTP model applied to COI gene indicated that Clade E comprised of a single species. However, application of bPTP model suggested four putative species for this clade. In order to distinguish which model delimited Clade E correctly, male genitalia (an important character for morphological diagnosis) were dissected from 100% ethanol-preserved bee specimens of Clade E and observed under microscope for comparison (male genitalia images within Figure 3). We detected no appreciable morphological difference between selected specimens compared to other Australian allodapines (e.g. Reyes 1993). Consequently, Clade E was considered as a single species. This result emphasises that multiple approaches for species delimitation should be used to best assess the number of putative species within phylogeny.

5.3. Geographical structure

Our haplotype network (Figure 4) and haplotype phylogeny (Figure 2) analyses reveal geographical structure of haplotypes for all clades except for Clades A and C.

Lack of haplotype diversity in the *Brevineura* clade (Clade A) surprised us because they are largely restricted to southern regions of Australia (Bull *et al*., 2003). Our finding suggests that they might have either been experienced a major population bottleneck in a distant past

due to the habitat destruction/limited gene flow or have had very small population size inherently, although other possibilities such as a recent mitochondrial sweep for example from *Wolbachia*, are also possible (e.g. Bazin *et al*., 2006).

Among study sites, Dandenong Ranges comprise the most common haplotypes, which support a previous study, suggesting that south-eastern of Australia was a primary centre for exoneurine diversification (Chenoweth & Schwarz, 2011).

5.4. Demographic effects of Pleistocene climatic oscillations

Periodic occurrence of the glacial and interglacial cycles during Pleistocene caused the environment of Australia to experience major climate changes (Byrne *et al*., 2008). Severe frost accompanied by regional increases in aridity during Pleistocene cycles would have provoked environmental stresses on many floral and faunal elements of the continent if they were not well adapted to such conditions (Byrne *et al*., 2008). Despite Pleistocene importance, few studies have reconstructed the historical demography of insects during this epoch.

Our study is the first attempt to explore the responses of Australian-temperate allodapine bees to Pleistocene climate fluctuations. Using Bayesian coalescent analyses, we found an unchanged pattern of effective population size until about 16 kya for Clades C/D and 8 kya for Clade E of *Exoneura* (Figure 5), though as we have noted above, inferred population sizes prior to the latest population change need to be treated very cautiously.

Our BSP analyses suggested increasing population sizes of the three *Exoneura* species since LGM, but no evidence of a major population crash at the last glacial maximum, as found by Groom *et al.,* (2013, 2014) for bees from tropical Pacific islands. Groom *et al*., (2013, 2014a) found that halictine bees of three South Western Pacific (SWP) archipelagos suffered from massive population declines during LGM.

The post LGM increases found in our study and the study by Dew *et al*., (2016) on *Ceratina australensis* could be due to declining aridity in post LGM Australia. More specifically, Dew *et al*., (2016) linked this increase in N_e to the reduced competition, expansion into new suitable habitat and increased resource availability due to the warming climate during post-

LGM. However, she indicated that lack of knowledge about detailed historical reconstruction of Australia can be problematic to examine these hypotheses. However, as we note above, our study did not find evidence for a dramatic decline in N_e of focal bees during the LGM, and this contrasts strongly with studies on SWP halictine bees (Groom *et al*. 2013, 2014a). We here put forward two possibilities that might explain the current outcome.

It is possible that since Australian *Exoneura* bees occur on higher latitude, they may have broader species ranges both altitudinally and latitudinally to inhabit according to the Rapoport's rule (Rapoport, 1975), suggesting that species towards tropics have smaller latitudinal ranges. Consequently, it seems likely that *Exoneura* species could migrate to new habitats with more preferable climate conditions during extreme weather events in their native habitats. However, tracking climate changes for bees occurring in isolated islands like Fiji is much less likely.

Unchanged population size of *Exoneura* species over the LGM also might be due to the connectivity in climate related habitats of *Exoneura* species. Given that *Exoneura* species distributed from Western Australia to South Australia, Victoria and Queensland (Figure 1) and that, these regions were sufficiently connected, we think that this connection may have prevented inbreeding and reduced genetic diversity (via genetic drift) in this faunal group due to the ecological processes like gene flow and migration and therefore, this may have preserved their past population sizes (e.g. Correa Ayram *et al*., 2016).

Previous studies found that animals are able to adapt to high/low environmental temperatures using mechanisms which have developed through evolution (Sørensen *et al*., 1999; Even *et al*., 2012; Zhao *et al*., 2012; Yampolsky *et al*., 2014; Sanin *et al*., 2016). We think that given *Exoneura* lineages adapted to a wide range of seasonal temperatures fluctuation (ranging from -2 ˚C in winter up to 45 ˚C in summer) since arriving in Australia, this may cause a higher selection on these species for expression of more tolerant genes/physiological adaptation to increase thermal tolerance. Whereas halictine bees of SWP experience almost uniform temperature over a year, ranging from 27˚C in winter to 34 ˚C in summer.

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Table 1. Collection localities of exoneurine bees used for COI barcode sequencing along with the number of barcoded specimens, identified genera and the number of unique haplotypes recovered from each location.

Sampling location		Collection dates	Specimens barcoded	Identified genera	Recovered haplotypes
South Australia	Chain of Ponds	May 2013	3	*Brevineura*	1 (*Brevineura*)
	Myponga	August 2013	1	*Brevineura*	
	Mylor	August 2013	1	*Brevineura*	
	Stirling	August 2013	2	*Exoneura*	
	Warrawong Sanctuary (Heathfield)	June 2013	2	*Exoneura*	
	Woorabinda	August 2013	7	*Exoneura*	3 (*Exoneura*)
	Bridgewater	October 2013	18	*Brevineura and Exoneura*	
	Mount Lofty	August 2013	9	*Brevineura and Exoneura*	
Dandenong Ranges, Victoria		February 2013	78	*Exoneura and Inquilina*	7 (*Exoneura*)
					3 (*Inquilina*)
Daylesford, Victoria		July 2013	44	*Exoneura and Inquilina*	3 (*Exoneura*)
					1 (*Inquilina*)
Warwick, Queensland		December 2013	4	*Exoneura*	2 (*Exoneura*)
South western of Western Australia		November 2013	70	*Exoneura*	9 (*Exoneura*)

Table 2. Divergence age estimates and 95% confidence intervals (CI) for focal exonerine clades using Beast analysis.

Node description	Age (Mya)	
	Mean	95% CI
Divergence of genera *Brevineura* /*Exoneura* and *Inquilina*	27	22-32
Divergence of *Inquilina* /*Exoneura*	21.92	14-29
Divergence of *Exoneura*	13.85	7-22
Divergence of *Inquilina*	13.58	7-21

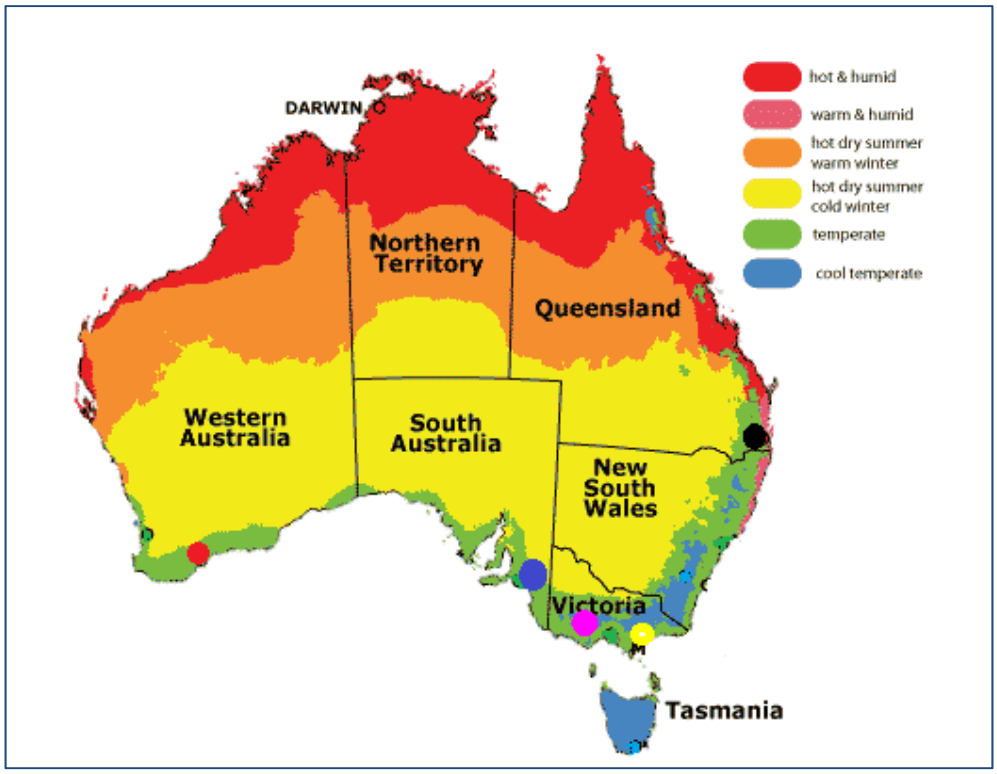

Figure 1. Collection localities of exoneurine bees on map of the climate zones of Australia. Colourful circles indicate sampling locations: Dandenong Range: yellow; Dayles Ford: Pink; Queensland: black; South Australia: blue; Western Australia: red.

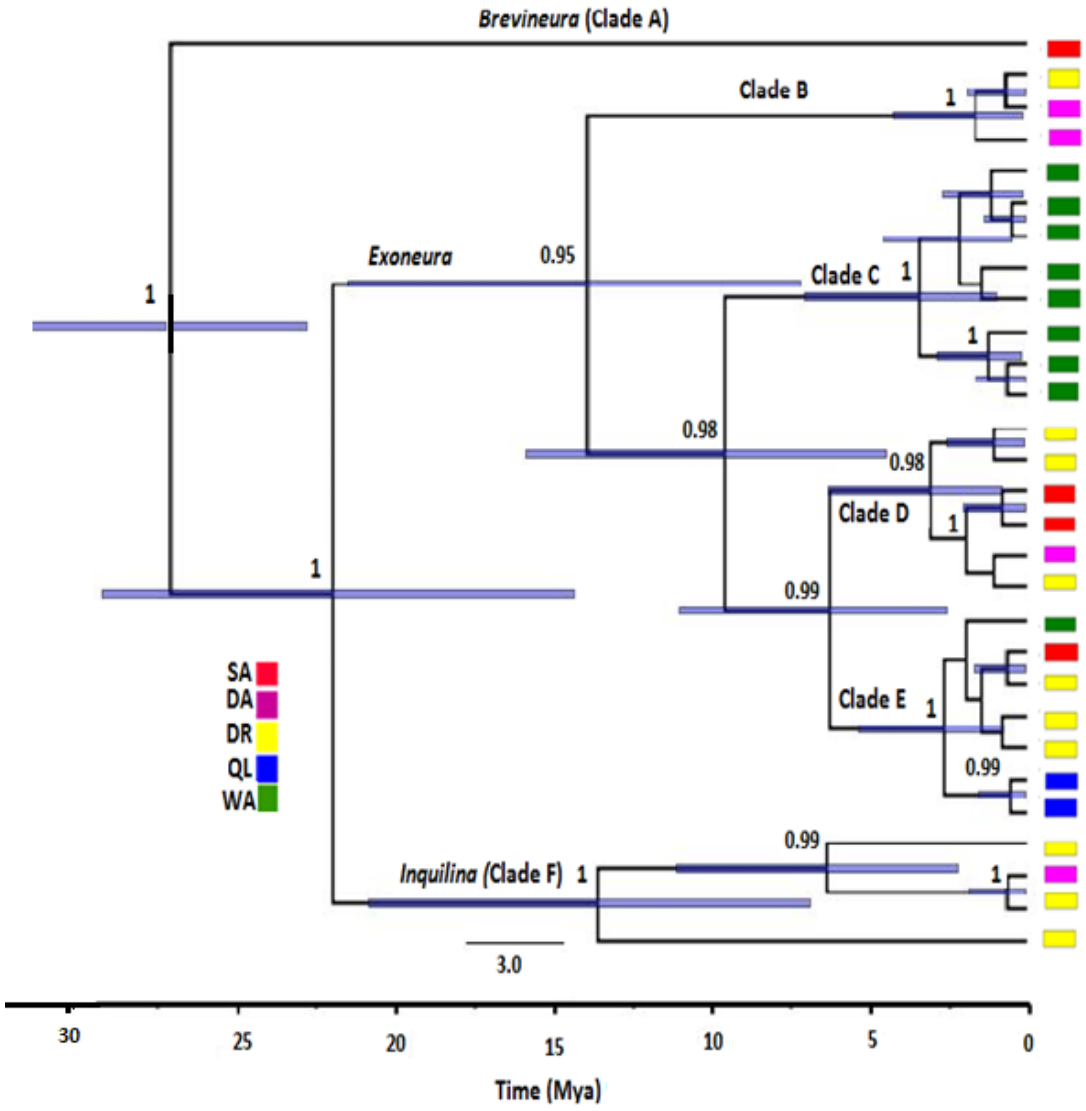

Figure 2. Maximum credibility tree resulted from Beast analysis for mtDNA COI gene of exoneurine bees. The numbers next to the nodes are posterior probabilities ≥ 0.95. Error bars indicate 95% confidenace intervals on the node dates. The horizontal axis depicts time before present in Millions of years (Mya). Identified clades were labelled in the tree: *Brevineura* (Clade A), *Exoneura* (clades B - E), *Inquilina* (Clade F). The colourful rectangles show the locations where each haplotype was recovered.

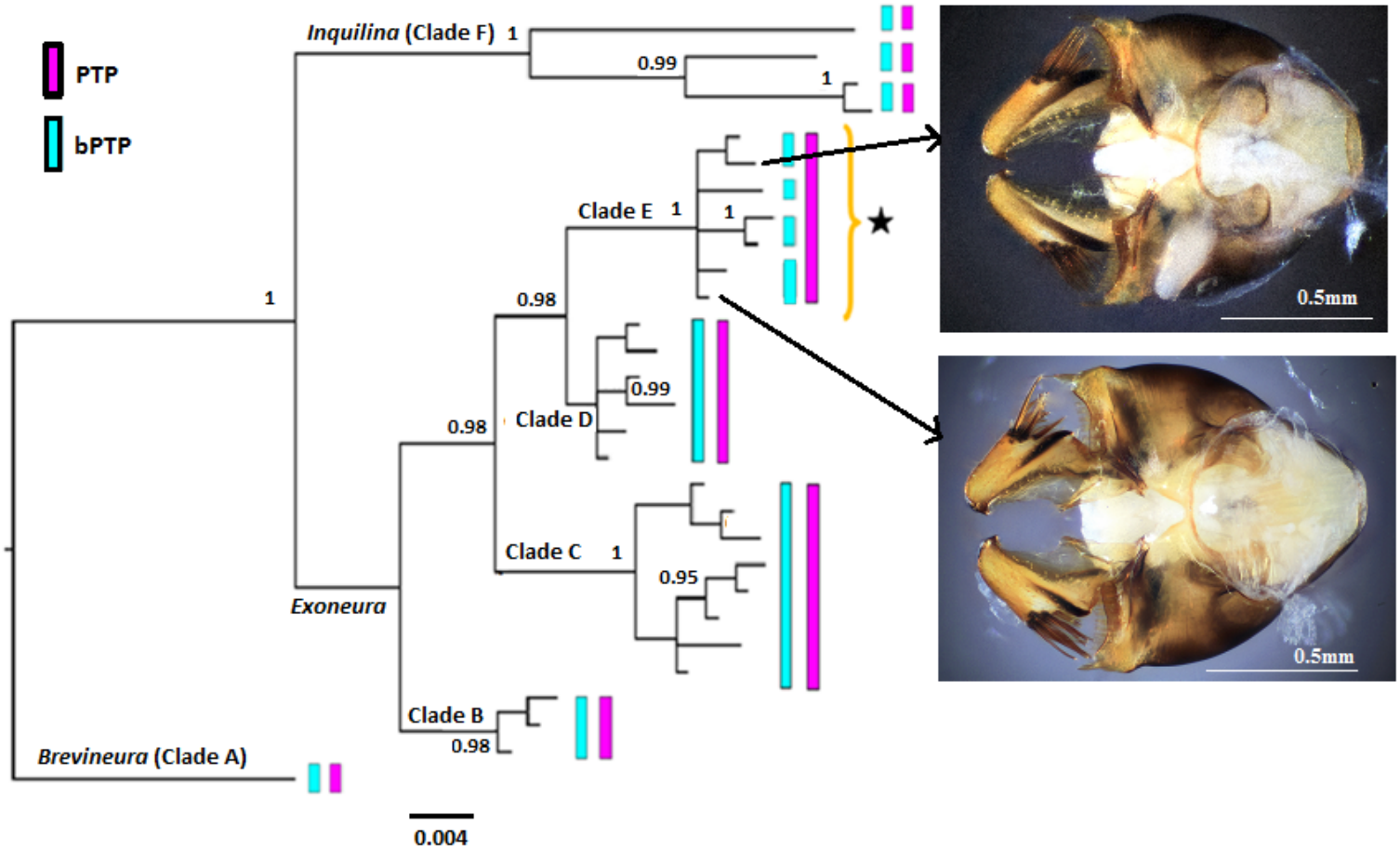

Figure 3. 50 % majority rule consensus Bayesian Inference tree resulted from MrBayes analyses based on the mtDNA COI gene. The numbers next to the nodes are posterior probabilities ≥ 0.95. Identified clades were labelled. The blue and pink bars show species delimitation using bPTP and PTP methods respectively. The black star denotes lineages considered to be the same putative species based on the PTP method. Images indicate ventral view of male genitalia of specimens from Clade E which were detected as the same species according to the PTP method.

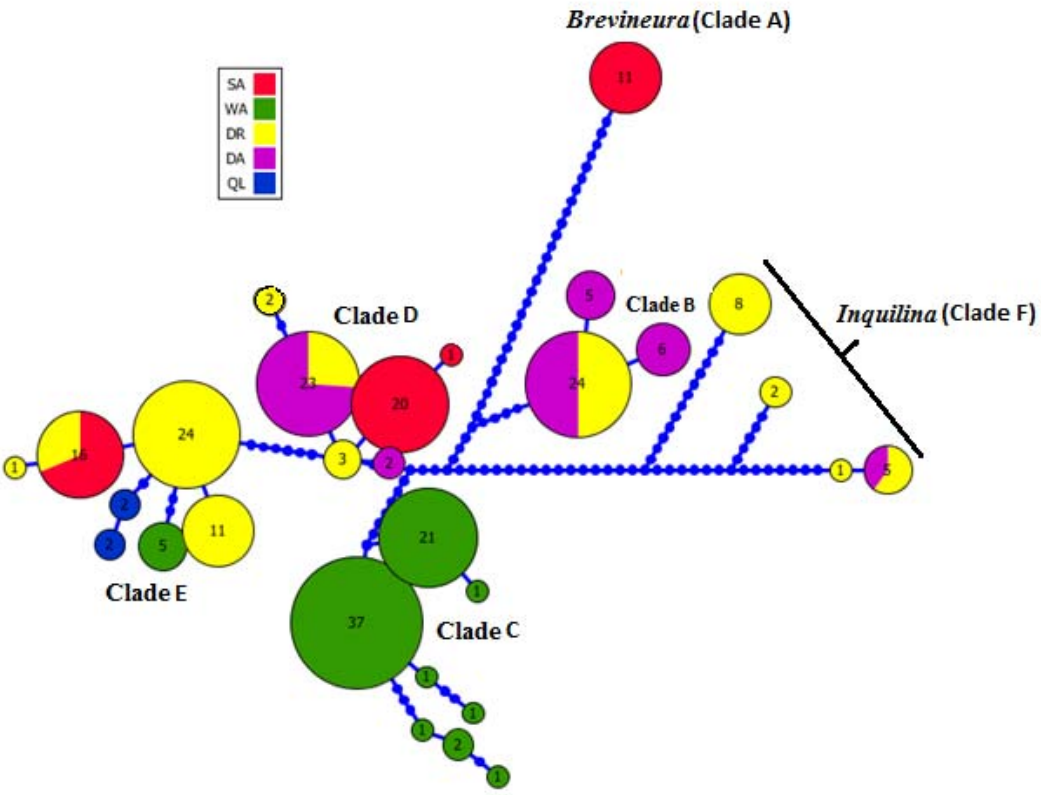

Figure 4. Minimum spanning tree constructed from sampled exoneurine bees. Each circle represents a unique haplotype and the numerals inside indicate the number of individuals sharing that particular haplotype. Each step between haplotype indicates one base pair substitution. The colourful rectangles show the locations where each haplotype was recovered.

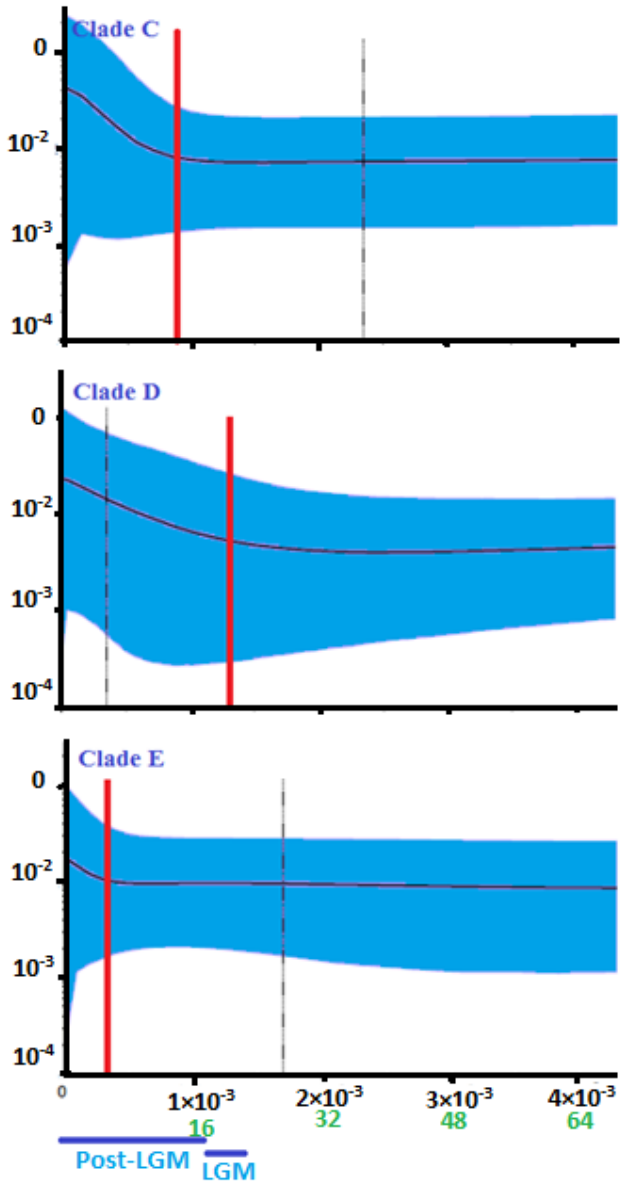

Figure 5: Corresponding Bayesian skyline plots (BSP) for Exoneura Clades (C, D and E). Maximum time is the root height mean, dotted vertical line represents lower 95% highest posterior density. Bold black line indicates mean plot values; blue shaded area represents upper and lower confidence intervals for mean estimates. Bold Red line indicates the approximate time of elevated/lessened effective population size. Numbers on y-axis represent effective population size. Black numbers on x-axis are mutations per site per generation and green numbers are time based on kya. Blue lines under plots indicate timing of LGM and post-LGM in Australia.

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General discussion

1. Synthesis

The general aim of my research program was to understand how social parasite species with very small effective population sizes (N_es) have been able to persist over extensive evolutionary time despite their putatively much slower rates of adaptive evolution compared to their hosts. I used the speciose group of Australian allodapine host and social parasite bees to examine this aim.

My colony-based collection data suggested that Australian allodapine host and parasite bee species differ by about an order of magnitude in their effective population sizes, with *Inquilina* only infesting about 5% of host colonies. However, phylogenetic studies of allodapine host and parasite bees revealed that parasite species have managed to survive over long periods of evolutionary time and also followed their hosts through multiple speciation episodes (Chenoweth & Schwarz, 2011; Smith *et al*., 2013). This finding creates a major paradox for traditional evolutionary theory (Kimura & Ohta, 1971), which suggested that rates of adaptive evolution are limited by effective population size, therefore species with very small population sizes should be less able to compete when locked into arms races with species that have much larger population sizes.

In the first chapter of this thesis, I estimated relative N_e of allodapine host and parasite bees based on incidences of parasitism, using very large sample sizes of *Exoneura robusta* Cockerell 1922 and its associated parasite *Inquilina schwarzi* Michener 1983. In addition, the effects of complicating factors on N_e such as of reproductive skew in host colonies, relatedness among host nestmates and sex ratio biases of host species, were also considered when estimating effective population sizes of focal host and parasite species. Nevertheless, even by including the effect of these factors, which are likely to greatly underestimate the relative N_e of host species compared to its parasite, I obtained a substantial disparity in relative N_e of host and parasite species, with inquiline species having N_es that are about an order of magnitude lower than their host species. I argued that there might be several mechanisms that allow long evolutionary persistence of allodapine parasite bees, which are locked in a tight coevolutionary arm races with their hosts. In my first hypothesis, I suggested that genomic mechanisms such as increased recombination rates (Hill & Robertson, 1966; Felsenstein, 1974) or mutation rates (Biémont & Vieira, 2006; Pritham, 2009; Biémont,

2010) might speed up the rates of adaptation of social parasites above those that are based purely on N_e.

For other hypotheses regarding the long-term persistence of inquilines, I pointed out the possibility of other mechanisms which don't involve evolution of genes in inquilines, but may permit them to survive along with their hosts despite their small N_e. One mechanism arises when inquiline species cooperate with the host species in nesting and defences and exploit only a small fraction of the host colony resources. This hypothesis is arised given cooperative nesting frequently observed in allodapine bees between unrelated conspecifics. In such situation, the association between host and parasite species could even approach symbiosis rather than arms race. Symbiosis between host and parasite species could limit the ability of hosts to evolve defences against inquilines and allow inquilines to persist in host colony. The low rate of virulence obtained for *I. schwarzi* here might be another possibility for lowering host evolutionary responses and allowing inquilines to persist. In such a situation, selection pressures on host species to prevent attack may be low due to the small population size and very low infestation rates of inquilines. However, selection pressure on hosts migh fluctuate, where there is strong selection pressure when inquilines are common, but low pressure when inquilines are rare (Erler *et al.*, 2014). Another possibility occurs if inquiline species are able to switch from a long-term host, which may have evolved effective defences, to a new host which has not had recent evolutionary 'experience' against that new infesting inquiline species and needs considerable time to improve its defences. In such a situation, once new host's defences become effective, the parasite may be able to switch hosts again. These possibilities have not been explored in other studies and may be areas for fruitful future research.

Given that N_e is affected by sex ratio in haplodiploid organisms like bees (Wright, 1933; Hedrick & Parker, 1997), in my second chapter I investigated the effect of sex ratio patterns of *I. schwarzi* (obligate social parasite of *E. robusta*) on the disparity of N_e between these two species which are locked in an evolutionary arms race. I found that the population-wide sex ratio of *I. schwarzi* is female-biased in the smallest brood, but becomes more even in larger brood, suggesting that this species is able to adjust sex ratios according to the number of its own brood. I then argued about the possible evolutionary scenarios for the inferred sex ratio pattern in inquiline species which are explained briefly here.

Previous studies on allodapine bees attributed inferred female biased sex ratios in allodapine bees to local resource enhancement (LRE) where daughters cooperate in brood rearing and play an alloparental role in the event of maternal death when the brood are still premature (Bull & Schwarz, 2001; Aenmey *et al*., 2006; Thompson & Schwarz, 2006). This scenario is rejected for the inferred sex ratio pattern in *I. schwarzi* because there is only one inquiline female remaining in host nest during brood rearing. I also argued that local mate competition (LMC) is not possible for the sex ratio of *I. schwarzi* as it predicts a male-bias when brood number is small (West, 2009), which is opposite to what I found for this species. I argued that local resource competition (LRC; Clark, 1978) is consistent with the *I. schwarzi* sex ratios where female inquilines compete to inherit their host colony and only one female inquiline which has higher fitness, could successfully inherit her host colony. This scenario predicts female bias in the smallest brood numbers, with increasing investment in males as brood numbers become larger which is consistent with the resultant sex ratio pattern in *I. schwarzi*.

Furthermore, my analyses showed that at the population level, the sex ratio of *I. schwarzi* is unbiased while that of its host species *E. robusta* is extremely female biased. Extreme female biased sex ratio of host species may decrease the relative N_e of the host as the genes passed on by the host males, are sampled from a smaller population than that of host females. On the other hand, the relative N_e of *I. schwarzi* increases up to 50% higher due to its overall sex ratio parity. This suggests that the relative disparity in N_e between focal host and parasite species in this study is less than that estimated from parasitism rates alone.

In my third chapter, I examined if allodapine social parasites have indeed faster evolutionary rates than their social host, because of their much smaller effective population sizes and the consequence of enhanced genetic drift on the parasite species. To do so, I compared nonsynonymous to synonymous substitution rate ratios (d_N/d_S) of 11 protein coding mitochondrial genes using two host/parasite pairs of Australian allodapines. My analyses showed that rates of molecular evolution of mtDNA genes are similar between the hosts and their associated parasite species.

I then discussed several possibilities that might explain why this study did not support the predicted differences in adaptive rates of molecular evolution between hosts and their social parasites, which are as follows: (1) Although the analyses in chapter one showed that N_e of

allodapine inquilines is about an order of magnitude lower than their hosts based on incidences of parasitisation, it is possible that the N_e of inquilines is not small enough in itself to affect the rate of molecular evolution in inquilines compared to their host species; (2) while it is clear that N_e may have considerable effects on the rate of evolution, estimation of the magnitude of that effect is not simple (Bromham & Leys, 2005; Woolfit, 2009). Previous studies (e.g. Bachtrog, 2008; Woolfit, 2009) suggested that the distribution of fitness effects of both slightly deleterious and advantageous mutations must be considered when the degree of the effect of a change in N_e on rate of evolution is determined. (3) In addition to the effect of N_e on the rate of evolution, fecundity also should be considered while comparing rates of molecular evolution as it is positively correlated with the rate of molecular evolution (Bomham & Leys, 2005, Welch & Bromham, 2005). Therefore, the impact of this feature on rate of evolution is opposite to that of the N_e effect, with higher rates for host species (which have high fecundities) relative to inquilines (which have low fecundities). (4) Another possibility could arise if the relationship between *Exoneura* Smith and its *Inquilina* Michener is verging on symbiotic rather than antagonistic coevolution. This situation could arise if inquilines cooperate with their host species in colony defense while it uses only a small fraction of the host colony resources. In such situation selection on both host and parasite genomes might be lower.

Previous studies have explored the effects of the last glacial maximum (LGM) on the geographical distribution and population size of terrestrial and marine organisms (Ruzzante *et al*., 2008; Palkopoulou *et al*., 2013). For my Forth chapter, I inferred historical population demography of temperate exoneurine bees back to the LGM by applying Bayesian skyline plot analyses to the barcoding region of the mitochondrial gene COI. Temperate exoneurine genera comprise the oldest clade of the allodapine bees in Australia and consist of the genera *Exoneura*, *Brevineura* and *Inquilina.* I restricted BSP analyses to only three clades of *Exoneura* due to the lack of haplotype diversity found for *Brevineura.* *Inquilina* also wasn't included in BSP analyses due to the very small sample sizes detected for this parasite clade. My analyses suggested that *Exoneura* species were largely unaffected by climate changes over the LGM compared to the responses of bees to glacial cycles with either tropical distributions (Groom *et al*., 2013, 2014a) or a principally xeri-zone bee (Dew *et al*., 2016). I suggested three scenarios which might explain a lack of major changes in the population size of this genus over LGM. One possible explanation is that *Exoneura* species are adapted to a wide range of seasonal temperatures fluctuation

(ranging from -2 ˚C in winter up to 45 ˚C in summer) and this may have buffered the against recent past climates. It is also possible that Australian *Exoneura* bees have broad species ranges that allow them to track changing climates both altitudinally and latitudinally without suffering substantial reductions in their population sizes. We also suggested that connectivity in climate related habitats may have stabilized past population sizes for this faunal group.

2. Future research directions

As discussed in the first chapter, smaller effective population sizes of allodapine social parasites compared to their host entails important evolutionary puzzles for how parasite species with low N_e have managed to persist over extensive evolutionary time. Although it is clear that N_e may have substantial effects on the rates of molecular evolution, my analyses here revealed that rates of molecular evolution between allodapine host and parasite is similar, at least for protein coding mitochondrial genes. However, further samples of other allodapine host-parasite species pairs will be required to confirm consistency of these results. Given that reduced N_e may affect mitochondrial and nuclear genes differently (Mitterboeck & Adamowicz, 2013), nuclear genes should be also analysed for comparison between host-parasite pairs, especially those for which an adaptive function are expected in parasite species.

On the other hand, it seems likely that the relationship between allodapine host and parasite species is a more benign relationship rather than antagonistic coevolution. At present, the evolution of more benign species interactions has not been explored in terms of rates of molecular evolution and I argued that my data calls for such an examination in future studies.

Host switching by inquilines might be another possibility that allows *Inquilina* species escape from long-term evolutionary races with an old co-evolved host to a novel host which still hasn't evolved effective defences against that infested *Inquilina*. Given the extensive evolutionary persistence of *Inquilina* species from 15 million years ago, I recommend that this possibility should be assessed beside other mechanisms which involve the evolution of

genes in inquilines. This hypothesis could be assessed by determining whether host switching is frequent in inquiline lineages.

The effects of past climate change on biodiversity of bees represent a 'natural experiment' that can allow us to anticipate the effects of future climate change on such pollinators. This issue is critically important for Australian allodapine inquilines, which have very small population size. In the fourth chapter, I couldn't apply Bayesian skyline analyses to *Inquilina* owing to the small sample size for this genus, which is very uncommon and difficult to sample. Given predicted climate change scenarios, studies are needed to determine how climate change may impact *Inquilina* species. In the future a more comprehensive sampling of allodapine inquilines needs to be carried out to predict the effect of climate change on their populations.

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