CONTRASTING EUSOCIALITY WITH CASTELESS ORGANISATION: ECOLOGY, GENETICS AND HISTORICAL DEMOGRAPHY IN AUSTRALIAN XYLOCOPINE BEES

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Cover photo: male specimen of *Exoneurella tridentata*

SUMMARY

This study explores multiple evolutionary aspects of the allodapine and ceratinine bees (family Apidae), utilising a comparative approach that incorporates phylogenetics, behaviour, ecology, biogeography and historical demography. My research focusses on the only Australian Ceratinini species, *Ceratina* (*Neoceratina*) *australensis,* and the genus *Exoneurella* from the tribe Allodapini. While *C. australensis* is subsocial, *Exoneurella* ranges from facultatively social to eusocial, with *E. tridentata* representing the only origin of true eusociality in the Xylocopinae.

My study is organized into six key research issues, each of which comprises a thesis chapter:

Firstly, I address the issues of terminology for social systems, where the lexicon is geared towards putative evolutionary pathways to eusociality, and therefore refers to societies based on development of hierarchies. This hinders social behaviour research by obscuring the identification of societies that lack hierarchies. To identify societies without hierarchies I propose the term 'casteless'. This is an important distinction from the terms communal and quasisocial, which imply a lack of hierarchies but are dependent on nest architecture and therefore taxonomically restricted. An explicit identification of casteless groups empowers social behaviour research and extends our understanding of social complexity.

Secondly, I examine the structure of social colonies of *Exoneurella setosa* and *Exoneurella eremophila*, in chapters two and three respectively, finding that both are casteless. Utilising Monte Carlo resampling techniques I find no evidence of hierarchies in social groups and only minimal benefits to social living for each species. Comparison with other casteless taxa

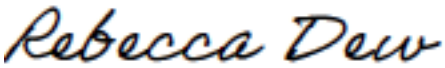
suggests that low barriers to dispersal coupled with only small benefits to social living may be the key to casteless group formation. I argue that, for the allodapines, casteless behaviour is an evolutionarily persistent and successful strategy.

Next I examine the biogeography and historical demography of *C. australensis* (chapter four), and *Exoneurella tridentata* and *Exoneurella setosa* (chapter five). By sequencing DNA 'barcodes' I explore whether differences in climate, nesting substrates or social behaviour may influence gene flow and historical demography in bees. *Exoneurella tridentata* and *C. australensis* show historical increases in population size after the Last Glacial Maximum, which is not apparent for *E. setosa. Exoneurella setosa* and *C. australensis* are mostly sympatric and share nesting substrates. While their historical demography differs they have strikingly similar haplotype networks, suggesting that more recently gene flow and dispersal in both species have been a common influence. Variable responses to historical climate change indicate that future responses may influenced by a matrix of climate, habitat reliance and social behaviour.

Lastly, I describe a new species named *Exoneurella micheneri,* in tribute to the late C.D. Michener. This study combines molecular and morphological data, recovering *E. micheneri* as basal to the other *Exoneurella*. The phylogeny also recovers the social parasites *Inquilina* and their hosts *Exoneura* as reciprocally monophyletic, reflecting other recent phylogenetic studies. I raise *Inquilina* **stat.r.** from a subgenus of *Exoneura* to full generic status. This distinction is important for future studies on the evolution of social parasitism. I also discuss the exciting prospects that *E. micheneri* provides for future social behaviour research.

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.



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GENERAL INTRODUCTION

Eusocial insect societies are characterised by extreme reproductive skew, often with complete reproductive dominance by one individual (the queen). Species with eusocial behaviour have been the focus of extensive research, partly due to the economic importance of many of these species, such as the honey bee *Apis mellifera* which is utilised for honey production and crop pollination services, but also because they present 'special difficulties' for classical theories of evolution. Indeed, the controversy over whether eusociality can even be explained by extension of classic Darwinian theory into inclusive fitness theory continues up until today (Nowak et al. 2010; Abbott et al. 2011; Allen and Nowak 2016). It is still unclear how these eusocial societies, with such extreme reproductive hierarchies, initially evolved. Part of the difficulty is that there are very few origins of eusociality, most occurring more than 60 Mya, so that closely related non-eusocial relatives have been lost (Engel et al. 2016; Cardinal and Danforth 2011; Moreau et al. 2006; Brady et al. 2003). Therefore, studies of most extant eusocial taxa can only infer factors influencing the maintenance of eusocial behaviour rather than those at the origin of eusociality. This has led to multiple calls for research on non-eusocial taxa that can illuminate transitions in social evolution (Rehan and Toth 2015; Kocher and Paxton 2014).

Given that bees are major pollinators for ecosystems and animal-pollinated crops (Klein et al 2007; Ollerton et al. 2011), our paucity of knowledge regarding their behaviour, ecology and life history limits our ability to safeguard critical pollination services. Rapid future climate change has been identified as a key risk to animal pollinators (Brown et al. 2016; Vanbergen et al. 2013) and shifts in some pollinator distributions in response to climate change have

already been identified (Aguirre-Gutiérrez et al. 2016; Burkle et al. 2013; Bedford et al. 2012), with species like the bumblebee *Bombus terrestris* in the Northern Hemisphere facing severe range reductions (Kerr et al. 2015; Cameron et al. 2011). Unfortunately studies on distribution changes are limited by often incomplete museum records, constraining our understanding of long-term climate change adaptations. We also do not know how differences in behaviour and ecology may influence a species' ability to adapt to climate changes.

This thesis aims to address these gaps in both our understanding of social evolution and how differences in social behaviour and ecology may influence climate adaptation. Firstly, the social behaviour of two non-eusocial species in the genus *Exoneurella* (Xylocopinae: Allodapini) are studied. I then leverage this knowledge, combined with that of another Xylocopine bee *Ceratina australensis* (Xylocopinae: Ceratinini), to make broad predictions using molecular analyses on how these bees were influenced by past climate change events.

The thesis takes the form of six chapters, each in an appropriate format for journal submission. A version of chapter I has been published by *Insectes Sociaux* (doi:10.1007/s00040-015-0435-1). Chapter II is currently under review at *Ecology, Ethology and Evolution*. The third chapter has been formatted for the journal *Apidologie*, to which it will be submitted. A version of chapter IV is published in the *Journal of Hymenopteran Research* (doi: 10.3897/JHR.49.8066). Chapter V has been formatted for the journal *Diversity and Distributions*, while chapter VI has been formatted for the journal *Systematic Entomology*.

As the primary author of all papers collated in this thesis I was responsible for most data collection and I performed all of the laboratory work including DNA sequencing and specimen dissections. Data analysis, manuscript and figure preparation were also primarily performed by myself, with assistance from co-authors. Specifically, the manuscript for Chapter I benefitted from discussions with with Michael Schwarz and Simon Tierney. Michael Schwarz and Simon Tierney also assisted with fieldwork for Chapters II and III, as well as giving guidance on statistical analyses and revision of manuscript drafts. Field samples from Queensland, used in Chapter IV, were collected by Sandra Rehan, who also commented on drafts of this manuscript along with Michael Schwarz. Revisions of chapter V were made with assistance of Michael Schwarz, Michael Gardner and Mark Stevens. Field work for chapter VI was assisted by help from Mark Stevens, and he revised drafts of the manuscript with Michael Schwarz.

A brief summary of each chapter is given below:

CHAPTER I: *Social evolution and casteless societies: needs for new terminology and a new evolutionary focus*

Societies lacking hierarchies have been largely overlooked in research on social behaviour. Studies overwhelmingly focus on eusocial species, or species with hierarchical behaviour as potential precursors to eusociality. This paper explores the benefits of studying groups lacking hierarchies. I argue that the current terminology obscures identification of societies that lack hierarchies despite the possible comparative power of such taxa to studies of social evolution. The term 'casteless' is proposed to define species where hierarchies are absent and methods for identifying such taxa are suggested.

CHAPTER II: *The evolution of equable nesting: casteless social behaviour in an allodapine bee*

Here I employ the methods suggested in chapter I to a study on the facultatively social species *Exoneurella setosa*. Wing length, wing wear and ovary size for each female were recorded as biometric proxies for body size, foraging activity and reproductive development. Utilising Monte Caro resampling techniques I found no evidence for hierarchies in social groups, and with only minimal benefits to social living. This is a striking finding given the well-developed hierarchical social behaviour of its congener *E. tridentata*. I discuss possible reasons why *E. setosa* has social colonies despite the low apparent benefits and hypothesise that differences in nest-site limitations may be a key driver of social evolution in this genus.

CHAPTER III: *Casteless behaviour in social groups of the bee Exoneurella eremophila*

This chapter delves further into the social behaviour of *Exoneurella*, presenting data on another facultatively social species, *E. eremophila*. Following the methods for Chapter II, I identify that *E. eremophila* is also casteless, completely lacking social hierarchies. Monte Carlo resampling techniques show that ovary size differences within social pairs are less than would be expected from random pairs forming among the population. I discuss the possible reasons for this and highlight the minimal apparent benefits for social living also found in this species. This is the second facultatively social *Exoneurella* species identified as casteless and I suggest that casteless behaviour is a persistent, successful evolutionary strategy for this genus and possibly the wider Allodapini.

CHAPTER IV: *Biogeography and demography of an Australian native bee Ceratina australensis (Hymenoptera, Apidae) since the last glacial maximum*

The previous chapters highlight that behaviour, ecology and evolution are often closely intertwined. In this chapter I go a step further to look at how these interacting factors may influence a species at a population level. I use mitochondrial COI sequences to explore the population genetics and historical demography of this species. My findings indicate that this species underwent a population expansion starting with the end of the Last Glacial Maximum, and coinciding with post glacial global warming. I discuss the implications of this in the face of rapid future climate change.

CHAPTER V: *Mixed responses of arid-adapted bees to the Last Glacial Maximum: The role of behaviour in long-term population stability*

The Last Glacial Maximum (LGM) was a period of increased aridification, so taxa already adapted to arid conditions may have responded differently to the climatic changes. I study the historical demography and population genetics of *E. tridentata* – a strictly arid to semi-arid species, and compare this to *E. setosa,* which also persists in semi-arid to arid regions, but ranges more broadly to temperate and subtropical areas as well. While *E. tridentata* shows a population expansion timed at the close of the LGM, *E. setosa* shows no population size fluctuation over the last 100-200kya. I suggest that behavioural flexibility may have provided *E. setosa* with long term population stability and discuss the surprising similarity in population genetic structure of this species to that of *Ceratina australensis*.

CHAPTER VI: *Taxonomy and generic status of the Australian allodapine bee genera Exoneurella and Inquilina (Apidae: Xylocopinae: Allodapini)*

This chapter formally describes a new species of the genus *Exoneurella*, named *Exoneurella micheneri* in honour of the late C. D. Michener. I present a phylogenetic reconstruction incorporating both molecular and morphological data, which supports the position of this species as basal to all other *Exoneurella*. The genus *Exoneurella* is redefined to incorporate this new species and a new species key is presented, which incorporates male genitalic traits. This paper also reinstates *Inquilina* to full generic status, reflecting its reciprocal monophyly with the genus *Exoneura* and reflecting the naming conventions used for the other parasitic Allodapini. These taxonomic revisions have important implications for future studies on both social behaviour and social parasitism.

I conclude this thesis with a *General Discussion*, exploring the overarching findings and conclusions of these studies.

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CHAPTER I

Social evolution and casteless societies: needs for new terminology and a new evolutionary focus

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Abstract

There has been considerable debate surrounding the evolution of eusociality, which has recently increased in vigour with regard to what actually constitutes eusociality. Surprisingly, there has been little discussion on terminologies for describing social systems that are more-or-less egalitarian, yet such societies form an obvious contrast to eusociality, and transitions between these two forms of social organization appear to be common. We argue that current terminologies and methods for dealing with non-hierarchical societies are not well suited for such comparative approaches to social evolution. We outline three problems for comparative approaches (identifying egalitarianism, implied egalitarianism and taxon specific terminology) and propose two solutions. The first solution is a re-sampling method to assess investment asymmetries, and the second is the introduction of the term "casteless" to encompass forms of social organization where there is no lifetime commitment to queen-like or worker-like roles, but where skew in reproduction or alloparental tasks may nevertheless be apparent at any one time. Our suggested terminology avoids the implied egalitarian nature behind the terms communal and quasisocial, which place undue emphasis on specific nesting biologies and which have the potential to impede 'bottom-up' comparative studies of social evolution. Such non-eusocial groups provide the best insights for understanding how social behaviour evolved and our suggested approaches should enhance future investigations.

Keywords: social evolution, communal, quasisocial, eusocial, egalitarian, despotic

INTRODUCTION

Studies on insect social evolution have long had a focus on the origins of eusociality, where some individuals assume roles that are primarily reproductive, or else highly altruistic. The problems that extreme altruism pose for evolutionary biology have been appreciated since the formulation of natural selection theory, but the selective mechanisms remain contentious (e.g. Nowak et al. 2010; Abbot et al. 2011; Nonacs & Hager 2011; Holman 2014; Rautiala et al. 2014; Gardner 2015). Considerable effort has been expended to identify the origins of eusociality and to then ask if these are associated with particular life history, ecological, developmental or genetic features (e.g. Wilson 1971; Lin and Michener 1972; Michener 1974, 1985, 1990; Pamilo and Crozier 1996; West-Eberhard 2003). More recently, focus has shifted to genomic bases of eusociality, and there is little sign that research in this area is abating (*reviewed by* Rehan & Toth 2015).

As molecular phylogenetic studies on social insects have increased, there has been a general decline in the number of inferred origins of eusociality in Hymenoptera and an increase in the number of inferred losses of eusociality (e.g. Danforth 2002, Cardinal and Danforth 2011; Gibbs et al. 2012; Rehan et al. 2012). It has been argued that losses of eusociality may be more informative for understanding selection for strong altruism, as opposed to *de novo* origins of eusociality, because losses of eusociality provide much greater comparative material (Rehan et al. 2012).

Whilst the comparative power provided by transitions to and from eusociality is well appreciated, there has been relative neglect of what the alternatives to eusociality actually are. Defining other social strategies has been non-trivial, yet these may be critical for

understanding social evolution. For example, one could ask the question 'when eusociality is replaced by other forms of sociality, are those hierarchical or egalitarian?' and 'if hierarchical, do those hierarchies result from dominance contests or might they simply arise from age-based or other kinds of reproductive schedules?' One could ask similarly interesting questions about the nature of ancestral societies that have given rise to eusociality. These are not idle questions because they deal with the issues of why lifetime commitment to reproduction or sterility could arise, or be lost. Here we argue that asking these kinds of questions is currently impeded by inadequate terminologies for non-eusocial societies. These terminologies arose from the 1960s and early 1970s, when kin selection theory was in its infancy, and well before reproductive skew theories made their first appearance. We believe it is time to 'trade-them-in' for terms that better serve current approaches to social evolution. We also think it is important that terminologies avoid implied knowledge of social systems, when such knowledge may actually be lacking.

In the sections below we highlight problems that are becoming apparent in how we deal with forms of social organization that are alternate to eusociality. These problems can compound, and we argue that they are substantial and pose significant impediments to understanding social evolution at broader scales. At the heart of these issues is the notion of 'egalitarianism', which was central to Vehrencamp's (1983) seminal paper and set the groundwork for later reproductive skew theories. Although the concept of 'egalitarianism' might appear to be clear-cut in everyday use, we believe that it is highly nuanced in social biology and these nuances are rarely explored in social evolution studies. To make things worse, there has been a long-term habit of using various terminologies, such as communal

and quasisocial, to imply egalitarian organization when such implications may be unjustified. These various, inter-related issues can be boiled down into three key problems, namely:

Problem 1 – Identifying egalitarianism. This involves our ability to determine what egalitarianism actually means in the context of life history complexity, and whether some forms of social organization really are egalitarian, or not. This is important because it involves the roles of direct and indirect fitness as drivers of sociality, as well the evolution of mechanisms to prevent intra-specific cheating and coercion.

Problem 2 – Implied egalitarianism. This problem arises from the use of terminology that may falsely imply egalitarianism when such an implication is either unintended or is not demonstrable. This is particularly relevant for terms such as 'quasisocial' and 'communal' (see Table 1) that may hide underlying social hierarchies that are not evident from cursory studies based on limited or 'snapshot' sampling.

Problem 3 – Taxon-specific terminology. Some social terminologies are only applicable to nesting biologies that are taxon-specific. This can obscure patterns of skew in parental and alloparental roles that may be critical in comparative studies, yet the importance of terminologies that encourage useful comparisons when assessing alternative evolutionary theories is well recognised (e.g. Crespi and Yanega 1995; Crespi 2009; Boomsma 2013).

History of social insect etymology

Attempts at categorizing insect social organization have been evolving for the past century (Table 1). Wheeler (1928) drew early categorical lines, but Batra (1966) was first to propose the term 'eusocial' in referring to halictid bee societies where three conditions were met: (i) generational overlap, (ii) cooperative brood care and (iii) reproductive castes. At the same time that the term 'eusociality' was becoming widely used, Michener (1969) developed a

series of terms that could be used to describe other forms of bee sociality, and these became increasingly important for comparative studies as researchers began to explore hypothetical evolutionary pathways, or 'routes', to eusociality (e.g. Lin and Michener 1972; Michener 1985, 1990).

Michener's (1969) study was strongly influenced by his knowledge of bee biology and systematics, particularly studies on social halictine (Halictinae) and allodapine (Xylocopinae) bees where social complexity varies enormously. Both of these bee groups contained many species where queen and worker castes were not morphologically distinct, but they differed in modes of brood provisioning (Michener 1974; Schwarz et al. 2007). Michener (1969) proposed the term 'communal' for social bees where multiple females share a common nest and where females individually construct and mass provision their own brood cells, as seen in numerous halictine bees. For allodapine bees, where brood are progressively reared in a single, unbranched communal tunnel, but where all females lay eggs, he proposed the term 'quasisocial'. Although the terms communal and quasisocial were not explicitly coined in relation to direct and indirect fitness, their subsequent uses have largely carried the implication that reproductive hierarchies are absent and that societies are more or less egalitarian (*e.g. Lasioglossum* (*Chilalictus*) *hemichalceum* - Knerer and Schwarz 1976, 1978; Ward and Kukuk 1998; *Andrena jacobi* - Paxton et al. 1996; *Exoneura robusta* - Schwarz 1986; *Sclerodermus harmandi* - Hu et al. 2012).

While Wilson (1971, 1975) attempted to advance proceedings by categorizing pre-social colony formation, devising a series of intermediate subsocial states (not included in Table 1), that system did not consider the issue of egalitarianism within communal or quasisocial

colonies. However, it did widen the taxonomic scope of definitions to incorporate all social insects and popularize the field. Subsequently, Crespi and Yanega (1995) boldly sought a lexicon that would incorporate all social animals, distilling Michener's parasocial concepts by: (a) isolating communal colonies as distinct from cooperatively breeding (quasisocial, semisocial) colonies; (b) transferring the focus from generational overlap to 'totipotency', 'alloparental care' and 'lifetime reproductive success' (*as per* Vehrencamp 1979) as watershed traits to delineate colony organization. Totipotency was also used to distinguish 'facultative' from 'obligate' eusociality, respectively differing from Michener's 'primitively' and 'highly' eusocial (Table 1).

Suggestions for a way forward

In this review, we argue that as currently defined the terms communal and quasisocial have the potential to be misleading unless detailed caveats on their intended meanings are made explicit (*problem 1* and *problem 2* above), with particular regard to how these problems influence approaches to reproductive skew theory. We then reiterate the etymological origins and dependence on nest architecture and mode of brood rearing (*problem 3*). Next, we offer a path beyond these complications, in the form of methodologies to assess asymmetric reproductive and alloparental investments (*solution 1*), and the use of the term 'casteless' to refer to colonies where variation in the lifetime fitness of individuals is stochastic and unimodal (*solution 2*). We then identify issues to be considered in future research and finally discuss the selective forces that might favour the evolution and persistence of casteless societies.

PROBLEM 1. IDENTIFYING EGALITARIANISM

Discriminating between egalitarian and hierarchical societies

Whilst Hamilton's inclusive fitness arguments predict the very broad conditions under which altruism can be favoured by means of kin selection (Hamilton 1964a,b), these conditions do not, in themselves, predict how reproduction should be apportioned among members of a group. The first major step towards understanding how patterns of reproductive skew could evolve within social groups was taken by Vehrencamp (1983) in her seminal paper on egalitarian versus despotic societies. Vehrencamp considered how various asymmetries among individuals, including their relatedness and competitive abilities, along with group-associated effects such as *per capita* output and effects of intra-group conflict, might influence reproductive skew. These early considerations were very important because they introduced the notion that predicting 'reproductive-shares' within societies involved much more than just relatedness and benefit/cost ratios.

Although Vehrencamp's (1983) paper contrasted 'egalitarian' with 'despotic' societies, it is important to keep in mind that for societies with high levels of reproductive skew, intra-colony relationships that could be viewed as 'dominant' and 'subordinate' need not involve coercion by a 'despotic' dominant. A seemingly dominant female could simply be an individual that is able to truthfully advertise her fecundity and other females may simply gain greater indirect than direct fitness by foregoing reproduction in order to be effective alloparents.

Vehrencamp's arguments were followed by an explosion of models that can be gathered under the banner of 'reproductive skew theory' (Reeve and Keller 2001; Johnstone 2000),

where the nuance of concepts such as ‘dominance’ were expressed in much more complex ways. The profusion of resulting skew theories were initially treated as falling into two broad categories, namely ‘transactional’ and ‘tug-of-war’ models, but the difficulties in measuring their key parameters empirically have had a very sobering effect on how skew theories could be assessed (e.g. Crespi 2009, but see Buston et al. 2007 and Buston & Zink 2009).

Crespi (2009) has contrasted two approaches for understanding social evolution and reproductive skew. ‘Top-down’ approaches are largely model-driven, wherein models are used to predict social parameters that are then measured empirically in organisms to determine which models are most appropriate. On the other hand, ‘bottom-up’ approaches measure social parameters, along with ecological, life history and genetic traits, within and across species, and these are then used to detect patterns that may reveal evolutionary processes. Phylogenetic convergences towards similar patterns, or divergences away from them, could then be used to assess theories that generate broad predictions. Crespi (2009) argued that top-down approaches have been highly problematic because predictions from skew theories frequently require the measurement of multiple parameters that are almost impossible to quantify for real organisms. Indeed, one could suggest that the resulting inability to discriminate between key skew models has lead to a malaise, where hopes of assessing even the most general models appears to have largely evaporated. Bottom-up approaches have the attraction that they may be able to identify evolutionary patterns based on measureable parameters, and are not constrained by model configurations that may ultimately be too simplistic or just inappropriate.

Bottom-up approaches certainly have attractions, especially when combined with increasingly sophisticated phylogenetic tools (*e.g.* Pagel & Meade 2006; Schwarz et al. 2011; Dew *et al*. 2012). However, they require that similarity or dissimilarity in forms of social organization can be compared across taxa, even when those taxa may have quite different biologies. This has been appreciated in studies that focus on origins of eusociality, where there has been very substantial debate over the meaning of this term (*e.g*. Crespi and Yanega 1995; Boomsma 2013). However, there has been no commensurate consideration of terms that describe societies without reproductive hierarchies and where reproduction may be more or less egalitarian. Yet these kinds of societies are critical if we wish to understand how eusociality arises, or is lost. We therefore need to be very clear about the terms that we use to describe non-eusocial colony organization.

Identifying egalitarian behaviour

Regardless of the merits of skew theories, it is important that we are able to identify egalitarian societies when they occur and that we are confident in this determination. We posit the following major issues that arise when trying to identify apparently egalitarian societies:

a) Whether 'snapshots' of reproductive skew at any one point in time (based on limited time frame samples) will reflect lifetime skew. This is a non-trivial issue because such samples may not take account of developmental differences between members of a society at the time of sampling. For example, a situation where females enter a reproductive queue based only on age may appear to be non-egalitarian for any one sampling period where an ovarian or work-related index is used, but such indices

may average out over individual lifetimes, such that temporally hierarchical societies are actually egalitarian over the life spans of individuals and colonies.

b) Where reproductive queues do occur, and where all colony members could expect to have equivalent lifetime fitness, we would have few problems in assigning the term egalitarian. However, how would we regard such societies if survival rates vary with position in a queue, for example if females occupying low positions in a queue have low probabilities of ever reaching the head of the queue?

c) Distinguishing between expected reproductive skew under a purely stochastic process, and skew expected from functional reproductive asymmetries. For example, in a scenario where two females are jointly only able to produce three brood, no outcome will indicate parity in reproductive success. For purely statistical reasons this problem will be greatest in species with very small colony and brood sizes. At the same time, small brood sizes are likely to co-occur with high levels of parental/alloparental care, so the problem arises that one may falsely conclude that high levels of reproductive skew are functionally associated with high levels of parental care. When this statistical problem is combined with age polyethism in reproductive activity, the problems of distinguishing between skew that reflects social hierarchies and skew that has a purely stochastic source becomes very difficult.

The above problems are non-trivial, but they are tractable given sufficient data and analysis.

PROBLEM 2. IMPLIED EGALITARIANISM

Unfortunately, most studies that have applied the terms communal or quasisocial to particular species lack the required detail to confidently infer egalitarian organization. The use of these terms can therefore encourage a sense that we know more about the existence of egalitarian/non-egalitarian structures in a species or colony than is actually justified. For example, both terms have frequently been used to implicitly or explicitly imply egalitarian societies, yet both terms are applicable to societies with effective reproductive skew but where caste-like behaviour may not be evident (*e.g*. some species of *Xylocopa* and *Ceratina*, Hogendoorn and Velthuis 1999) or where reproductive skew and skew in alloparental care arises despite egg-laying by all colony members (*e.g. Ceratina japonica* - Sakagami and Maeta 1984; *Lasioglossum (Chilalictus) hemichalceum* - Kukuk *et al*. 1998; Ward and Kukuk 1998; *Xylocopa sulcatipes* - Stark *et al*. 1990).

PROBLEM 3. TAXON-SPECIFIC TERMINOLOGY

As we noted above, bottom-up approaches to social evolution require that we can detect patterns in sociality across taxa. Unfortunately, the original coining of the terms 'communal' and 'quasisocial' (Michener 1969) explicitly depended on nest architecture and modes of brood provisioning in bees (summarized in the section *History of social insect terminology* above, and in Table 1). Communal organization generally refers to species where females share a common nest entrance but individually mass provision their own brood cells (such as halictine bees), whereas quasisocial organization mostly refers to species that live in undivided burrows, progressively provisioning brood that are in a shared nest lumen (such as in allodapine bees). Because allodapine brood are not physically separated from each other and jointly feed from food brought into the nest, parental care spills over into alloparental care when multiple reproductive females share a nest.

Since Michener's (1969) raising of the terms 'communal' and 'quasisocial', both have subsequently been applied to species where nest architecture and brood rearing may not be relevant (Crespi & Yanega 1995). For example, the term communal has been used broadly in social animal research for a wide variety of cooperative nesting or brood rearing behaviour, including direct shared brood care between reptilian/amphibian females (Doody et al. 2009; see also Gardner et al. 2015). In another case, the term quasisocial has been applied to cooperative subduing and protection of host larvae in a parasitic wasp, even though this does entail shared use of a nest or feeding of brood per se (*Sclerodermus harmandi* - Tang et al. 2014).

Problems arise as nesting type can be easily distinguished but behavioural complexity requires in depth study and few species have been studied in sufficient detail (Augochlorini - Danforth and Eickwort 1997; *Augochlora phoemonoe* - Dalmazzo and Roig-Alsina 2015; Euglossini - Cameron 2004). Halictines seal their brood in individual cells, which they have mass provisioned. Traditionally they are thought to have no further interaction with the brood, supplying no direct brood care and simply acting as guards of the nest as a whole. Seventeen species of halictines, however have now been found to open cells to remove parasitized or dead brood (Plateaux-Quénu 2008; Quiñones and Wcislo 2015). Extended contact between adults and their brood is similarly found in ceratinines which also mass-provision cells (Rehan et al. 2009; Rehan and Richards 2010). To further complicate matters, African allodapines in the genus *Halterapis* mass provision individual eggs (Michener 1971; Tierney et al. 2008b), and in one Malagasy allodapine, clutches of eggs are mass provisioned with a very large food store, cutting off contact between adults and developing brood (*Hasinamelissa minuta* - Schwarz et al. 2005). Combined, these various studies blur the distinction between communal and quasisocial nesting.

SOLUTION 1. MORE DATA AND BETTER ANALYSES

Assessment of asymmetry in reproduction and risk-associated behaviour

The lack of a benchmark metric for enabling comparisons of sociality across different taxa has not gone un-noticed (Beshers & Fewell 2001; Aviles & Harwood 2012). Division of labour metrics have been developed in an attempt to 'normalise entropy' (Gorelick *et al*. 2004), and provide standardized tools so that social systems of varying complexity which involve different behavioural repertoires can nevertheless be compared.

However, the crux of the issue discussed in our manuscript is less complex and we offer a relatively simplified method to deal with a similar problem. As mentioned above, it can be difficult to determine whether colonies are truly hierarchical or whether variation in reproduction or task allocation is just stochastic, a problem that increases as colony sizes decrease. This is a question of Type I and Type II errors. We believe that use of Monte-Carlo procedures can be invaluable here because they make the limits of available data very evident and they are distribution-free when null hypothesis distributions are not available. Such methods have been successfully used to assess reproductive asymmetries, with data including ovary size, body size and wing wear (*Amphylaeus morosus* - Spessa et al. 2000; *Ceratina* - Rehan et al. 2009; *Allodpaula (Dalloapula) dichroa* - Tierney & Schwarz 2009; *Megalopta genalis* - Tierney et al. 2013; *Braunsapis puangensis* - da Silva et al. 2016). Assessment of asymmetry in risky alloparental roles is more problematic, but could be measured by time spent in extra-nidal environments (e.g. *Cerceris rubida* - Giovanetti & Jacobi 2013), guarding behaviour, or by the use of wing damage, though caution is required here (*Anthidium manicatum* - Mueller & Mueller 1993; *Xenochlora nigrofemorata* - Tierney *et al*. 2008a; Tierney & Schwarz 2009).

In all cases, there is a clear need for repeated sampling across seasons/years because of the nature of facultative social nesting. A multitude of extrinsic factors relating to resource availability can influence the formation of group living and the hierarchies that may or may not eventuate (*reviewed by* Purcell 2011; Rehan et al. 2011; Tierney et al. 2013; Kocher et al. 2014). For example, two populations of a halictid bee species exhibit substantially different life-histories on alternate flanks of the Central American isthmus (*Lasioglossum (Dialictus) umbripenne* - Willie & Orozco 1970; Eickwort & Eickwort 1971), where a Pacific coast population was highly seasonal with regard to brood rearing and development of social hierarchies, in contrast to a Caribbean slope population that was relatively aseasonal. The latter example suffers from the fact that these populations were also studied at different seasonal periods of the year and thus the comparative conclusions must necessarily remain holstered. However, such studies provide incentive for more detailed investigations with increased and standardized sampling across seasons, although we acknowledge that this is not always possible. Census-based sampling is attractive in that one can obtain relative large sample sizes for field effort, but the limits of such snapshot data for inferring lifetime reproductive skew or fitness are severe.

SOLUTION 2. USE OF THE TERM 'CASTELESS'

In order to resolve the above problems we now propose that the term 'casteless' be used for species where individuals cooperate in managing resources needed for brood care, but where there are no apparent hierarchies in either reproduction or assumption of risky alloparental roles. The term 'casteless' would not specify how brood care is delivered or the generational composition of social groups, but it requires cooperation in the management of resources that are used for brood rearing. Furthermore, it would not apply to species where reproductive queues or age-based polyethism lead to predictable skew in direct reproduction. In this sense, castelessness would cover species where the *prospects* for reproductive shares are egalitarian, even if stochastic factors preclude parity in realized reproductive output.

We argue that the term 'casteless' captures an important feature in lifetime fitness that is different from societies where hierarchies are more-or-less permanent or else based on queues or other conventions that lead to predictable skew in fitness. We summarize this in Figure 1 by comparing measures of reproductive skew based on 'snapshot' samples of reproductive output (left hand panels) with lifetime direct fitness (right hand). Snapshot measures of reproductive output could be based on various proxies, such as ovary size or maternity of brood within a colony, whereas lifetime-fitness would be measured by the number of offspring successfully reared to maturity (adult eclosion). In the top row we represent solitary breeding females, where reproduction proxies at any one time need not indicate eventual lifetime fitness, but where social interactions do not occur and variation in lifetime fitness is purely stochastic. In the bottom row we represent hierarchical structures with more-or-less permanent castes (i.e. eusocial), which could be behavioural and/or

morphological. For these societies, snapshot measures of reproductive skew at any one time largely mirror eventual skew in lifetime fitness. In the third row we represent societies where reproductive hierarchies are present but not always fixed over the lifetime of an individual, and we use Crespi and Yanega's (1995) term semisocial for this situation. Here, snapshot measures of reproduction will indicate reproductive skew, but gradual changes in the position of individuals within a hierarchy will tend smear the degree of skew when measured as lifetime fitness, leading to a more platykurtic distribution than for solitary breeding females. Lastly, the second row in Figure 1 represents casteless societies, where social interactions do not influence lifetime fitness, such that snapshot proxies of reproduction will have a similar dispersion as the distribution of lifetime fitness, though with a lower mean.

The scenarios we represent in Figure 1 are very important for models of social evolution. For casteless societies the decisions that females make to join or leave a group will depend on the benefits (or costs) of social living and relatedness within groups, but not on power asymmetries. On the other hand, for hierarchical or queue-based societies, decisions to join or leave a group will also depend on an individual's position within that hierarchy. Such decisions are likely to involve an individual's ability to assess their rank within a group and its consequences for lifetime fitness. In other words, hierarchical societies should entail more complex information processing than casteless societies.

ISSUES FOR FUTURE RESEARCH

Phylogenetic comparative approaches to social evolution

As molecular techniques for studying phylogenetics and genomic changes associated with phylogeny increase, we can expect to see ever larger taxon sets being used in single studies. To date, genomic studies of eusociality have mostly involved very limited numbers of taxa (e.g. Woodard et al. 2011; Kapheim et al. 2015), but this will change and will also see greater emphasis on the inclusion of non-eusocial species where genomic changes associate with a much wider variety of social transitions can be explored (Kocher and Paxton 2014; Rehan and Toth 2015). These will include species that have previously been categorized as communal, quasisocial, semisocial, etc. Less ambiguous terminologies are likely to promote more effective comparisons by allowing equivalent forms of sociality to be identified across taxa. However, such comparative studies will also need to be cautious in applying labels to species or colonies so that the absence of reproductive skew, or the source of skew when present, is clear.

Unfortunately, as social insect research increasingly moves into genomic approaches there has not been concomitant attention paid to accurately describing the nature of sociality in non-eusocial species. This could become a serious impediment for comparative studies that might, for example, try to infer transitions between egalitarian and hierarchical organization, as we now briefly outline.

Origins of casteless societies

There is evidence that for the Halictidae, at least, casteless behaviour has evolved from both solitary living and eusocial ancestors (*Halictus* & *Lasioglossum*, Gibbs et al. 2012). Why

two such very different starting points could both give rise to apparently casteless organization has not been explored, yet is likely to hold some very interesting insights. For example, if casteless behaviour is derived from eusocial antecedents, we may expect traits associated with eusociality to be co-opted for living in egalitarian societies; this could involve capabilities for kin recognition (pheromonal or other signaling) to assess the reproductive status or vigor of nestmates. Alternatively, if casteless species have evolved from solitary ancestors we might expect societies where information transfer is much less sophisticated, or where the mechanisms that prevent despotic behaviour, to be very different. To date, these issues have not been flagged for investigation, yet we would argue that they are important for understanding social evolution if we are interested in more than just origins of eusociality.

CONCLUSIONS

Over the last half century, casteless and semisocial insects have been very much neglected as the 'poor cousins' of eusocial species. Part of this is due to the enormous ecological success of some eusocial clades, but there are a very large number of eusocial clades that have not had equivalent success. Studying evolutionary transitions in sociality, *per se*, will require that we have a clear understanding of more than just ecologically successful eusocial species and their extant solitary-living relatives. To this end, we advocate for two steps to be taken in future 'bottom-up' comparative studies, the first would be to avoid misleading terminology, and the second is to be cautious in assuming that species are egalitarian or hierarchical unless we have undertaken the requisite foundational natural history studies.

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Table 1. Social terminology. Key historical introductions of new terms (in capitals) and their definitions, along with the authors, year of publication and taxa for which the terms were devised

Wheeler						**SOCIAL INSECTS**	
(1928) social insects						Progeny that are protected & fed by mother, ultimately co-operate with mother to rear subsequent sibling brood.	
Batra						**EUSOCIAL**	
(1966) halictid bees						Nest founding adult female survives long enough to co-operatively rear brood with her mature daughters acting as worker caste.	
Michener	**SOLITARY**	**SUBSOCIAL**	**PARASOCIAL** COMMUNAL	QUASISOCIAL	SEMISOCIAL	**EUSOCIAL** PRIMITIVELY	HIGHLY
(1969) (1974) social bees	1 adult Fem	1 adult Fem	2+ adult females same generation	2+ adult females same generation / overlap	2+ adult females same generation	2+ adult females adult generations overlap	2+ adult females adult generations overlap
	mass provisions brood cells	progressively provisions brood cells	each female independently mass provisions brood cells	co-operative provisioning of brood/cell-construction	co-operative provisioning of brood/cell-construction	co-operative provisioning of brood/cell-construction	co-operative provisioning of brood/cell-construction
	no contact between generations	continual contact between generations (Apidae)	facultatively share common burrow	>1 adult attending each brood or cell construction	>1 adult attending each brood (sequential cell construction/provisioning)	>1 adult attending each brood (non-sequential cell construction/provisioning)	>1 adult attending each brood (non-sequential cell construction/provisioning)
	adult dies before brood mature	adult dies before brood mature			division of labour between egg layers and workers	division of labour between egg layers and workers	division of labour between egg layers and workers
						castes externally similar (some allometry)	castes externally distinct (especially queens)
Wilson						**EUSOCIAL**	
(1971) (1975) social insects						adult generations overlap and contribute to colony labour; co-operative care of brood; division of labour between egg layers and sterile workers.	
Crespi & Yanega			**COMMUNAL**	**COOPERATIVELY BREEDING** QUASISOCIAL	SEMISOCIAL	**EUSOCIAL**	
(1995) social animals			all adults are totipotent	all adults are totipotent	all adults are totipotent	overlap of generations not required	
			lack alloparental care	alloparental care	alloparental care	division of labour with lifetime trajectories related to behaviour & reproduction, established prior to sexual maturation (developmental)	
			no castes	lifetime reproductive success of the population is unimodal (see our Figure 1) and indicative of lack of castes	lifetime reproductive success of the population is bimodal (see our Figure 1) and indicative of reproductive division of labour	lifetime trajectories identifiable by empirical patterns of variant behaviour, that enable the designation of individuals into caste-groups (along definable axes)	
						transitions from one behavioural group to another are not possible, because they are developmentally-based	
						existence of alloparental care by one caste to the reproductive benefit of another caste	
						FACULTATIVELY	OBLIGATELY
						most reproductively active caste is totipotent, while other castes are not	no castes are totipotent, presence of alternate castes are required to rear brood
Dew, Tierney & Schwarz			**CASTELESS**				
(2015) social animals			2+ totipotent adult females in a facultative nesting association				
			cooperation in management of resources used for brood rearing				
			lifetime reproductive success of the population is a unimodal normal distribution (see our Figure 1) and indicative of lack of castes				

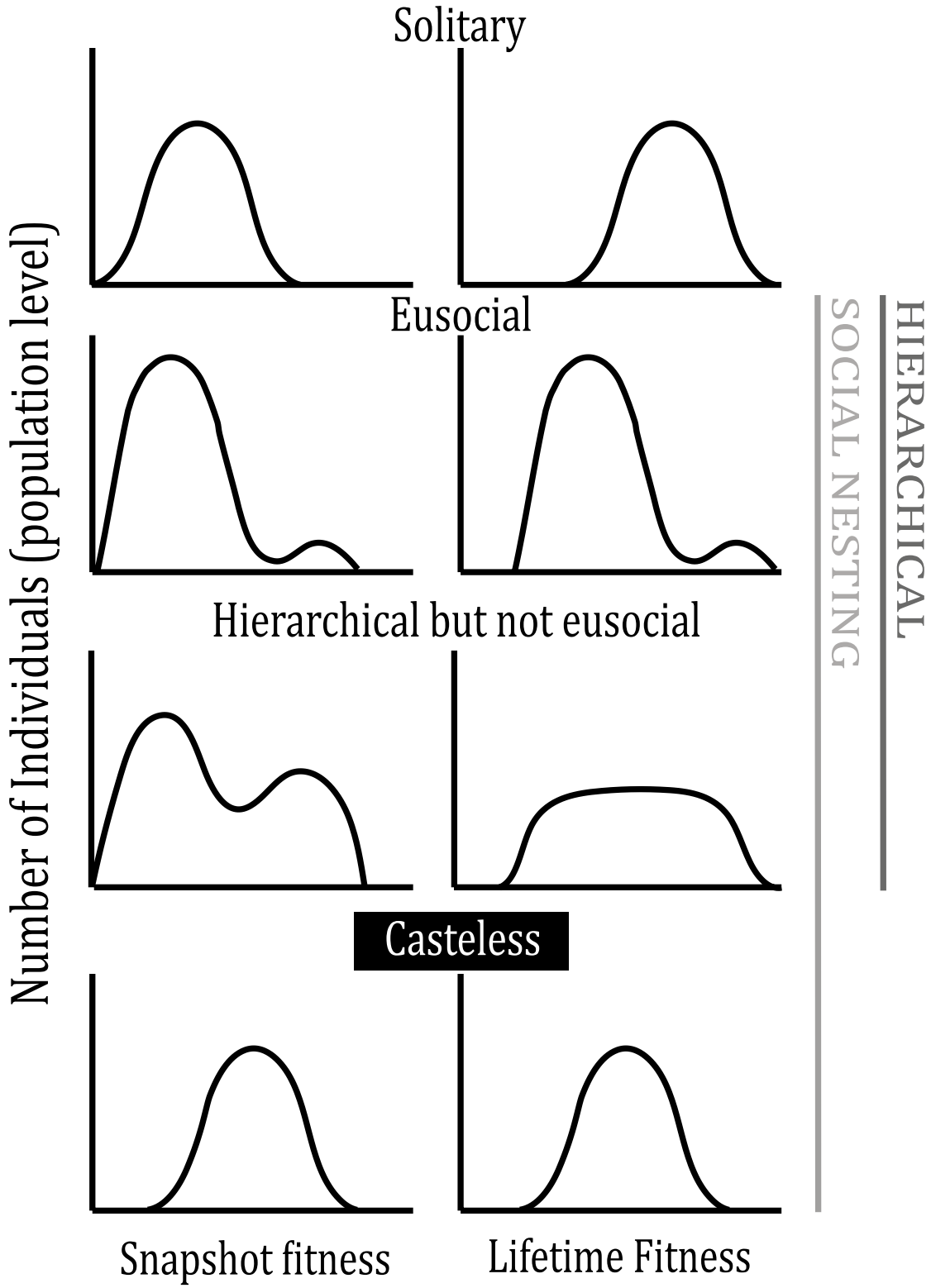

Figure 1. Idealized fitness distributions for solitary, casteless, semisocial and eusocial species. The left hand panels represent 'snapshot' measures (at any one point in time of the life cycle) and the right hand panels represent 'lifetime' fitness.

CHAPTER II

The evolution of equable nesting: casteless social behaviour in an allodapine bee

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ABSTRACT

Facultatively social species exhibit varying degrees of reproductive skew that provide valuable insights to the possible evolutionary forces shaping the origins of obligate eusocial colony organisation, wherein the majority of individuals (workers) forego direct reproduction. Here we report the nesting biology of a semi-arid population of the allodapine bee *Exoneurella setosa*, which forms social colonies that lack reproductive hierarchies and are therefore 'casteless'. An intriguing discovery given that a congeneric eusocial species exhibits the greatest morphological distinction between queen and worker castes in the entire subfamily Xylocopinae (Apidae). *Exoneurella setosa* exhibited a modal colony size of two females per nest and we analysed nest-mate differentiation in ovarian development, body size and wing wear (proxy for foraging activity), contrasting empirical results with Monte Carlo simulated colonies to ascertain that multifemale nests lack evidence for reproductive skew. Our results support a nest-site limitation hypothesis as the key driver for eusocial organisation within the Xylocopinae and that the absence of such environmental limitations, combined with minor benefits for group nesting, can select for casteless social organisation.

Keywords: social evolution, facultative behaviour, casteless organisation, reproductive skew, dominance hierarchies, per capita brood production, dispersal limitations, eusociality.

INTRODUCTION

There has been extensive research into the origins of eusociality in insects. Molecular phylogenetic approaches have been useful for identifying origins of eusociality (see reviews in Schwarz et al. 2007; Tierney et al. 2008; Danforth et al. 2013; Kocher & Paxton 2014), but the selective mechanisms underlying these origins remain controversial. The predominant theories mostly rely on inclusive fitness arguments and associated reproductive skew models (e.g. Reeve & Keller 2001; Buston & Zink 2009; Nonacs & Hager 2011; Boomsma 2013; Holman 2014) but these have also been recently challenged (e.g. Nowak et al. 2010; Rautiala et al. 2014).

It has been suggested that a fertile approach to understanding the evolution of eusociality is to examine cases where eusociality is not fully developed or has been lost (Rehan & Toth 2015). Molecular phylogenetic studies have gradually been revising the number of origins of eusociality downwards, at least for bees, and increasing the number of inferred losses (e.g. Danforth et al. 2003; Gibbs et al. 2012; Rehan et al. 2012). Eusociality has been lost approximately twelve times in the halictine bees (Halictidae: Halictinae, Gibbs et al. 2012), and recent phylogenetic reconstructions suggest there has also been one loss leading to the bee tribe Euglossini (Apidae: Apinae, Cardinal & Danforth 2011; but see Almeida & Porto 2015). Origins of eusociality have occurred approximately once in the halictines, once in the corbiculates and twice in the allodapines (Apidae: Xylocopinae: Allodapini, Cardinal & Danforth 2011; Gibbs et al. 2012; Rehan et al. 2012). Unfortunately studies utilising these social transitions are severely hampered by the sheer lack of robust behavioural data for many species coupled with phylogenetic uncertainties within some

groups. One shortcoming of comparative social evolution studies concerns the imbalance of detailed contrasting knowledge for eusocial versus non-eusocial species which impedes our ability to understand transitions in social states involving (i) altruism, (ii) behavioural/reproductive castes, and (iii) the complexity of societies that are not eusocial (Rehan & Toth 2015).

Non-eusocial allodapine bees often have hierarchical social groups where one or a few females are the reproductive dominants but specific social structure varies between species (Tierney et al. 1997, 2000, 2008; Schwarz et al. 2007; Tierney & Schwarz 2009). A number of halictine bees are facultatively eusocial, with larger females often monopolizing reproduction within the nest (Richards et al. 2005; Schwarz et al. 2007). On the hand, in some species the timing of adult eclosion, rather than size, determines reproductive dominance in a number of bees and wasps (Schwarz & Woods 1994; Torres et al. 2014). Primitive worker like roles are seen in *Ceratina* species where dwarf daughters are coerced into foraging for their mothers and sisters (Apidae: Xylocopinae: Ceratinini, Maeta et al. 1992; Michener 1990; Rehan & Richards 2010). However, in some bee species the dominant female is the sole forager, while subordinates wait in a reproductive queue before simultaneously assuming both egg-laying and foraging roles (Prager 2014). More elaborate hierarchies are seen in many extant allodapine taxa including the Australian exoneurine genera (*Brevineura*, *Exoneura*, *Exoneurella*). Within this group *Brevineura* and *Exoneura* have well-formed hierarchies with complex social interactions including pheromonal control of ovarian development and coercive responses to mated individuals (O'Keefe & Schwarz 1990; Bull et al. 1998; Hogendoorn & Schwarz 1998). Social caste development is most

extreme in the genus *Exoneurella. Exoneurella tridentata* displays the only morphologically distinct queen and worker castes in the entire tribe (Houston 1977; Hurst 2001). Only one other allodapine species from Madagascar exhibits clearly discrete size-based caste differentiation (Schwarz et al. 2005; Chenoweth et al. 2008)

The Australian allodapine genus *Exoneurella* consists of four species (Houston 1976; Reyes et al. 1997). As for most other allodapines these are stem-nesting bees, which utilise dead stalks and twigs of plants for nesting tunnels. Commonly allodapines associate with plants with soft pithy centres which the bees can excavate themselves. The exception being *E. tridentata*, which founds nests in pre-existing burrows in hardwood trees (Hurst 2001). *Exoneurella* are progressive provisioners, and continuously supply resources to brood through to maturity. In contrast to the eusocial *E. tridentata,* the three other species exhibit less complex social organisation, with high levels of solitary nesting (Michener 1964a; Neville et al. 1998; Hogendoorn et al. 2001). However, previous studies on these species did not statistically compare reproductive activity among nestmates in relation to body size and foraging activity.

Neville et al. (1998) studied a temperate coastal population of *E. setosa* that exhibited low levels of social nesting during the peak brood rearing season, but lacked the statistical power required to discern whether any reproductive patterns were present or not. In this population, the majority of overwintered nests exhibited group nesting, which then disperse in spring and by the beginning of summer all nests contain solitary females. Less than 19% of nests contain multiple females throughout the remainder of the summer

season and these proportions increase through autumn and peak in winter (88% multifemale nests).

Here we detail the nesting biology and natural history of a previously unstudied semi-arid population of *Exoneurella setosa*. Our specific goal was to determine whether reproductive hierarchies were present in social nests. Our analyses directly compare nestmate ovarian development with body size and wing wear (as an estimate of foraging activity), and incorporate simulated expectations to test differences from null assumptions. We test the hypothesis that social nests of *E. setosa* should exhibit reproductive hierarchies as an evolutionary precursor to the highly eusocial organisation displayed by its congener *E. tridentata*.

METHODS

Nest Collections

Collections were undertaken near Mildura, Victoria, Australia (34° 09'16.4"S 142° 09'23.9"E) from areas of open semi-arid woodland with a chenopod understory surrounding the banks of the Murray River. The main course of the Murray River runs through this region, and all of our collecting sites were within 2km of its banks. Mildura has very hot, dry summers and temperate winters. Nests were collected over four periods, which broadly cover the seasons of winter (26-27 June 2013), spring (11-13 October 2013), summer (21-23 January 2014) and autumn (12-14 April 2014).

Nests were mostly in dead stems of annual plants from the genus *Senecio* (Compositae), predominantly in open sandy areas. Post collection, nests were stored on ice in a cool box at ~4ºC for transportation to the laboratory. We then recorded the number of eggs, larvae, pupae and adults for each colony. Comparisons of brood and adult numbers across the year were used to determine colony life cycle for the population and the number of generations per year. Nests contents were stored in 99% alcohol and transferred to 70% ethanol 24 hours prior to adult dissection.

Dissections

Females were dissected to determine if they were inseminated and measure ovarian development. Insemination status was determined by observation of the spermatheca,

which is opaque when sperm is present. In some cases the spermatheca was not found, most likely due to its almost complete transparency when females are not inseminated. Ovarian development was evaluated by summing the lengths of the three largest terminal oocytes to produce a measure of ovary size (Schwarz 1986; Cini et al. 2013). Wing length has a linear relationship to pupal weight in allodapines and was used as a proxy for body size, measured from the tip of the submarginal cell to the axilliary sclerites (Schwarz 1986). Wing wear is in an indicator of foraging activity and was scored by the number of wing nicks on the outer edge of both forewings. Greatly worn wings were recorded as 21 because it was impossible to identify individual nicks and tears in badly worn wings with more than 20 nicks (n = 18). We were unable to recognise callow (newly emerged) females, as many females with no wing wear were inseminated with developed ovaries. Colouration of individuals, which is an indicator in some species, was also too variable to be reliable.

Data Analyses

Data were analysed in SPSS version 19.0.1 and R version 3.1.0 (R Development Core Team 2015). When assumptions of normality were not met, we used non-parametric analyses.

Monte Carlo re-sampling procedures were used to explore whether reproductive hierarchies occur within social nests (*per* Tierney & Schwarz 2009; Tierney et al. 2013). This is performed by comparing empirical differences in nestmate metrics (ovary size, wing length, wing wear) with simulated distributions derived from randomly coupled females. Empirical samples are derived from the peak season of ovarian development. One thousand simulated nestmate pairs are used to produce expected distributions for each of the three

metrics, which derive from pooled social nests; and are subsequently compared with a solitary pool to account for any potential biometric differences between social and solitary nesting individuals. If hierarchical structures are absent from social colonies, then variation among nestmates in metrics of wing length, ovary size or wing wear, should not differ from individuals randomly sampled from the population to create ‘virtual’ colonies.

RESULTS

Nesting Biology

A total of 358 nests were collected throughout the study, with a combined mean colony size of 1.97 females per nest, a modal social colony size of two females per nest and a maximum of 12 females in one winter nest. Fig. 1 presents colony size as a proportion of the total nests collected during each collection period (winter n = 53; spring n = 75; summer n = 180; autumn n = 50), and Fig. 2 presents brood phenology over the four collection periods. Brood were present in solitary and social nests from spring to autumn, while three winter nests contained pupae. In spring there were only eggs and larvae present but no pupae. During summer all brood developmental stages were present (eggs, larvae and pupae), suggesting that brood
rearing is continuous from spring onwards. By autumn egg laying had ceased and brood within nests were largely mature (larvae and pupae).

The number of social nests (relative to solitary nests) varied significantly between seasonal collection periods (Chi-square $X^2_3 = 34.284$, $P < 0.001$). Social nesting was lowest in spring (28%; Fig. 1), when many females disperse to found their own nests and gradually increased over the brood rearing season (summer 36%; autumn 48%) with the greatest proportion observed in overwintering colonies (76%). Samples collected during the brood rearing seasons of spring, summer and autumn did not differ from each other in the number of social nests when winter nests were excluded ($X^2_2 = 5.231$, $P = 0.73$). During these brood

rearing periods 38% of nests were social and mean colony size was 1.61 ± 0.06 SE (n = 305; Fig. 1).

Reproductive Development

Dissections of adult females were performed on 304 individuals from 142 randomly sampled nests (winter n = 55; spring n = 44; summer n = 105; autumn n = 95). In this dissected population 75.8% of females were inseminated. At the population level, ovarian development differed between seasons (Kruskal-Wallis $H_3 = 156.44$, $P < 0.001$). Ovarian development (sum of the lengths of the three largest terminal oocytes) was significantly greater in summer (all pairwise Mann-Whitney U test $P < 0.001$) when the mean ovarian indices 1.84 mm (± 0.07 SE) were an order of magnitude greater than all other seasons. Spring, autumn and winter were not significantly different from each other at Bonferroni adjusted alpha values.

Metrics of Solitary versus Social Nesting Females

We then compared morphometrics of females from solitary and social nests to see if there were differences among females from either nesting category. Winter samples were excluded from all subsequent analyses due to reproductive diapause while overwintering. We explored seasonal differences throughout the brood rearing seasons (spring, summer & autumn) among females from social nests compared with solitary females.

Because social nests contain multiple females, this leads to a disproportionately large sample size *cf*. solitary nests (social n = 179; solitary n = 65). To address the potential

effects of pseudo replication we repeated the aforementioned analyses, whereby social females were down weighted by the number of females in the nest (*e.g.* in solitary nests the female was weighted 10, in two-female nests each female was weighted at 5, three-female nests individuals weighted at 3.3). Re-weighted analyses corroborated unweighted analyses. Ovary size of social females was significantly larger than solitary females in autumn ($U = 19195$, $N_1 = 238$, $N_2 = 250$, $P < 0.001$), but not in spring or summer. Wing length was not different in any season. Wing wear of solitary females was significantly greater during summer ($U = 15695$, $N_1 = 160$, $N_2 = 294$, $P < 0.001$) and autumn ($U = 21760$, $N_1 = 250$, $N_2 = 238$, $P < 0.001$) but not during spring.

Social Colony Structure

Our next analyses compared individuals within two female colonies too see if there was a reproductively dominant individual. These analyses were performed independently for summer and autumn (spring was excluded due to the small sample of 2-female nests). We ranked individuals within each nest according to relative (a) ovary size, (b) wing length and (c) wing wear and compared morphometrics (excluding tied ranks). Individuals ranked by ovary size did not differ in body size (U-test P values ≥ 0.38); nor did they exhibit different levels of wing wear (U-test P values ≥ 0.62). Comparably, individuals ranked by body size showed no differences in ovary sizes (U-test P values ≥ 0.52) or wing wear (U-test P values ≥ 0.60). Lastly, individuals ranked by wing wear showed no differences in ovary size (U-test P values ≥ 0.07), however individuals with greater wing wear were marginally larger in body size in summer ($U = 13$, $N_1 = N_2 = 8$, P values = 0.05), but no difference was found in autumn ($P = 0.32$).

Simulated social nesting

The results above suggest an absence of clearly identifiable reproductive hierarchies. In order to comprehensively explore this lack of social nestmate differentiation we compared the empirical differences in nestmate metrics with simulated distributions derived from randomly coupled females. We restricted resampling analyses to the summer sample because this was identified as the sample with peak ovarian development across the population (see *Reproductive Development* section above). Dissections from the summer sample included data from 12 two-female nests and 16 solitary nests. We first simulated 1,000 nestmate pairs to produce expected distributions from the pooled two-female nests and then compared results derived from solitary nests to examine effects of social nesting.

Out of the 1,000 simulated nestmate pairs derived from the pool of 2-female social nests, 19.3% exhibited differences in ovary size greater than the observed summer mean difference between 2-females nestmates of 1.2 mm (Fig. 3). When the simulated pairs were drawn from a pool of solitary females 7.7% of the simulated distribution exceeded the observed mean difference. Both simulations suggest that observed ovarian differentiations do not lie outside of expectations derived from random assembly of 'nestmates', and provide no support for reproductive caste differentiation in the empirical data. Similarly for body size, 19.0% (2-female nest pool, Fig. 3) and 16.1% (solitary pool) of the simulated distributions exceeded the mean difference observed between empirical pairs. Again for the wing wear analyses, the simulated distributions exceeded the mean difference observed

between empirical social pairs (16.3% of the 2-female nest pool, Fig. 3; and 44.8% of the solitary pool).

The above analyses were repeated with 3-female nests from the summer collection (n = 12). In this case the most extreme difference between the metrics of three individuals in each nest was recorded (e.g. difference in body size of the largest and smallest females), and simulated triplets were drawn from a pool of individuals derived from 3-female nests as well as an independent pool derived from solitary females. Of the 1,000 simulated triplets, 25.3% (3-female pool) had greater differences in ovary size than the observed empirical mean. However, when drawing from the solitary pool only 1.2% of the simulated triplets exhibited greater differences in ovary size compared with the empirical three-female nest mean. This result is likely due to the fact that (a) most solitary females have developed ovaries and therefore exhibit less variation between individuals, and (b) our inability to identify newly eclosed callow females (remembering that the modal social colony size is two); rather than an indication of socially-mediated reproductive skew within social nests. For body size, 44.8% (3-female pool) and 50.1% (solitary pool) exhibited greater differences in body size than the observed mean from empirical 3-female nests. For wing wear, 28.0% (3-female pool) and 89.9% (solitary pool) of the simulated nestmates had greater differences than the observed mean.

Efficacy of Social Colonies

We calculated the *per capita* brood production (PCBP = adult females/total brood) of colonies during each season to explore whether brood rearing efficiency improves with

colony size, as exemplified by many eusocial species (e.g. Schwarz et al. 2007; but see Michener 1964b). There were no significant differences in PCBP when comparing solitary and social nests, indicating there are no *per capita* benefits to group nesting (Fig. 4). Autumn was the only season to exhibit significant differences in PCBP (Kruskal-Wallis H_3 = 9.667, P = 0.022) and the only significant pairwise comparison showed that 2-female colonies were more productive than 3-female colonies (Mann-Whitney U = 8.0, N_1 = 12, N_2 = 6, P = 0.004; Bonferroni adjusted alpha = 0.008). However, this result is likely a by-product of recent adult eclosion leading up to overwintering, noting that pupal numbers are greatest in the autumn sample (Fig. 2) and that we were unable to distinguish callow females in this study.

We then used a generalized linear model to explore maximal reproductive investment per nest (i.e. ovary size of the most developed female per nest). We aimed to ascertain if (i) the presence of current nestmates (adult females), or (ii) the likelihood of future nestmates (female pupae), was associated with egg laying activity. Mature brood (pupae) were only present in nests during summer and autumn, so only these seasons were considered and analysed independently. Additional covariates - number of larvae, pupae, brood (number of eggs, larvae and pupae combined) and male pupae - per nest were also considered in the model. The model was run with a Gaussian error distribution, and model reduction was assessed by Bayesian Information Criteria (BIC) to avoid false positives that are more likely with AIC. Interactions were also considered but did not improve model fit. Departure of residuals from a normal distribution as assessed via chi-square tests. The fitted models and their associated BIC are given in Supplementary Table 1.

The best fit model indicates that during summer the number of adult females (B = 0.27, t = 3.1, d.f. = 1, P = 0.0039) and total brood (B = 0.15, t = 2.9, d.f. = 1, P = 0.0062) were significant indicators of increased maximum ovary size, while the quantity of larvae (B = -0.17, t = -2.7, d.f. = 1, P = 0.011) and female pupae (B = -0.28, t = -2.2, d.f. = 1, P = 0.033) showed significant negative effects. Total pupae and male pupae were excluded from the model. In autumn, adult females was the only significant predictor of maximum ovary size (B = 0.13, t = 5.3, d.f. = 1, P < 0.001; Fig. 5). Including female pupae reduced model fit and did not indicate a significant relationship with ovary size (Supplementary Table1). This suggests that the presence of other females in the nest may positively influence reproductive development, but that development is not increased at the prospect of future helpers (female pupae).

DISCUSSION

Casteless Social Colony Structure

Our results provide evidence that reproductive hierarchies are absent from riparian semi-arid colonies of *E. setosa.* Comparisons of ovarian development, body size and wing wear indicated no dominance hierarchies within social nests, nor are there any *per capita* benefits to social brood rearing. The only Monte Carlo simulation where empirical differences in nestmate ovarian development fell outside expected distributions (triplets derived from a pool of solitary females) is a likely artifact of not being able to identify newly emerged females. The modal social colony size was 2-females per nest, the mean colony size was 1.97, and in all months the mean PCBP of 2-female nests is greater than 3-female nests, significantly so in Autumn. The combined evidence suggests that colony sizes larger than 2-females are likely comprised of newly eclosed females that do not contribute to nest productivity. The largest colony sizes were found in winter (up to 12-females per nest), but in spring we found the highest proportion of solitary nesting of any collection period, suggesting that the majority of overwintering females disperse to found new nests and that all subsequent small colonies (mainly consisting of 2-females) are indeed casteless. The only apparent benefit to group nesting is greater ovarian enlargement among summer and autumn colonies (*cf*. solitary females), which could be related to reproductive competition within social nests or the security of added nest defence inherent to multifemale nests. However, there was no evidence for increased ovarian development based on the likelihood of future alloparents (presence of female pupae), suggesting the younger generation are not relied on to fill worker-like brood care roles and may not act as 'insurance' for future brood.

This is in stark contrast to the morphologically distinct queen and worker castes of the congeneric *E. tridentata* and, therefore, *E. setosa* provides no evidence for the presence of a precursory hierarchical state in an *Exoneurella* common ancestor that would set the blueprint for *E. tridentata*. We therefore consider social nests of *E. setosa* to be 'casteless' as described in the review of Dew et al. (2016).

Evolution of Casteless Societies

Figure 6 presents a generic level cladogram of the Xylocopinae indicating the most recent common ancestor of the Allodapini, the origin of lineages that contain documented examples of casteless organisation and the only origin of eusociality with discrete morphological castes. The casteless social structure of *E. setosa* is highly surprising given the origin of eusociality in the genus *Exoneurella* and the reproductive dominance hierarchies that are well-characterised in the sister genera *Exoneura* and *Brevineura* (*reviewed by* Schwarz et al. 2007; Tierney et al. 2008; Schwarz et al. 1998). Given that *E. tridentata* is the sister lineage to the remaining described species of *Exoneurella* (Chenoweth & Schwarz 2011), the casteless behaviour of *E. setosa* could represent a loss of hierarchies. However, *E. tridentata* has undergone rapid and unique colony size evolution compared to other allodapines (Dew et al. 2012), so it may not necessarily be representative of the ancestral state for the entire genus. Ancestral reconstruction of social traits in the most recent common ancestor to the tribe Allodapini suggests an absence of caste specialization, but a presence of reproductive skew due to asynchronous overlap of generations in colonies (Schwarz et al. 2011). It was argued that these supernumerary females 'wait' to transition into either (a) worker-like roles, or (b) delayed reproductive roles with equable fitness

returns (measured over a lifetime); therefore ancestral allodapine societies could have been casteless. Further support for this interpretation is that casteless behaviour has also been discovered in the sister genus to all remaining allodapines - *Macrogalea* (Tierney et al. 2002; Thompson & Schwarz 2006; Butler et al. *in review*). Whether societies with reproductive queues are casteless largely depends on survival rates and the probability of reproducing over an individual's entire lifetime, which is difficult to assess in a longitudinal manner (see Dew et al. 2016). Consequently, the prevalence of castelessness throughout the tribe makes determination of the directionality of social evolution, including within *Exoneurella*, ambiguous.

As the number of taxa recognised as casteless increases we are more likely to be able to make evolutionary inferences about phylogenetic pathways – see Figure 6. Casteless behaviour has been identified in other allodapines - a species of *Braunsapis* (da Silva et al. 2016) and in three species of *Macrogalea* (Tierney et al. 2002; Thompson & Schwarz 2006; Butler et al. *in review*). The orchid bee *Euglossa hyacintha* has also been found to lack reproductive castes (Soucy et al. 2003), as does the colletid *Amphylaeous morosus* (Spessa et al. 2000) and a number of wasp species (West-Eberhard 1979, 1987, 2005). Casteless behaviour is also suggested for a number of communal halictine bees (Michener 1969; Danforth 1991; Schwarz et al. 2007), though care has to be taken not to assume reproductive skew based on seemingly communal colony organisation. The absence of hierarchical structure in other bee and wasp species is very possible, but the lack of detailed natural history data combined with appropriate analyses make this difficult to determine (Dew et al. 2016).

Attributes of casteless social nesting

There may be some minor differences associated with casteless social colonies compared with solitary nesting *E. setosa.* Social females had larger ovary sizes in autumn compared with solitary females, which may indicate reproductive competition within nests or an extended brood rearing period. However, PCBP of social and solitary nests were very similar in all seasons. Our study did not collect longitudinal data on predation and parasitism rates so we cannot directly assess nest failure or survival. However, because brood are not enclosed within brood cells social colonies are very likely to experience reduced predation and parasitism due to the presence of guards in the nest, as for other exoneurine bees (Chenoweth et al. 2007; Zammit et al. 2008). Therefore, any benefits to social nesting in *E. setosa* are likely to be minimal and typical of what have previously been described as 'communal' and cooperatively breeding 'quasisocial' colonies (Michener 1974), that are comprised of two (or more) totipotent females in a facultative nesting arrangement; that engage in cooperative management of brood rearing resources; with lifetime reproductive success of the population represented by a unimodal distribution (semisocial and eusocial populations should exhibit bimodal distributions – *see* Dew et al. 2016). From a functional standpoint, it may well be that casteless societies achieve the same aim of even the most complex eusocial colonies, namely to establish a secure abode in which to rear brood – see Wcislo and Tierney (2009) for a comprehensive review.

Evolution of Social Behaviour

The casteless behaviour of *E. setosa* suggests that the establishment of reproductive

social hierarchies may not be essential stepping-stones towards the evolution of eusociality despite the inferred behavioural links (*reviewed by* Lin & Michener 1972; Michener 1985). Small colony sizes have greater evolutionary stability (Fu et al. 2015), so in order to transition to eusociality, extreme ecological circumstances may be required to overcome the demographic constraints. Broad modelling of cooperatively breeding animals suggest that reproductive skew in societies should increase as limits to dispersal for independent brood production become stronger (Buston et al. 2007). Such limitations to independent breeding could be due to environmental factors such as nesting substrate scarcity or high risk of mortality during dispersal, and have been argued to represent sufficient selective forces to enable the evolution of eusociality (Avila & Fromhage 2015). Assimilation of these models suggests that dispersal costs due to limited nest-site availability could be a key factor driving the formation of eusocial colony organisation. Conversely, we may expect societies that exhibit very low (or minimal) reproductive skew to thrive under environmental conditions where there are relatively small costs to dispersal for independent nesting.

Empirical evidence from the genus *Exoneurella* are consistent with the predictions of the aforementioned models. All allodapines are stem nesting bees that exhibit mandibular adaptations for wood nesting (Michener 2007), however, the eusocial *E. tridentata* are unusual in the habit of nesting in hard wood trees (*Acacia papyrocarpa* and *Alectryon oleifolius*), these pre-formed burrows are initiated by beetle larvae, but once established, bee colonies last for upwards of five years (Hurst 2001). A variety of other arthropods (especially arachnids and orthopterans) utilise these pre-formed tunnels, and so there may be considerable inter-order level competition for this resource (Dew & Schwarz 2013). In

comparison, *E. setosa* is able to nest in a wide variety of abundant and annually renewed plant species (e.g. Neville et al. 1998), which might reduce barriers to dispersal. The characteristics of *E. setosa* nesting substrates are representative of the majority of allodapine species: dead plants stems with soft pithy centres, that the bees excavate themselves. A trade-off of utilizing such readily abundant soft plant tissue is substrate senescence, because such nests have limited durability and rapidly deteriorate over 1-2 years, forcing nest disbandment. The two remaining species of *Exoneurella* (*E. lawsoni* and *E. eremophila*) nest in many of the same plant species as *E. setosa* (Michener, 1964a; Houston, 1977; Hogendoorn et al. 2001). Like *E.* setosa, these other species are facultatively social and may also lack castes, but more in-depth investigations enabling comparisons between nestmates are required.

Conclusion

Comparisons of nestmate metrics indicates that social groups of *E. setosa* completely lack hierarchies and exhibit minimal benefits to group nesting. Dispersal related costs appear to be a determining factor of social biology within the genus *Exoneurella*, demonstrating the extrinsic selective force of an organism's environment in social evolution trajectories. It is probable that casteless behaviour is more common than has been identified in the literature due to the focus on traits leading to eusocial evolution. The alternative evolutionary pathway represented by casteless social groups may allow us to understand drivers of social group formation and altruistic behaviour, in a parallel manner to those that lead to hierarchical societies. Our study therefore provides a comparative foundation for broader considerations of behavioural evolution among facultatively social insects and cooperatively breeding animals generally.

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Disclosure Statement

The authors declare no potential conflict of interest.

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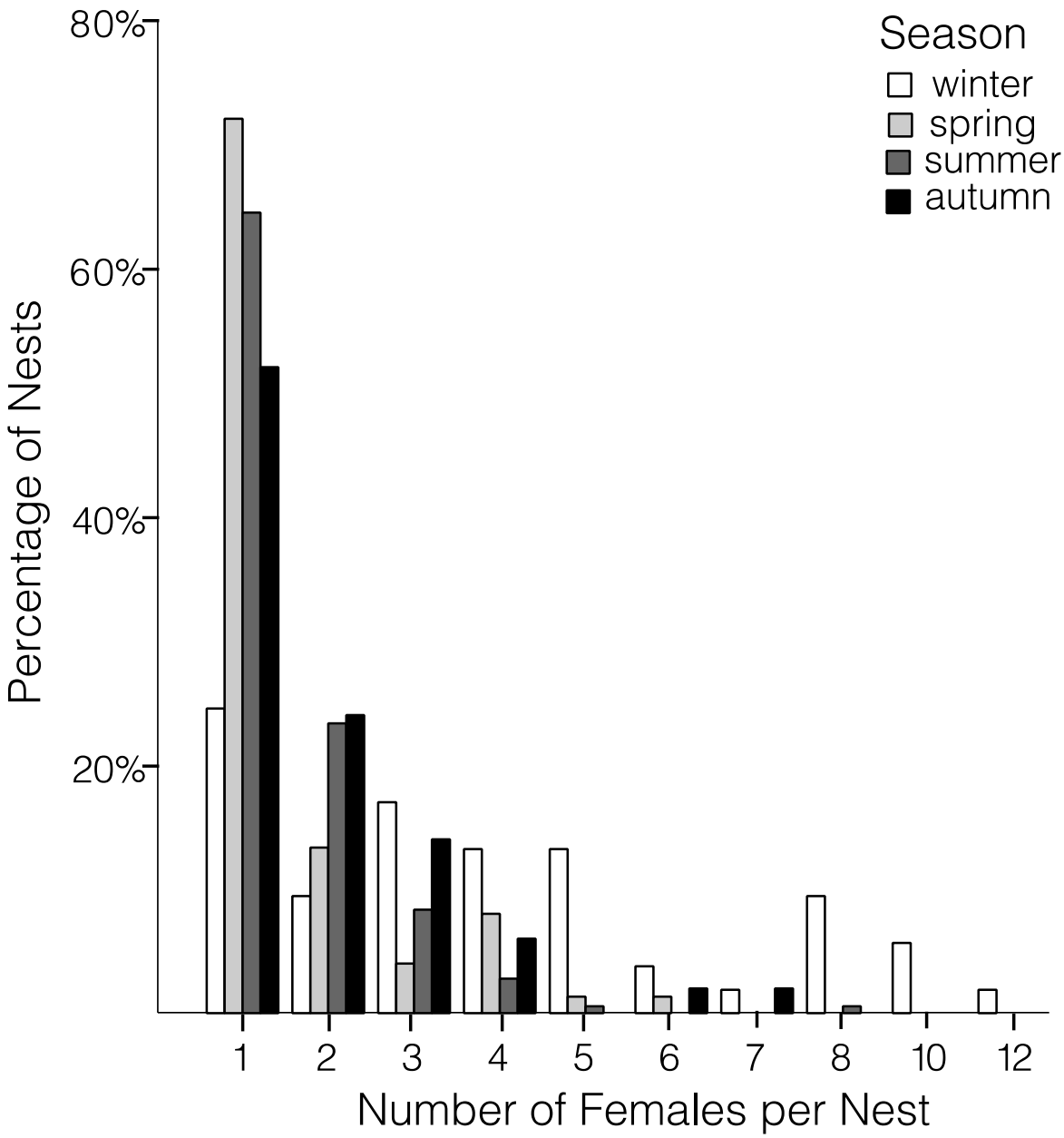

Fig. 1 - Colony size. Number of adult females per nest represented as a proportion of the population sampled across the seasons of winter, spring, summer and autumn.

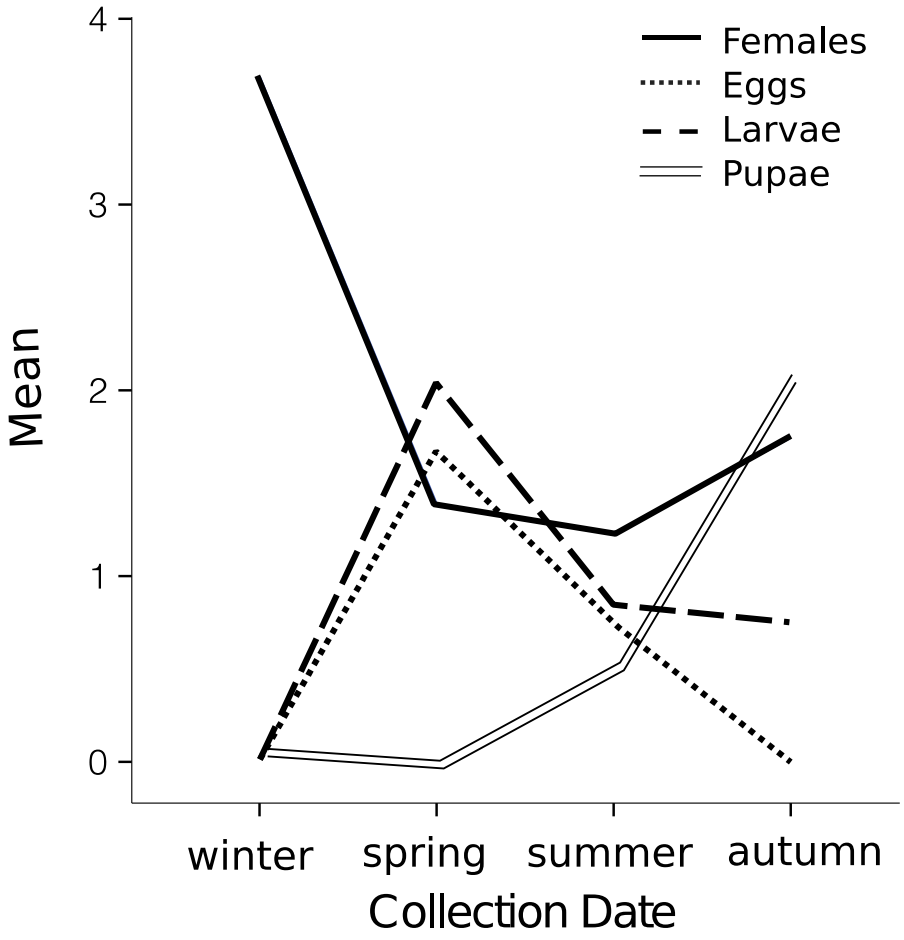

Fig. 2 - Nest phenology. The mean number of immature brood and adults present in winter, spring summer and autumn nests: eggs (dots), larvae (dashes), pupae (double line) and adults females (solid line).

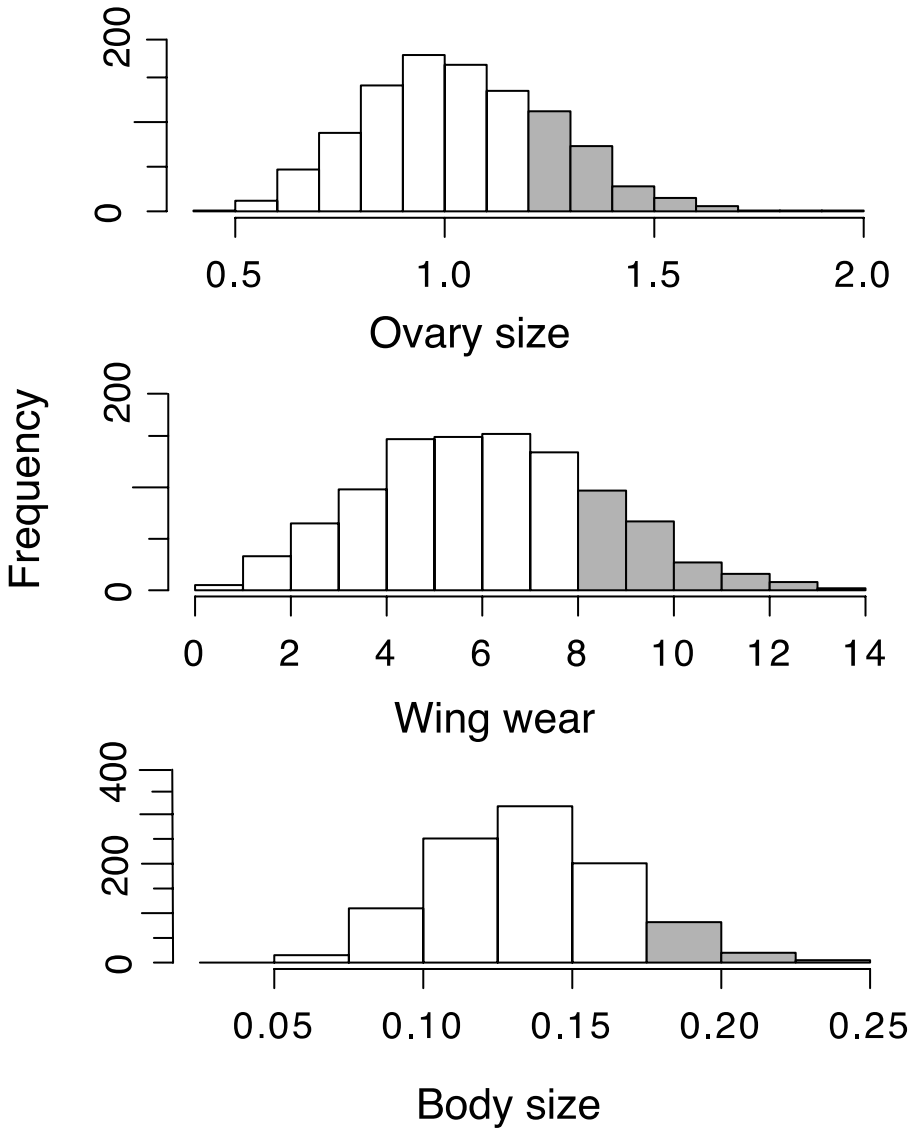

Fig. 3 - Expected distributions of nestmate differences in ovarian development, body size and wing wear. Simulated distributions of the differences between nestmate pairs (derived from individuals in 2-female nests) for the metrics of ovary size, body size (wing length) and wing wear (wing nicks). Grey histogram bars indicate the proportion of the simulated data that exhibit greater differences than the observed mean derived from 2-female social nests.

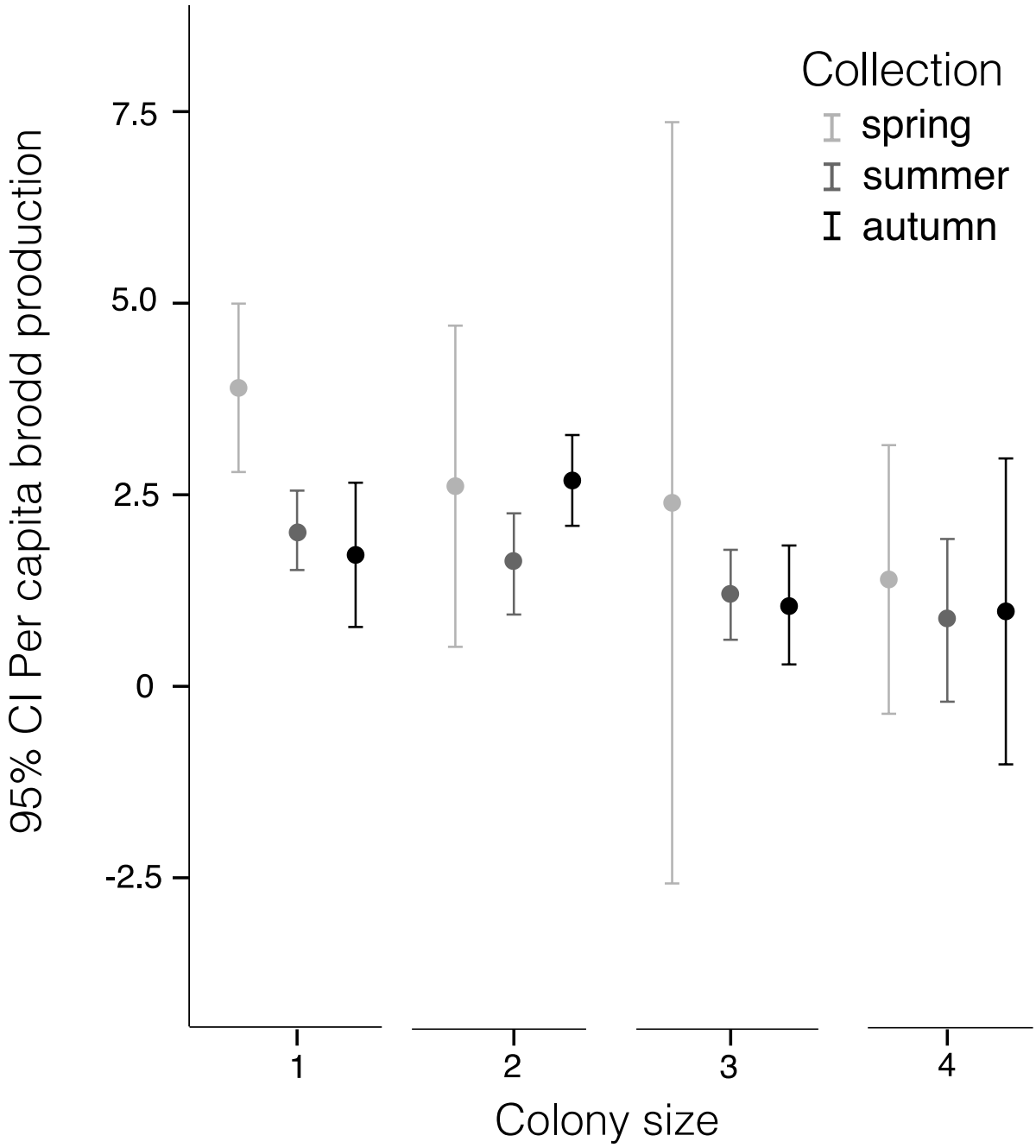

Fig. 4 - 95% confidence intervals of the per capita brood production of different sized colonies during the brood rearing seasons of spring, summer and autumn.

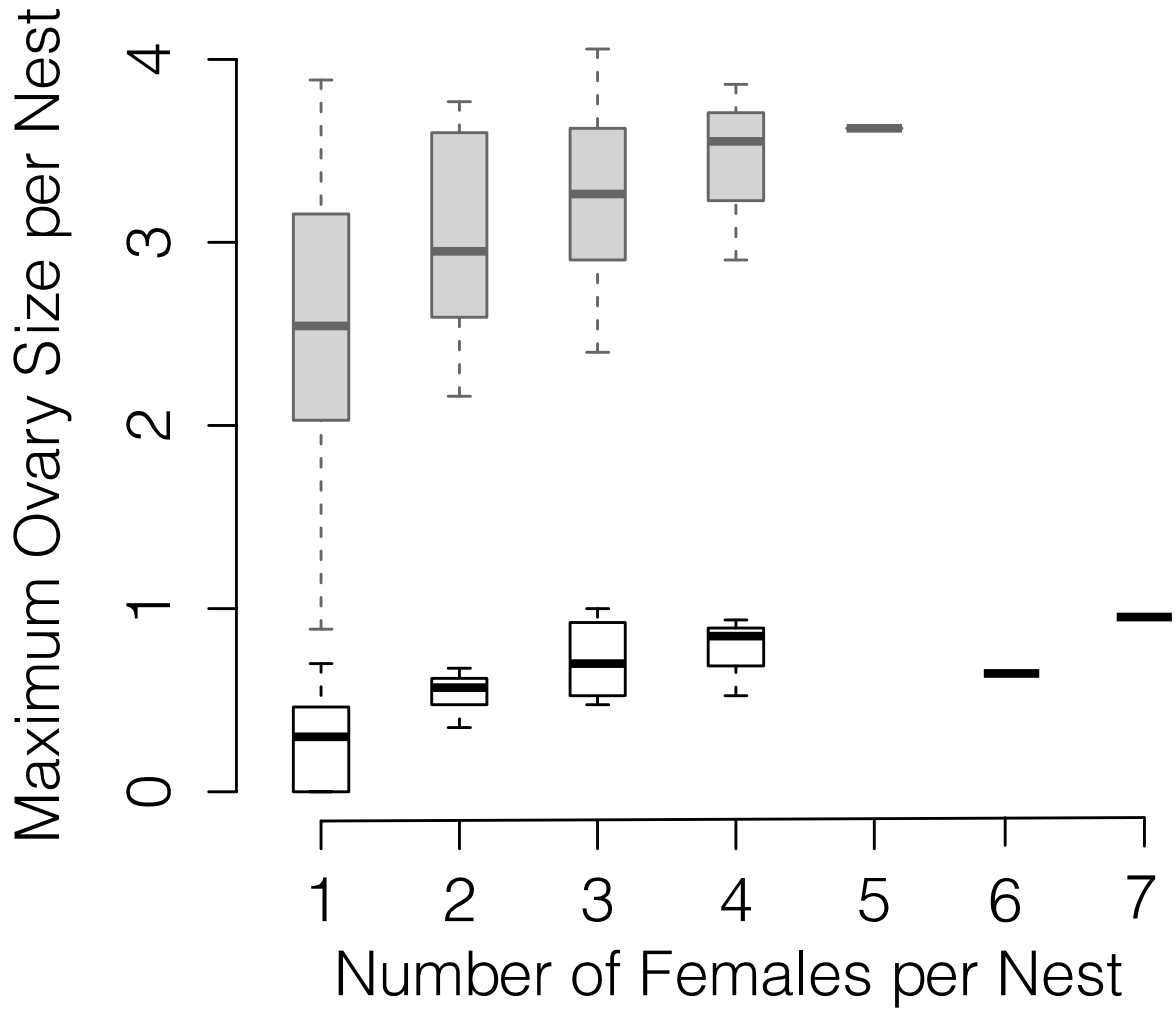

Fig. 5 - Boxplot of the maximum ovary size (mm) within each nest against colony size. Grey boxes (summer), white boxes (autumn).

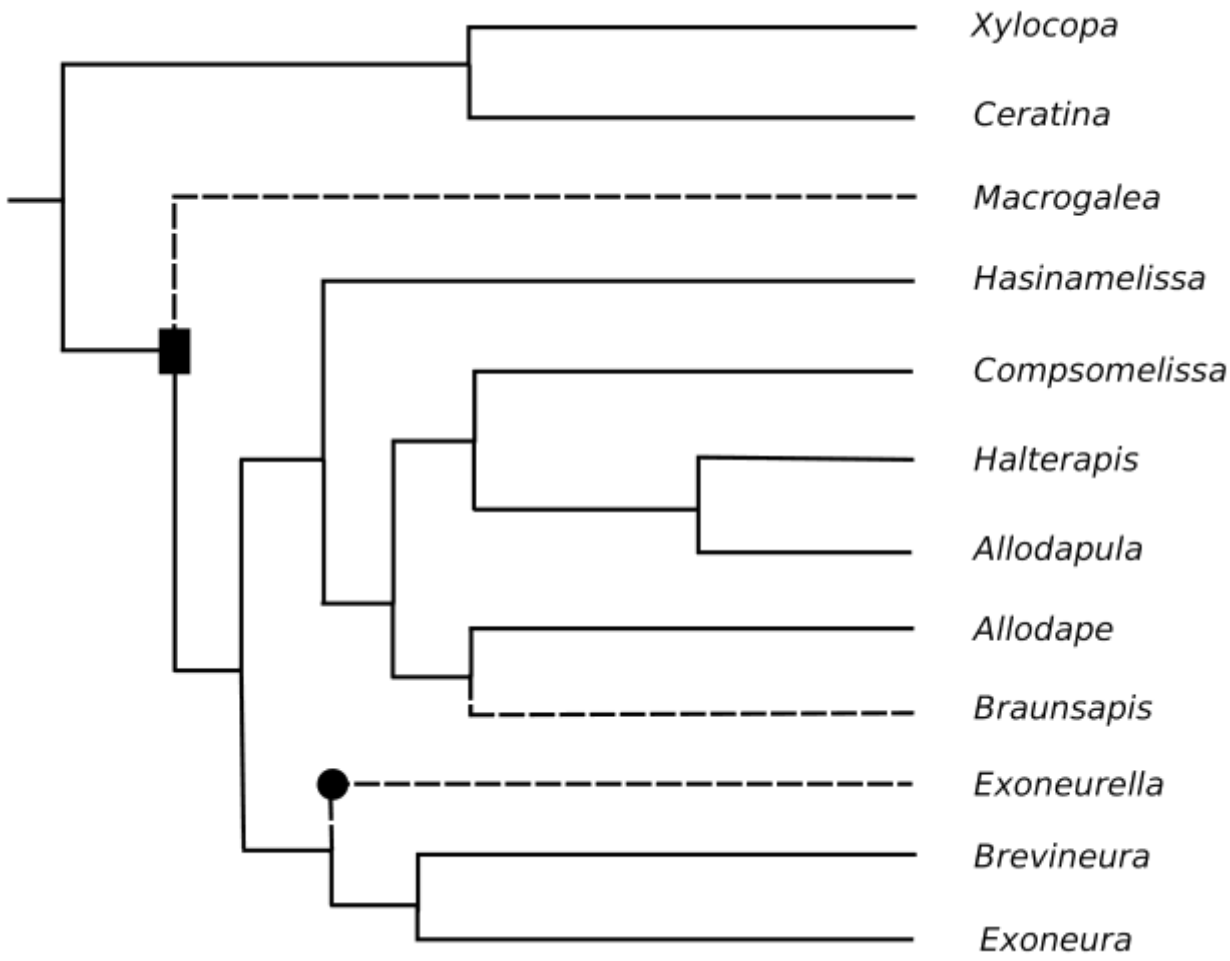

Fig. 6 – Genus-level cladogram of the Xylocopinae. Stippled branches indicate lineages containing casteless taxa. The solid rectangle indicates the most common recent ancestor node of the allodapines. The circle denotes the only origin of eusociality within the Xylocopinae to contain discrete morphological castes. Toplogical relationships among the allodapine genera (Allodapini) are adapted from Smith et al. (2013), and include the sister tribes *Xylocopa* (Xylocopini) and *Ceratina* (Ceratinini).

Supplementary Table 1: Generalised linear models of predictors of maximum ovary size in summer and winter. Best fit models for each season are in bold. Model formula follows conventions in R.

Summer				
Model	Residual Deviance	Degrees of Freedom	AIC	BIC
~ female pupae + total larvae + adult females + total brood	**13.205**	**1**	**84.53**	**93.78**
~ female pupae + total larvae + adult females + total brood + total pupae + male pupae	13.028	1	85.93	97.03
~ female pupae + total larvae + adult females + total brood + total pupae	13.028	1	85.93	97.03
~ female pupae * total larvae * adult females * total brood * total pupae * male pupae	21.097	1	167.62	234.22
Autumn				
~ adult females	**3.5076**	**1**	**-4.43**	**-0.77**
~ female pupae + adult females	2.0794	1	-3.90	1.59
~ female pupae + total larvae + adult females	2.0558	1	-2.43	4.89
~ female pupae + total larvae + adult females + total pupae	2.0192	1	-1.252	7.89

~ female pupae + total larvae + adult females + total pupae + male pupae	2.0192	1	-1.252	7.89
~ female pupae + total larvae + adult females + total brood + total pupae + male pupae	2.0192	1	-1.252	7.89
~ female pupae * total larvae * adult females * total brood * total pupae * male pupae	0.7502	1	1.201	54.86

CHAPTER III

Casteless behaviour in social groups of the bee *Exoneurella eremophila*

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This chapter has been formatted for submission to *Apidologie*.

Abstract

The allodapine bee genus *Exoneurella* contains the only eusocial species within the entire subfamily Xylocopinae (Apidae) with discrete queen and worker morphology. Here we show that the non-eusocial congener, *Exoneurella eremophila*, is casteless. Nest collection and dissection data show no evidence of hierarchies, and there were no *per capita* benefits to group nesting in terms of brood production in any collection period. The casteless behaviour exhibited by *E. eremophila* appears to be common among very diverse lineages of the bee tribe Allodapini, and as such represents an evolutionarily persistent behavioural strategy. This suggests that castelessness is not a transitional stage towards eusociality, rather it may represent a separate 'evolutionary endpoint'.

Keywords:

casteless / allodapine bee / social behaviour / eusocial / nest-site limitations

Introduction

Eusociality is the most extreme form of social organisation and the evolution of this trait often involves a major increase in behavioural complexity and interdependence of individual organisms (Szathmary and Maynard Smith 1995). Evolutionary transitions to eusociality are the subject of a large body of research but direct studies aimed at identifying how it has evolved are limited as most eusocial lineages have ancient origins, with no closely related extant non-eusocial taxa (Brady et al. 2006). This has led to an increasing recognition of the importance and power in studying those taxa that contain recent origins of eusociality, as well as more simple forms of organisation (Rehan and Toth 2015; Kocher and Paxton 2014). Traditionally, comparative studies have focused on societies with 'primitive' hierarchical organisation, perceived as potential precursory states leading to eusociality.

Not all social groups form hierarchies, with some societies appearing to form more-or-less egalitarian societies that can be regarded as 'casteless' (Wcislo and Tierney 2009; Dew et al. 2016). Casteless societies are those that exhibit no hierarchies, such that members of a society have no life-time commitment to specific behavioural roles such as reproduction, foraging, or guarding (Dew et al. 2016). Understanding the social biology of casteless species can provide insights into incipient stages of social evolution without assuming that social complexity evolved in a step-like manner through ever increasing levels of hierarchical complexity (Lin and Michener 1972, Michener 1985).

Identifying casteless societies is not straightforward and requires careful analyses in order to distinguish lifetime fitness from temporal snap-shots of nesting biology.

However, the number of social insects identified as casteless has been growing. Recent studies on facultatively social bees utilise statistical resampling techniques to compare morphometrics of colony nestmates against null expectations (random pairs drawn from the population as a whole), in order to determine differentiation in reproductive skew and foraging effort. This is a powerful technique for capturing non-hierarchical structuring within social groups (Dew et al. 2016). Studies utilizing this methodology have indicated a lack of castes in the Australian temperate colletid bee *Amphylaeus morosus* Smith (Spessa et al. 2000), the tropical Asian-Pacific allodapine bee *Braunsapis puangensis* Cockerell (da Silva et al. 2016) and *Exoneurella setosa* Houston from Australian semi-arid riparian regions (Dew et al. *in review*).

The identification of casteless societies within a population of *Exoneurella setosa* (Dew et al. *in review*) illuminates an interesting dichotomy in the social evolution of this genus. Its congener, *E. tridentata* Houston, is eusocial with reproductive queens and sterile workers that are morphologically differentiated (Hurst 2001; Houston 1976). All of the *Exoneurella* species outside of eusocial *E. tridentata* are facultatively social, including *E. lawsoni* Rayment (Michener 1964), *E. eremophila* Houston (Hogendoorn et al. 2001) and the casteless *E. setosa* (Neville et al. 1998; Dew et al. *in review*). It is unknown if casteless behaviour is unique to *E. setosa* or if it occurs in the other facultatively social *Exoneurella* as well. If castelessness is a common social trait of *Exoneurella* this would have important implications for our understanding of social evolution.

The only previous study of *E. eremophila* examined populations in severely arid regions of central South Australia (Hogendoorn et al. 2001), and this suggested that social hierarchies

might be absent via comparisons of mated and unmated females in the population. However, further evidence for reproductive or dominance based hierarchies was not explored; such as comparisons between nestmate's relative ovarian development and body size.

The aim of the current study is to determine the social structure of colonies of the allodapine bee *E. eremophila* in a riparian semi-arid population where it lives in sympatry with casteless colonies of *E. setosa*. Specifically, we tested the presence or absence of hierarchies in social groups, utilising statistical resampling techniques. We discuss the ecological factors that may have facilitated the evolution of castelessness and look at the prevalence of this behaviour across the allodapines as a whole.

Materials and Methods

Sample Collection

Collections were taken from Mildura, Victoria, Australia (34° 09'16.4"S 142° 09'23.9"E) in areas of chenopod shrubland, within 2 km of the banks of the Murray River. There were three separate collection dates covering spring (11-13 October 2013), summer (21-23 January 2014) and autumn (12-14 April 2014). Whole nests were collected during early morning or late evening, when all colony members would be present in the nest, mostly from dead stems of annual Compositae plants in the genus *Senecio*. Entrances to the nests were taped to prevent bees escaping and nests were placed in a cool box for transport to Flinders University for processing. We recorded nest census data including the number of adult females and males, type and number of brood, sex of pupae, nest length and any evidence of predation or parasitism. All nest contents were preserved in 100% ethanol. Adult females were later transferred to 70% ethanol to allow hydration of tissues to facilitate dissection. Numerical sex ratios (r = male pupae / total pupae) were calculated to compare sex allocation between social and solitary nests.

Dissections

Wing length was measured from the tip of the submarginal cell to the axilliary sclerites. In allodapines wing length is proportional to pupal weight (Schwarz 1986) and is an appropriate proxy for body size. Both forewings were scored for the number of nicks on the outer edge. In badly worn wings individual nicks are impossible to discern, so these were

given a score of > 20. A metric of ovarian size was calculated for each female by summing the length of the three largest oocytes (Cini et al. 2013; Schwarz 1986). Individual spermathecae were observed for evidence of insemination, indicated by opalescence or transparency when not inseminated (Schwarz 1986).

Statistical Analyses

Data were analysed in SPSS v19.0.1 and R v3.1.0 (R Development Core Team 2015). Our variables of interest did not match normal distributions, so non-parametric analyses were used to compare metrics of ovary size, body size and wing wear among nestmates. Per capita brood production (total brood / adult females per nest) was compared between social and solitary nests as a measure of colony rearing efficiency (Michener 1964, 1974; Tierney et al. 1997; Schwarz et al. 2007).

Metrics of ovary size, body size and wing wear were further compared between nestmates and simulated random pairs of females in the population, using Monte Carlo resampling procedures (Spessa et al. 2000; Rehan et al. 2009; Tierney and Schwarz 2009, 2013; da Silva et al 2016; Dew et al. 2016). These analyses determined whether actual nestmates differed from each other in key traits, such as body and ovary size, more than would be expected from random pairings of non-nestmate or solitary nesting females. In theory, consistently larger differences in ovary size, body size or wing wear between nestmates, relative to random couplings, should be indicative of hierarchical nesting (e.g. Spessa and Schwarz 2000). We calculated the empirical mean difference between 1st and 2nd rank females for each morphometric. We then simulated random pairs from a pool of all the 2-female

nesting individuals (with sample replacement) to get 'expected' nestmate differences. This was replicated 1000 times to generate null distributions for each morphometric. The empirical mean differences were then compared with the null distributions.

These analyses were then repeated using a pool of solitary nesting females from which 'virtual' pairs were drawn. Our rationale here is that individuals in a casteless nest should be performing all tasks (or a portion of all tasks), in the same way that a solitary female would, but in a shared space. Therefore, we would not expect empirical differences in morphometrics to differ from expected 'virtual' pairs of randomly sampled solitary nesting females.

Results

A total of 238 nests containing adult females were collected during summer (N = 39) and autumn (N = 195). Only four nests were found in spring and were excluded from statistical comparisons due to the low sample size. Modal colony size in both samples was one female per nest (Figure 1), but group nesting was more prevalent in autumn. During summer mean colony size was 1.41 with a maximum of three females per nest, and 33% of nests contained more than one female. In autumn, mean colony size rose to 2.46 with a maximum of nine females per nest, and the proportion of social nests was 61%; which likely represents the recruitment of recently emerged adults that will disperse in spring.

Morphometrics of solitary *versus* social nesting females

Solitary females were compared to those in social nests to get a baseline comparison of females choosing either nesting strategy. Dissections were performed on 143 individuals from 80 randomly sampled nests. The dissected samples included 40 solitary females and 103 females from social nests. There were seasonal differences in ovarian development, with females possessing larger ovaries in summer than in autumn (Mann-Whitney, U = 3953, P < 0.001), hence all subsequent analyses were performed separately for each collection sample.

Mating behaviour was inferred from insemination status. The proportion of solitary and social females that had mated was not different in either summer (Chi-square $X^2_1 = 1.384$, P = 0.222) or autumn (Chi-square $X^2_1 = 3.017$, P = 0.082; Figure 2). During summer solitary and

social females did not significantly differ in ovary size (Mann-Whitney U = 262, P = 0.49), wing length (U = 328, P = 0.94) or wing wear (U = 286, P = 0.35). However, in autumn social females did exhibit significantly larger ovaries (0.58 mm ± 0.026 s.e.) than solitary females (0.37 mm ± 0.046 s.e.; U = 343, P = 0.001). Social females also had on average larger body sizes (2.37 mm ± 0.014 s.e.) than solitary females (2.25 mm ± 0.020 s.e.; U = 321, P < 0.001). While solitary females had more worn wings (mean wing nicks = 3.6 ± 1.32 s.e.) than their social counterparts (0.99 ± 0.37 s.e.; U = 914, P = 0.016). These autumn differences in wing wear (between solitary and social nests) is most likely due to the recent eclosion of callow females that remain and overwinter within the natal nest.

Each social nest has multiple individuals, which could create pseudoreplication problems when compared to solitary individuals (solitary nests – summer N = 19, autumn N = 21; social nests – summer N = 35, autumn N = 68). To address these potential problems these samples were down-weighted by their colony size as a fraction of the maximum colony size out of the dissected nests for that season (five individuals in autumn, three in summer). For example, in summer this gave solitary females a weight of 3, 2-female nest individuals weights of 1.5 and 3-female nest individuals weights of 1. The weighted analyses supported the results from the unweighted tests.

In summer, social and solitary females did not have significantly different ovary sizes, body sizes or wing wear (U-tests, $P \geq 0.15$ for all three tests). But social females in autumn were found to have significantly greater ovary sizes (U = 3423, P < 0.001) and body sizes (U = 2823, P < 0.001), corroborating the unweighted analyses. Solitary females in autumn were likewise found to have more wing wear than social females (U = 4973, P < 0.001).

Social group structure

Next we compared members of social nests, to determine whether within-nest social differentiation was present. The proportion of inseminated social females did not differ in summer and autumn (76% and 78% respectively, Figure 2). In summer mated social females had larger ovaries (2.28mm) than unmated (0.82mm; $U = 8.5$, $P < 0.001$). Mated and unmated females did not have significantly different ovary sizes in autumn ($U = 197$; $P = 0.167$). This equivalence in ovary size is due to decreased ovarian development in mated females at this time, with ovary size of mated females in autumn (0.06mm) showing a large drop form mated females in summer (2.28mm, $U = 1027$, $P < 0.001$). Wing length and wing wear showed no significant pattern with insemination in either season (Wing length U-tests $P \geq 0.36$, Wing wear U-tests $P \geq 0.20$).

Individuals from 2-female nests only (due to small sample size of larger colonies) were then compared for evidence of hierarchies in both summer and autumn. Females in each nestmate pair were ranked based on ovary size, body size and wing wear separately. Females ranked by ovary size did not have significantly different body sizes (both U-tests, $P \geq 0.73$) or wing wear (both U-tests, $P \geq 0.21$) in either season. Similarly, females ranked by body size did not have significantly different ovary sizes (both U-tests, $P \geq 0.77$) or wing wear (both U-tests, $P \geq 0.36$). Again, when ranked by wing wear nestmates did not exhibit significantly different body sizes (both U-tests, $P \geq 0.16$) or ovary sizes (both U-tests, $P \geq 0.47$).

Monte Carlo simulations

Monte Carlo simulations were used to further explore patterns in morphometrics among 2-female nests. Only the summer collection sample was used for simulated analyses, as this period represents the peak of reproduction and brood rearing in our samples and should allow us to capture any patterns in reproductive skew among nestmates, were they to exist. Random nestmate pairs were simulated 1,000 times in two different ways: (social-pool) from the pooled 2-females nests (N = 13); and (solitary-pool) from the pooled solitary females (N = 20). We did this because social nests could have non-random morphometric compositions not detected in the previous comparisons of social and solitary individuals (*see section above* - 'Morphometrics of solitary *versus* social nesting females').

Results from social-pool (2-female nests) indicate that 97% of the 1,000 simulated pairs showed greater differences in ovary size than the mean difference between empirical nestmate pairs of 0.59mm (Figure 3). Therefore individuals in 2-female nests were more similar than expected from the simulated distribution. When this analysis was repeated with simulated pairs drawn from solitary-pool (solitary nests), 84% had greater differences in ovary size than the observed mean difference, strongly suggesting an absence of reproductive castes. This indicates that ovary size differences in social pairs are less than one would expect based on random assignment of females to virtual nests. For body size, 58% (social-pool) and 34% (solitary-pool) of the simulated pairs had greater differences than the observed social mean, again providing no evidence of hierarchies Similar results were obtained for wing wear with 32% (social-pool) and 69% (solitary-pool) of the simulated distribution having greater differences than the mean difference between observed pairs.

Brood Production

The majority of nests had brood present in both summer (69%) and autumn (72%), though the composition of the brood varied across seasons (Figure 4). Of the four nests collected in spring, three had brood, consisting of both eggs and larvae, indicating that brood production started as early as late September. Eggs, larvae and pupae were present in summer. In autumn, only one nest was found to have an egg, with larvae and/or pupae present in all other nests that were rearing brood. There were a total of three nests (one solitary and two social) found with brood parasitised by encyrtid wasps, all in the autumn sample.

Per capita brood production (PCBP) was compared between solitary and 2-females nests with Mann- Whitney U tests (sample sizes of larger social nests were too small for meaningful comparison). For both summer and autumn there were no significant differences in PCBP between social and solitary nests ($U = 97$, $P = 0.21$; $U = 1353$, $P = 0.055$ respectively).

Sex allocation did not vary significantly between seasons ($U = 1148$, $P = 0.32$) with an overall mean sex ratio of 0.45 ± 0.020 s.e. Sex ratios in social and solitary nests did not vary in summer ($U = 45$, $P = 0.72$). But during autumn sex ratios were significantly more female biased in solitary nests ($r = 0.29$) than social nests ($r = 0.52$; $U = 947$, $P < 0.001$).

Discussion

Our results provide strong evidence that social colonies of *E. eremophila* are casteless. There was no skew between social nestmate pairs in insemination status, ovary size, body size or wing wear other than expected from randomly assembled 'virtual nestmates'. Females in social nests were in fact more similar in ovary size than that expected from randomly assembled pairs (Figure 3). There are a number of possible explanations for this. Firstly, females may co-found nests and represent breeding cohorts at similar reproductive stages, although nest co-founding is not known to occur in any species of *Exoneurella*. Second, environmental resources may standardize ovarian development across the population, such as the availability of protein resources from pollen. Third, female nestmates may be highly related and ovarian development is genetically conserved.

In addition to a lack of castes we also found only minor benefits to group nesting. One third of nests are social during summer (the peak brood rearing period), so we might expect fitness benefits to social nesting, however, there were no differences in PCBP between social and solitary nests. There may be minor benefits associated with social nesting as the brood rearing season comes to a close because autumn social females had larger ovary sizes than solitary nesting females. A similar result was found for the sympatric species *E. setosa* (Dew et al. in review). Social individuals may experience a slightly extended reproductive period compared to solitary females, perhaps due to co-operative nest defence. Social females in autumn also had larger body sizes, which could be due to better provisioning of newly eclosed adults in social nests. Wing wear was reduced in social females compared to

their solitary counterparts in autumn, although this result could be due to the emergence of recently eclosed adult brood.

Sex ratios were considerably male biased in social colonies at the end of the brood rearing season. Such seasonal variation in sex ratio bias is consistent with species that exhibit caste differentiation, whereby initial broods are female biased to increase the potential for alloparental care (worker castes) to arise. Similar results were discovered in a study of facultatively social *E. lawsoni* from subtropical tableland savannahs of eastern Australia (Michener 1964). It is therefore speculative as to whether (*i*) non-eusocial species of *Exoneurella* derive from a eusocial common ancestor and have lost worker-like behaviour to become casteless (*per* Michener); or whether (*ii*) these casteless *Exoneurella* species (Michener 1964; Dew et al. 2016; in review) have in fact set social preconditions allowing the only truly eusocial allodapine species to arise?

Sociality and Ecology of *Exoneurella*

Fitness gains via improved brood rearing efficiency in social nests is thought to represent one of the main selective agents for the formation of insect societies generally and are commonly found among allodapine bees (e.g. Michener 1974; Schwarz et al. 1998; Tierney et al. 1997, 2000, 2002; Joyce and Schwarz 2006; Thompson and Schwarz 2006). However, social evolution in the genus *Exoneurella* does not appear to be influenced by brood rearing efficiency, as none of the *Exoneurella* studied to date show improved PCBP with increasing colony size, not even in the eusocial species *E. tridentata* (Dew et al. *in* review; Hurst 2001; Michener 1974).

Benefits to group living may consist of improved nest guarding, enabling temporally extended brood rearing deeper into autumn. Intracolony relatedness is exceedingly high in eusocial *E. tridentata* (r = 0.75; Hurst 2001) and is presumed to be high in *E. setosa* and *E. eremophila*, with mother-daughter or sister-sister associations most likely developing within natal nests (Hogendoorn et al. 2001; Neville et al. 1998). Therefore, when coupled with high relatedness even minimal benefits to group living may prove sufficient selective pressure to promote social nesting regardless of the structure of the resultant social organization.

While even meager benefits to group living may promote sociality within *Exoneurella* generally, evolution of variant social structures may have evolved in response to the relative costs of dispersal. Differences in both the availability and longevity of stem nesting substrate appear to be associated with large differences in dispersal opportunity, and it is therefore plausible that nest substrates may have been key determinants in social behaviour. The evolution of eusociality in *E. tridentata* has been linked to extreme nest site limitations (Dew et al. 2012), because of their reliance on pre-formed burrows in two species of hardwood trees, for which there is competition from a variety of other arthropods (Dew et al. 2013). Dispersal from the natal nest is therefore likely to be very risky because the likelihood of finding suitable nesting habitats are very low. We argue that this is likely to facilitate the evolution of eusocial organization via the formation of long-term colonies that are further enabled by the durability of the hardwood nesting substrate.

A similar dispersal linked hypothesis – the aridity food distribution hypothesis - has also been proposed to explain the variation in social behaviour of different mole rat species

(Jarvis et al. 2005; Spinks et al. 2000; Sichilima et al. 2008). Mathematical modelling suggests that nest-site limitations or similarly high dispersal risks may be sufficient to promote eusocial evolution on their own, even in the absence of brood production benefits (Avila and Fromhage 2015).

In comparison, the casteless *Exoneurella* species are able to nest in a wide variety of plant substrates that are in ample supply and renewed annually. In this sense there are few limitations to dispersal, however the nesting substrates are of an ephemeral durability and less likely to persist across multiple seasons. Indeed, the high level of solitary nesting reported in these species indicates that dispersal from the natal nest is not restricted, nor is it an overwhelming risk (Michener 1964; Neville et al. 1998; Hogendoorn et al. 2001; Dew et al. in review). Minimal restrictions to dispersal have also been noted in casteless *Braunsapis puangensis* and *Amphylaeus morosus* (da Silva et al. 2016; Spessa et al. 2000). It appears that low barriers to dispersal, with small benefits to social living are key factors in casteless society formation in *Exoneurella* and possibly other casteless groups.

Evolution of casteless societies among Apiformes

Casteless behaviour occurs in diverse allodapine genera and is potentially ancestral to the allodapine bees as a whole. Reconstruction of the most recent common ancestor of the Allodapini indicates social groups were present but true castes were not, rather there was temporal reproductive skew due to inter-generational delay in reproduction (Schwarz et al. 2011; Rehan et al. 2012). Notably, reproductives in ancestral allodapines were also foragers, permitting the possibility of primitive casteless societies. The casteless behaviour of

Macrogalea, which forms the sister clade to all other allodapines, likewise suggests that castelessness has ancestral origins (Tierney et al. 2002; Thompson and Schwarz 2006; Butler et al. in prep.). But whether ancestral reproductive queues are viewed as casteless or not hinges on whether individuals attained reproductive roles later in life and thereby had equal lifetime reproductive opportunities. This is difficult to assess (see Dew et al. 2016), and it is quite possible that some 'waiting' females did not attain full reproductive status, forming rudimentary hierarchies. Or maybe this natal philopatry simply allowed flexibility in social structure, enabling the diversity of social forms seen in the Allodapini today.

If the allodapines are ancestrally casteless then this trait may have simply been retained in most *Exoneurella*. Currently, however, the behaviour at the root of *Exoneurella* has not been established. An undescribed Exoneurella species from Western Australia is phylogenetically the closest to the sister group Brevineura (Chenoweth and Schwarz 2011, but see chapter VI). The social behaviour of this species is unknown, but it is does not have dimorphic females like those of *E. tridentata* (pers. obs. R Dew). The possibility of a socially hierarchical common ancestor to the genus *Exoneurella* cannot be ruled out until the social behaviour of this western species is known (Chenoweth and Schwarz 2011).

While the ancestral condition for *Exoneurella* is still ambiguous, a loss of hierarchies to casteless behaviour is known for the allodapine *Braunsapis puangensis*, which lost facultative castes upon its recent introduction to Fiji from India (da Silva et al. 2016). Similarly, the casteless behaviour of *Euglossa hyacintha* Dressler (Apidae: Euglossini) likely represents a loss of hierarchies to castelessness (Cardinal and Danforth 2011; Soucy et al. 2003), because the Euglossini form part of the corbiculate bees (Apidae), of which most

tribes are eusocial (Michener 1974). The colletid bee *Amphylaeus morosus* (Colletidae: Hylaeinae), however, evolved casteless behaviour from solitary living, as the vast majority of colletids are solitary (Spessa et al. 2000).

The number of casteless lineages now identified and their inherent similarity to communal nesting lineages (*reviewed by* Dew et al. 2016) suggest that the absence of castes in social insect organization has been a persistent and successful nesting strategy over significant periods of evolutionary time. Which raises the question of whether casteless and eusocial societies are simply an alternate means of achieving the same end – the establishment of a secure abode in which to rear brood (*see* Wcislo and Tierney 2009).

So while the broader comparative evidence suggests castelessness is not necessarily a transitional behavioural state on some rigid evolutionary trajectory towards a hierarchical eusocial optimum, the *Exoneurella* evolutionary scenario remains opaque. Do non-eusocial *Exoneurella* represent the loss of castes from a eusocial common ancestor (similar to *E. tridentata*), or did the absence of castes in combination with (*i*) severe limitations to natal-nest dispersal and (*ii*) a highly durable nesting substrate enable extreme social hierarchies? If the latter is true, then extrinsic chance events have created a platform for the evolution of morphologically distinct behavioural castes and the development of truly eusocial colony organization. In short, did these bees need to lose castes in order to comprehensively develop them?

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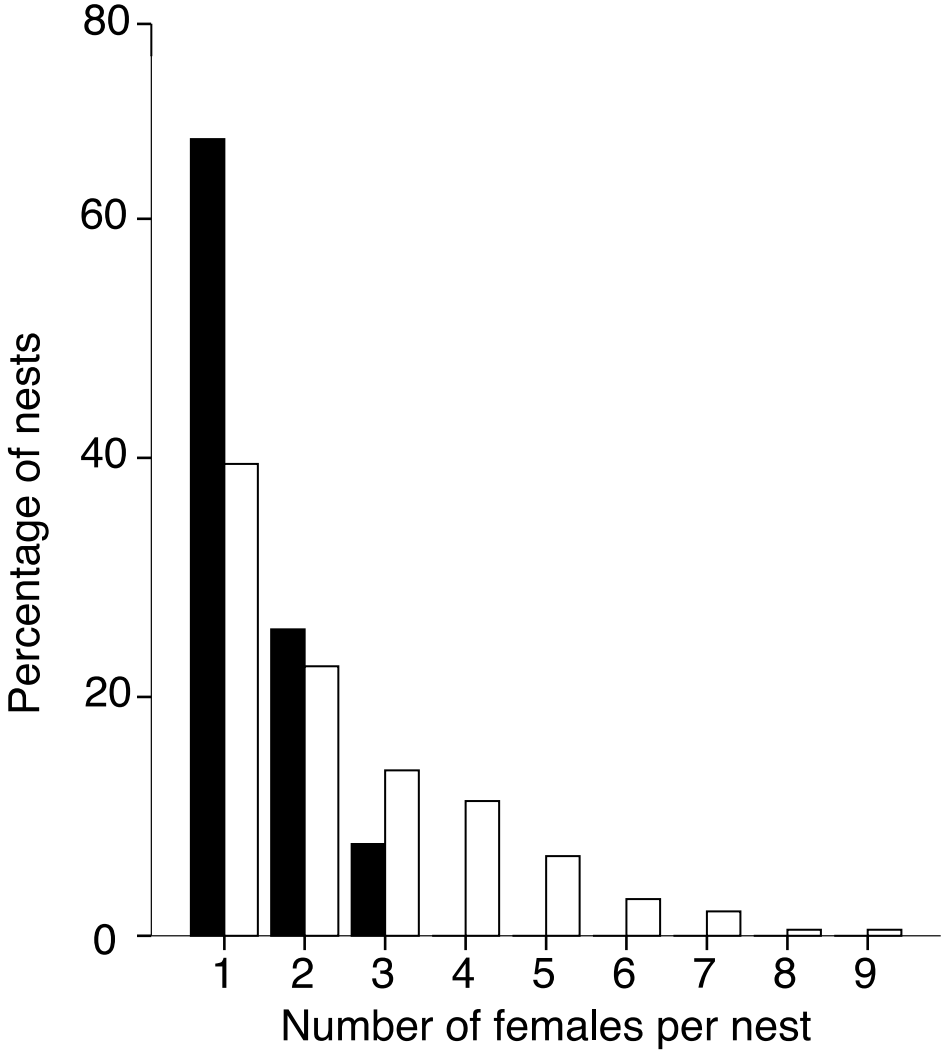

Fig. 1 Colony size distribution as a percentage of all nests of *E. eremophila* collected per season. Summer: black, autumn: white

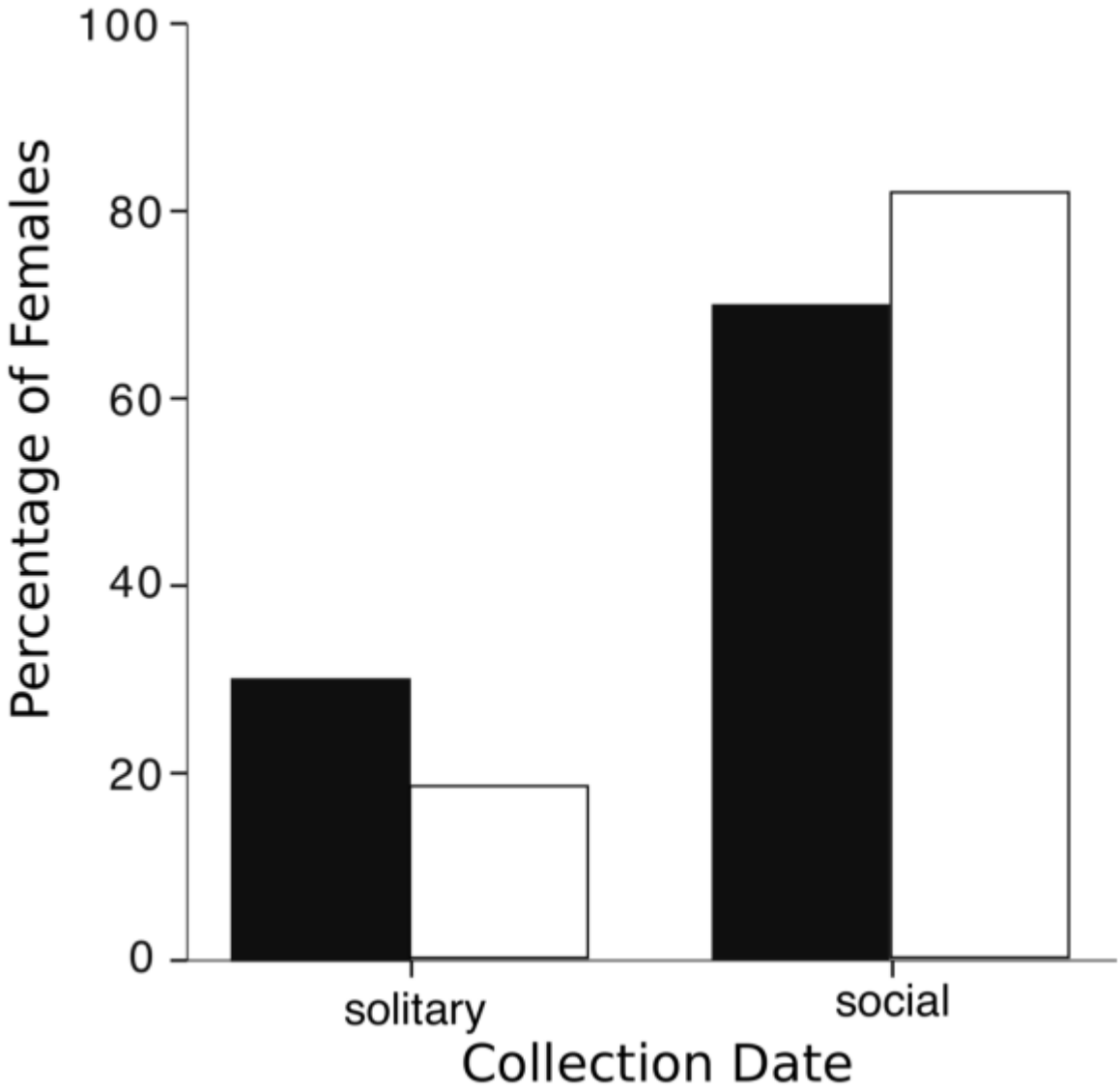

Fig. 2 Percentage of mated females of *E. eremophila* in solitary nests and social nests during.

Summer: black, autumn: white

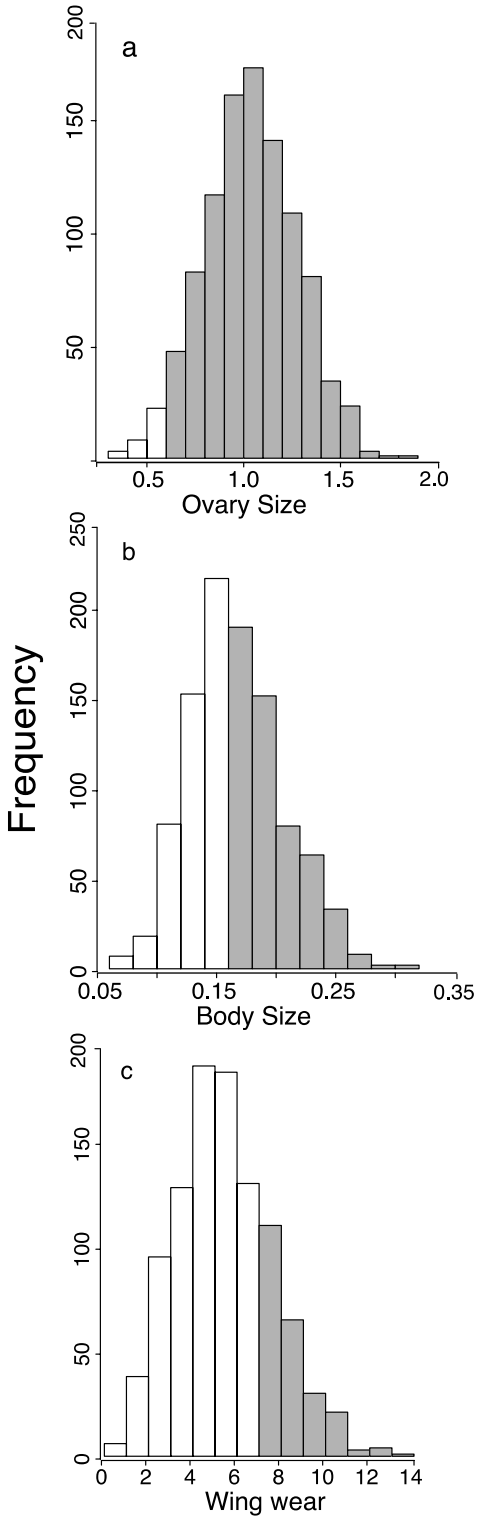

Fig. 3 Frequency of differences in ovary size (a), body size (b), and wing wear (c) between virtual nestmate pairs. Pairs were randomly drawn from summer social nests (pool-a) in a Monte Carlo resampling procedure. Grey bars indicate the proportion of virtual pairs exhibiting greater differences than the observed mean derived from actual nest pairs.

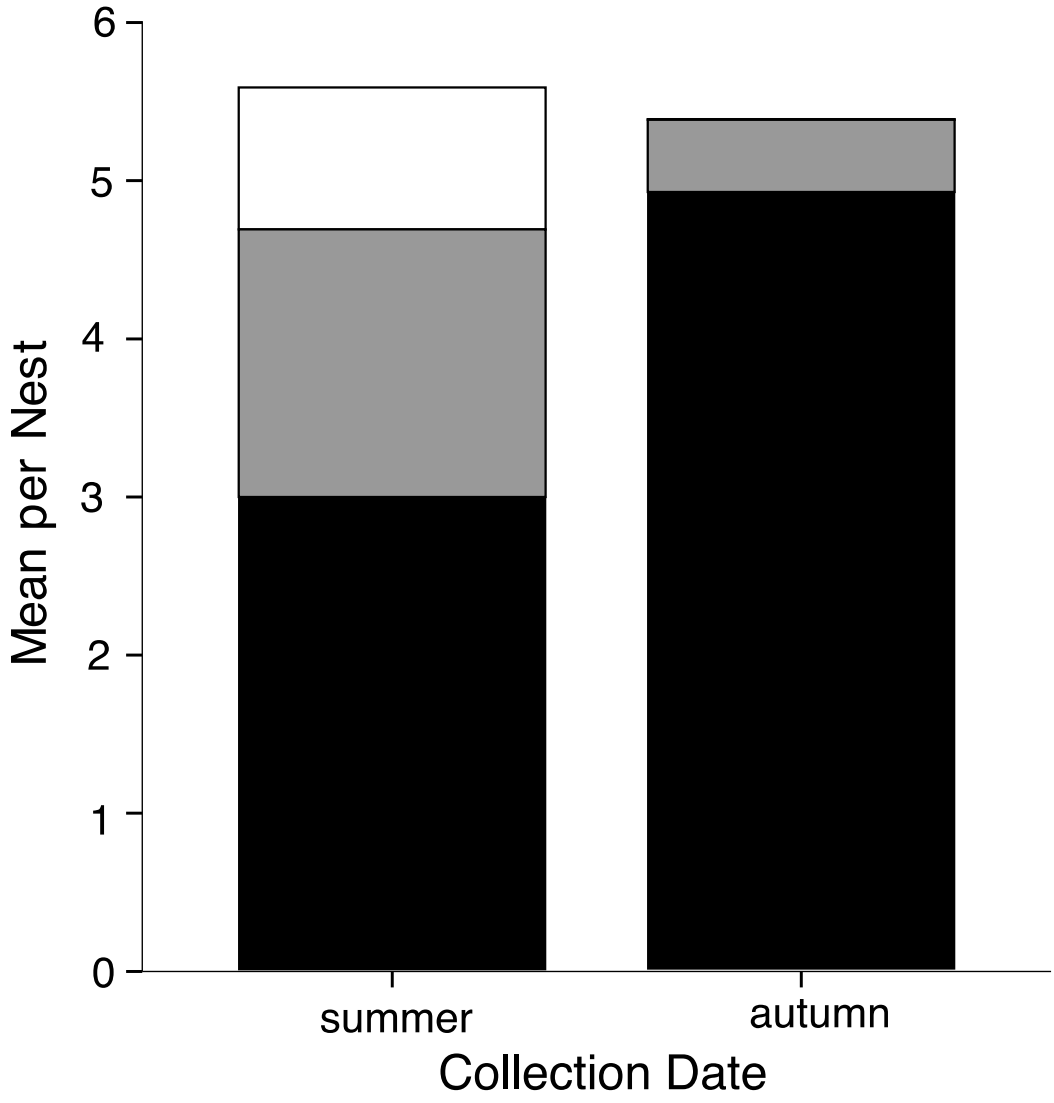

Fig. 4 Stacked histogram showing the mean number of eggs (white), larvae (grey) and pupae (black) in nests of *E. eremophila* in summer and autumn.

CHAPTER IV

Biogeography and demography of an Australian native bee *Ceratina australensis* (Hymenoptera: Apidae) since the last glacial maximum

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Abstract

The small carpenter bees, genus *Ceratina,* are highly diverse, globally distributed, and comprise the sole genus in the tribe Ceratinini. Despite the diversity of the subgenus *Neoceratina* in the Oriental and Indo-Malayan region, *Ceratina (Neoceratina) australensis* is the only ceratinine species in Australia. We examine the biogeography and demography of *C. australensis* using haplotype variation at 677 bp of the barcoding region of COI for specimens sampled from four populations within Australia, across Queensland, New South Wales, Victoria and South Australia. There is geographic population structure in haplotypes, suggesting an origin in the northeastern populations, spreading to southern Australia. Bayesian Skyline Plot analyses indicate that population size began to increase approximately 18,000 years ago, roughly corresponding to the end of the last glacial maximum. Population expansion then began to plateau approximately 6,000 years ago, which may correspond to a slowing or plateauing in global temperatures for the current interglacial period. The distribution of *C. australensis* covers a surprisingly wide range of habitats, ranging from wet subtropical forests though semi-arid scrub to southern temperate coastal dunes. The ability of small carpenter bees to occupy diverse habitats in ever changing climates makes them a key species for understanding native bee diversity and response to climate change.

Keywords

climate change; dispersal; DNA barcoding; bayesian skyline plot; haplotype network; population structure

Introduction

Molecular studies have greatly increased our understanding of the antiquity of bees and their historical biogeography, especially with respect to centres of origin and subsequent dispersal routes (e.g. Fuller et al. 2005; Hines 2008; Chenoweth and Schwarz 2011; Rehan and Schwarz 2015). Other studies using museum collection data have implicated very recent climate change as a possible factor underlying changes in bee abundances (e.g. Cameron et al. 2011; Burkle et al. 2013), but there are surprisingly few studies that have attempted to infer changes in bee abundance beyond the last 200 years (but see Wilson et al. 2014). In the face of likely future climate change, it is important to understand how bees have responded to past climates so that we may better predict future trends.

Two recent studies (Groom et al. 2014a; López-Uribe et al. 2014) have used phylogeographic and coalescent Bayesian Skyline Plot analyses to examine changes in bee abundances for tropical halictine (Halictidae) and euglossine (Apidae) bees respectively. Both studies found a strong response to Pleistocene climates, suggesting that these two faunal groups have been impacted by glacial cycles despite their tropical distribution. Small carpenter bees, *Ceratina* (Apidae: Ceratinini), of the subgenera *Zadontomerus* in eastern North America also showed a rapid population expansion approximately 20kya, linked to post-glacial cycles (Shell and Rehan 2016). However, no studies have examined possible impacts of historical climates on bee species spanning temperate to xeric zones, beyond those using museum records.

The small carpenter bee genus *Ceratina* has a nearly global distribution, occurring on all continents except Antarctica (Rehan and Schwarz 2015). This includes recently colonized remote islands in the Southwestern Pacific and southern Indian Ocean, probably via accidental human agency (Rehan et al. 2010a; Groom et al. 2014b). *Ceratina* originated in Africa in the late Paleocene or early Eocene and showed rapid long distance dispersal events allowing it to eventually colonize the New World by the late Eocene (Rehan and Schwarz 2015). Interestingly, patterns of radiation in this tribe suggest that major long distance dispersal events have been rare and tended to occur more frequently in the early history of this tribe rather than later on, despite there being few geographical barriers to later dispersal events (Rehan and Schwarz 2015). Physical impediments to dispersal, such as water barriers, are actually believed to have decreased in the more recent history of this tribe (Rehan and Schwarz 2015).

Ceratina (*Neoceratina*) is the sister clade to all other *Ceratina* subgenera and originated from a dispersal from Africa to the Oriental region in the early Eocene, with some species extending into the Palearctic (Rehan and Schwarz 2015). Surprisingly, there is only a single ceratinine species in Australia, *Ceratina (Neoceratina) australensis* (Perkins, 1912). This species forms the sister lineage to all other *C.* (*Neoceratina*) species, and its stem age is dated to the middle Eocene. *Ceratina australensis* has become a model species for understanding simple forms of sociality where both solitary and social forms remain in sympatry (e.g. Rehan et al. 2010b, 2011, 2014). Solitary nests comprise about eighty-five percent of the population and are founded by females that disperse from their natal nests (Rehan et al. 2014). Dispersal of females could facilitate gene flow between populations across Australia.

Michener (1962) recorded *Ceratina australensis* from subtropical and temperate regions of eastern Queensland and New South Wales but there have been no further studies of its distribution. Here we use haplotype variation at 677 bp of the mitochondrial 'barcoding' region of cytochrome *c* oxidase subunit 1 (CO1) from 102 specimens of *C. australensis*, obtained from southern Queensland, mid-New South Wales, northern Victoria and southern South Australia, to examine historical demography and geographical patterns in population genetic structure. Based on the dispersal of females from natal nests we predicted that there would be gene flow between populations, influencing the genetic structure and historical demography of the species.

Methods

Collecting Localities for Genetic Samples

Specimens of *Ceratina australensis* were collected from four populations: (i) Mildura in northwestern Victoria; (ii) West Beach in metropolitan Adelaide, South Australia; (iii) the Cowra region in central New South Wales; and (iv) the Warwick region in southeastern Queensland (Figure 1). Our four study sites represent a substantial proportion of the geographic and climatic conditions for the species, covering warm temperate forest, semi-arid riverine woodland, mediterranean coastal dunes and central tablelands. GPS coordinates, collection dates and the number of barcoded specimens from each population are shown in Table 1. Nests, predominantly found in dead stems of plants from the genera *Cakile* (Brassicaceae), *Senecio* (Asteraceae)*, Ferula* (Apiaceae) and the species *Verbena bonariensis* L. (Verbenaceae) were collected during early mornings or late evenings. This ensured that the bees would be present in the nest and not out foraging. Nests were collected, as this species is rarely observed on flowers (Michener 1962). Nests were stored on ice or at 4°C until processing. One randomly chosen adult from each nest was stored in 100% ethanol for DNA sequencing.

DNA Extraction and Sequencing Techniques

One leg of each specimen was incubated overnight in arthropod lysis solution with proteinase K. Extractions proceeded using a glass fibre plate and a vacuum manifold to pull the eluates through the membrane, following the procedures detailed in Ivanova et al. (2006). The DNA extract was stored in 50µl TLE (10mM TRIS, 0.1mM EDTA pH8). A forward primer combining M13/pUC (Messing 1988) and LC01490 (Folmer et al. 1994; 5'-GT TTT CCC

AGT CAC GAC CCT TTT ATA ATT GGA GGA TTT GG-3') and a reverse primer comprising the reverse M13/pUC with primer M399 (Schwarz et al. 2004; 5'-CA GGA AAC AGC TAT GAC TCA TCT AAA AAC TTT AAT TCC TG-3') were used for PCR amplification of a 700bp region of CO1. We used 27.5 µl reactions with 0.1 µl immolase as the active enzyme, 1 µl of each M070 and M080, 5 µl of MRT Buffer, 15.4 µl water and 5 µl DNA. The PCR cycle began with 10min of 94°C. The annealing stage had 5 cycles consisting of 60s at 94°C, 90s at 45°C and 90s at 72°C followed by 35 cycles of 60s at 94°C, 90s at 50°C and 60s at 72°C. Elongation was 10min at 72°C and the reaction was terminated with a final 2min at 25°C. The PCR products were cleaned using a vacuum plate with 100 µl TLE, with the cleaned products stored in 30 µl TLE. Final forward and reverse sequencing of the cleaned PCR products was performed by the Australian Genome Research Facility.

Alignment and Phylogenetic Reconstruction

Sequences were imported into GENEIOUS v.6.1.6 (Kearse et al. 2012) for editing and alignment. Reverse and forward sequences were combined into a consensus sequence for each sample. As we were comparing individual base pair changes, no ambiguous or unknown base pairs (including at the end of sequences) could be left in the final alignment. We aimed to maximize both the sequence length and the number of samples. The sequence length was shortened, so that all samples had base pair data covering the same read length without any missing or ambiguous nucleotides, since missing data can lead to spurious results for coalescent analyses (Ho and Shapiro 2011). The final alignment consisted of 102 sequences of 677 bp in length, with 28 unique haplotypes. All edited sequences are submitted to GenBank (accession numbers KR824844-KR824934 and KU664337-KU664347).

An undescribed *Neoceratina* species from the Solomon Islands, *Ceratina (Neoceratina)* "Solomons sp." was included in the alignment as the outgroup to determine the root of the tree. This species has been shown to be phylogenetically distinct to *Ceratina australensis* (Rehan and Schwarz 2015). The sequences available for this species did not cover the full length of the 677 bp alignment, so the *C. australensis* sequences were shortened to 639 bp for this analysis. This sequence attenuation did not remove any of the unique haplotype information present within the *C. australensis* sequences. The phylogenetic tree was reconstructed in BEAST v.1.8.1 (Drummond and Rambaut 2007) with a Yule Process tree prior. The substitution model HKY+I+Γ model was identified as the most appropriate based on Akaike information criterion in JMODELTEST (Guindon and Gascuel 2003; Darriba et al. 2012). The analysis ran for 5 x 10^7 generations, logged every 1,000 trees, using a fixed mutation rate of 1.0, with all other parameters set to default. The log files were viewed in TRACER v.1.5 to confirm that the posterior had stabilized. A consensus tree was constructed with TREE ANNOTATER v.1.8.1 with a burn-in of 10,000 trees (i.e. 10 million iterations; Supplementary Figure 1). The BEAST analysis was run three separate times to confirm convergence.

The full alignment was then pruned to contain only unique haplotypes (28 sequences). The analysis was run following the conditions described above. TRACER again confirmed the posterior of the analysis had stabilised and a consensus tree with a burn-in of 10,000 trees was generated (Figure 2).

Haplotype networks

Analysis of Molecular Variance (AMOVA) was implemented in ARLEQUIN 3.11 (Excoffier et al. 2005) to compare genetic variation within and among Victoria, New South Wales, South Australia and Queensland populations. For these analyses we used all sequences and included all codon positions. All four populations were compared in the full model followed by pair-wise comparisons of each possible pairing in subsequent AMOVA analyses. The full alignment was imported into NETWORK (Fluxus Engineering 2016) and a haplotype network was constructed using a median-joining analysis with epsilon set as zero (Bandelt et al. 1999; Figure 3). HAPLOVIEWER confirmed the final network and was used to generate a publication quality figure (Salzburger et al. 2011).

Historical Demography

We used Bayesian Skyline Plot (BSP) analyses implemented in BEAST and TRACER to explore historical changes in effective population size of *Ceratina australensis*. For these analyses we included all sequences available including duplicate haplotypes, as analyses of only unique haplotypes can give erroneous results (Grant 2015). BSP analyses assume that genetic markers evolve neutrally (Ho and Shapiro 2011). The very small number of amino acid changes in our alignment suggests that purifying selection may be operating on 1st and 2nd codon positions, so we restricted BSP analyses to 3rd codon positions only. In these analyses we used a GTR model for nucleotide substitutions, but did not include an invariant sites parameter (I) since 3rd codon positions should not be constrained by selection. Analyses were run for 100 million iterations, sampling every 10,000th iteration to reduce autocorrelation, and were repeated three times to check for convergence. We implemented a strict molecular clock with rate of 1.0, which allows us to readily convert mutations per site per generation into chronological years.

We converted the Bayesian Skyline plot scale to chronological years through dividing it by mutation rate and the number of generations per year. We used the mitochondrial mutation rate observed in *Drosophila melanogaster* Meigen, viz. 6.2 x 10^{-8} mutations per site per generation as an estimate of the mutation rate for *Ceratina australensis* (Haag-Liautard et al. 2008). This method follows a previous study on demographic history in Fijian halictine bees in the genus *Lasioglossum* (Groom et al. 2014a) and North American *Ceratina* species (Shell and Rehan 2016). We note that the mitochondrial mutation rate for *Caenorhabditis elegans* (Maupas) is estimated as 9.7 x 10^{-8} mutations per site per generation, close to the rate for *D. melanogaster*, and that they have mitochondrial AT biases of 76% and 82% respectively. Previous studies have reported an AT bias of 74% for the same barcoding region as in our study (Groom et al. 2014a; Shell and Rehan 2016), and our *Ceratina* haplotypes had an AT bias of 78%. The number of generations was determined as two per year based on nest contents data from the Victorian and South Australian sites (Dew and Rehan, unpublished data), which also corresponds to detailed studies on the Queensland population (Rehan et al. 2010b, 2011, 2014).

In order to determine whether inferred changes in historical population size in the BSP analysis were significant we also carried out another coalescent analysis using the same parameter settings as our BSP analysis, but implementing a constant population size model. This was then compared to our BSP analysis using a Bayes Factor test.

Inferring Ancestral Distributions

Ancestral distributions were inferred using BEAST ancestral traits reconstruction. The full alignment of 102 sequences was used. Sample location for each sequence was coded as a discrete trait (either New South Wales, Queensland, South Australia or Victoria). The analysis ran for $2x10^8$ generations, logged every 1,000, with a Yule process tree prior. A HKY+I+Γ site model with a strict clock of rate 1.0 was employed. All parameters for phylogeny and ancestral trait reconstruction had reached stability, as viewed in TRACER with a burnin of $1x10^8$ generations. Using this burnin a consensus tree was constructed in TREE ANNOTATER.

Results

Haplotype Lineages

In total 28 haplotypes of *Ceratina australensis* were found across the four field sites. The haplotype tree along with posterior probability values for node support from our BEAST analysis is given in Figure 2. This analysis indicates the presence of a clade comprising one New South Wales and two Queensland haplotypes, which is highly supported (PP = 1.0) as sister clade to the remaining haplotypes. Our haplotype network analysis (Figure 3) indicates that this clade, which we will refer to as NTH1 (due to their relative northern location), is separated from the common ancestor for the remaining haplotypes by seven nucleotide substitutions, none of which involve amino acid changes.

Both the haplotype tree in Figure 2 and the haplotype network in Figure 3 indicate geographical structuring of haplotypes. Firstly, the haplotypes in NTH1 were not recovered in any of the 99 specimens genotyped from the more southwestern sampling locations of Victoria and South Australia. Secondly, there was another clade comprising five haplotypes that were only recovered from the more northeastern localities of Queensland and New South Wales, and we refer to this clade as NTH2. Lastly, the haplotype, which we refer to as STH1 (due to its southern location), mostly comprised specimens from South Australia, but also some from Victoria, and it was not represented in any of the Queensland or New South Wales specimens. Two haplotypes are shared between Queensland and New South Wales, with one shared between Queensland and Victoria.

Population Structure

Pair-wise comparisons among all individuals revealed significantly greater sequence divergence between populations than within populations for Victoria to New South Wales and Queensland, and for South Australia to all other populations (Table 2). Queensland and New South Wales were not significantly different from one another. These results are mirrored by pairwise F_{ST} calculations (Table 2), suggesting that all populations except New South Wales and Queensland are genetically differentiated. There was only one fixed base pair difference identified between any of the populations. This was at base 486, which was a thymine in all Queensland and New South Wales samples but an adenine in all South Australia samples (Victorian samples varied at this base). Tajima's D value indicated neutral evolution between populations (Table 3).

Historical Demography

Our Bayesian skyline plot (BSP) analysis is summarized in Figure 4 where it is temporally aligned with a graph summarizing two temperature proxies taken from Pahnke et al. (2003). In Figure 4 we have used two x-axis scales, one using mutations per site per generation and the other using years before present, based on a mutation rate of 6.2×10^{-8} mutations per site and two generations per year. Based on the current best estimate for mutation rate these plots suggest an increase in effective population size beginning approximately 20–18ka, with a peak rate of increase at about 15–8kya, and a plateauing after about 6kya. Our BSP plot shows a long period of apparent stasis from approximately 64–20kya. This is an artifact of the analysis, where signals prior to the last major effective population size change are lost (Grant et al. 2012; Grant 2015). As this section of the graph is largely uninformative this region was trimmed in the final figure to show just the plot from 32kya.

The 95% Highest Posterior Densities for the BSP plot in Figure 4 indicate a substantial level of uncertainty in how population size may have changed over time, although a general trend for logistic growth is clear. A Bayes Factor test comparing our BSP model with a constant population size model gave a Bayes factor of 6.164, indicating strong support for increasing population size over time.

Ancestral Distribution

The reconstructed ancestral distribution of haplotypes is shown in Figure 5 with only the probability of the location reconstruction displayed for those branches with a probability below 0.99. The analysis supports a migration from further northeast moving southwest into South Australia. It suggests that there have been multiple dispersals between Victoria and Queensland but one strongly supported dispersal event between the New South Wales and Victorian populations. There is very low support for the ancestral distribution on all branches preceding the Queensland and New South Wales' clades, so we cannot infer directionality of dispersal between these clades and Victoria.

Discussion

Geographic Structure

Our haplotype phylogeny, haplotype network and AMOVA analyses suggest geographic structure in haplotypes between the four sample sites. The NTH1 clade consisting of specimens from New South Wales and Queensland forms a sister clade to all other lineages (Figure 2), separated by a minimum of nine base pair mutations (Figure 3). Clade STH1 is restricted to the more southwestern populations of South Australia and Mildura. It is interesting that the South Australian population comprises only a single haplotype. It seems unlikely that a population bottleneck would remove all but one matriline in the population, however without further gene regions we cannot rule out this possibility. Another explanation is that the population has not been in place long enough for new haplotypes to arise and/or that there has been insufficient maternal gene flow from northern populations to promote haplotype diversity above that from a small founder population. The haplotype data are indicative of a southwestwards population expansion.

Interestingly, the second-most common haplotype in our sequences was found in both the Queensland and Victorian samples, and it has given rise to further haplotypes in both regions and NSW. Our BSP phylogeny (Figure 4) suggests that these haplotypes arose recently, and this might indicate that vagility in *Ceratina australensis* has not been sufficient to completely erode geographical assortment of matrilines.

Historical Demography

Our BEAST traits analysis also supports a more northeastern origin with a subsequent introduction into South Australia (Figure 5). The analysis is not able to discern between New South Wales, Queensland and Victoria as the likely origin of *Ceratina australensis* into Australia, but given that the subgenus *C.* (*Neoceratina*) is a primarily Oriental and Indo-Malayan clade (Rehan and Schwarz 2015), an origin in Queensland seems most likely. Pairwise comparisons indicate that the New South Wales and Queensland populations are not genetically distinct (Table 2). Interestingly the New South Wales population is about equidistant from both the Queensland and Victorian sites, however the Victorian population shows significant genetic distinction from New South Wales. The difference in gene flow between these populations may be due to fragmentation of suitable habitat for *C. australensis* in the semi-arid to arid zone separating the New South Wales and Victorian populations (Figure 1). The traits analysis indicates that multiple dispersals of matrilines between the Queensland-New South Wales populations and Victoria have occurred but the direction of movement between populations could not be resolved (Figure 5).

Regardless of when and where *Ceratina australensis* entered Australia, our BSP analyses provide strong support for an increase in effective population size beginning about 2.5×10^{-3} mutations/site ago then plateauing about 0.8×10^{-3} mutations/site ago. Assuming a mutation rate of 6.2×10^{-8} and two generations per year (Shell and Rehan 2016), these values correspond approximately to 20 kya and 6.5 kya respectively.

Our timescale indicates an increase in effective population size approximately 20 kya. This increase could be linked to reduced competition, expansion into a new niche or increased

resource availability. To investigate these possibilities we need detailed historical reconstructions, which are not presently available for Australia. Climate reconstructions from 20 kya are available for the southern hemisphere (Pahnke et al. 2003). Climate change may act directly on species, or indirectly, for example by increasing flowering plants and therefore resource availability. In Figure 4 we have contrasted the BSP curve with two climate proxies (δ^{18} O isotopes and estimated sea surface temperatures, SST) for the southern hemisphere reported by Pahnke et al. (2003). These graphs suggest that the major period of increasing N_e for *Ceratina australensis* coincides with a major period of post-glacial warming, and that the more recent leveling off in N_e could correspond to a plateauing or slight decline in temperature since 6 kya.

Unfortunately, there are very few detailed studies of paleoclimates in Australia beyond a very small number of sites, limiting further analyses. In one of the most thorough studies, Ayliffe et al. (1998) reconstructed climate in the Naracoorte region, in the South East of Australia, over the past 500,000 years. This geographical location, however, is well removed from our study sites. Reconstructions of Australia-wide paleoclimates are summarized by Byrne et al. (2008), and while this provides evidence for very broad changes in Australian climates, those studies do not permit reconstruction of paleoclimates in a way that permit refugial areas for *Ceratina australensis* during the last glacial maximum (LGM) to be identified with confidence. However, it seems likely that during the LGM, climatic regimes that now occur in southern Queensland would have had a more northerly distribution.

The inferred increase in N_e for *Ceratina australensis* coincides closely with the timing of dramatic increases in population size for three independent halictine bee clades in Vanuatu,

Fiji and Samoa, each of which corresponded to interglacial warming (Groom et al. 2013, 2014a). It is difficult to imagine a factor other than global climate that would be able to influence isolated bee populations in a similar way across the southwestern Pacific (SWP) and Australia, especially when it is considered that *C. australensis* is in a different family to the SWP halictine bees and has very different nesting and floral-adaptation biologies to halictine bees (stem nesting versus ground nesting, and long-tongued versus short-tongued, respectively). On the other hand, we did not find evidence for a dramatic decline in N_e of *C. australensis* at the LGM, and this contrasts strongly with studies on SWP halictine bees (Groom et al. 2014a). It is possible that this contrast is due to *C. australensis* occurring on a continent, where it may have been able to persist in a wide range of refugial habitats, which would have not been as abundant for bee species restricted to small islands.

The expansion of population sizes in *Ceratina australensis* in the current interglacial is consistent with expectations for a Mediterranean or subtropical adapted species responding to warming climates in the southern hemisphere, where southern latitudes retreated from glacial conditions experienced at the LGM. This is also concordant with our historical biogeography analyses, which suggests a northeastern origin, followed by accumulation of haplotype diversity in the semi-arid population in northern Victoria and a recent dispersal to South Australia, indicated by the lack of haplotype diversity and BEAST ancestral traits reconstruction.

Conclusion

Our results suggest that *Ceratina australensis* has responded in major ways to climatic changes since the LGM, but there are two important questions that need resolution. Firstly, because bees are pollinators, historical changes in their diversity and abundance are likely to have impacted angiosperm reproduction in the past, and this may help understand current angiosperm communities. Secondly, if past climates have had large impacts on bee populations in the past, it is important to understand these so that we can anticipate the effects of future climate change. We can only interrogate museum records for impacts of climate change to very limited extents: for Australian insects this will be mostly limited to the last 200 years. In contrast, genetic methods can be used to examine changes well before the recent past and for species that were not covered by early collectors. Our results suggest that genetic approaches to historical demographics of native bees may hold important insights for understanding how climate change has impacted pollinating biota and plant-pollinator relationships.

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Table 1. Summary of samples collected from each population of *C. australensis*. Includes GPS coordinates, collection dates, total number of specimens sequenced and the number of unique haplotypes recovered.

Population	Latitude (S) / Longitude (E)	Collection Dates	Specimens Barcoded	Haplotypes
Cowra, New South Wales	33° 52.78’ / 148° 45.73'	October 2015	11	8
Mildura, Victoria	34° 09.25’ / 142° 09.58’	June 2013, October 2013, January 2014, April 2014	42	9
Warwick, Queensland	28° 12.85’ / 152° 02.10’	January 2010	30	14
West Beach, South Australia	34° 56.28’ / 138° 29.95’	June 2012, July 2014	19	1

Table 2. *Ceratina australensis* regional population structure. Diagonal indicates average pairwise differences within populations, number in parentheses indicates total number of sequences for that region; above diagonal are average pairwise differences between populations; below diagonal are pairwise F_{ST} values. Significant values ($p < 0.05$) indicated in bold.

Population structure	Queensland	New South Wales	Victoria	South Australia
Queensland	6.88736 (30)	6.5303 (0.49)	**6.93968 (<0.0001)**	**6.5 (<0.0001)**
New South Wales	0.0598 (0.19)	5.49091 (11)	**5.03247 (<0.0001)**	**5.09091 (<0.0001)**
Victoria	**0.35579 (<0.0001)**	**0.26487 (<0.0001)**	2.5331 (42)	**3.78571 (<0.0001)**
South Australia	**0.42823 (< 0.0001)**	**0.55586 (<0.0001)**	**0.58507 (<0.0001)**	0 (19)

Table 3. Tajima's D and Fu's F_S tests of neutrality within populations. Segregating sites (S), Tajima's D score and significance value (D p-value), and Fu's F_S value and significance values (F_S p-value) are presented. Values in bold are statistically significant ($p < 0.05$).

Neutrality tests	Queensland	New South Wales	Victoria	South Australia
S	19	16	16	0
Tajima's D	1.36657	0.02304	-1.01646	0
D p-value	0.937	0.558	0.186	1.000
Fu's F_S	**-25.15767**	**-6.57395**	**-26.633**	3.4×10^{38}
F_S p-value	**0**	**0.001**	**0**	1

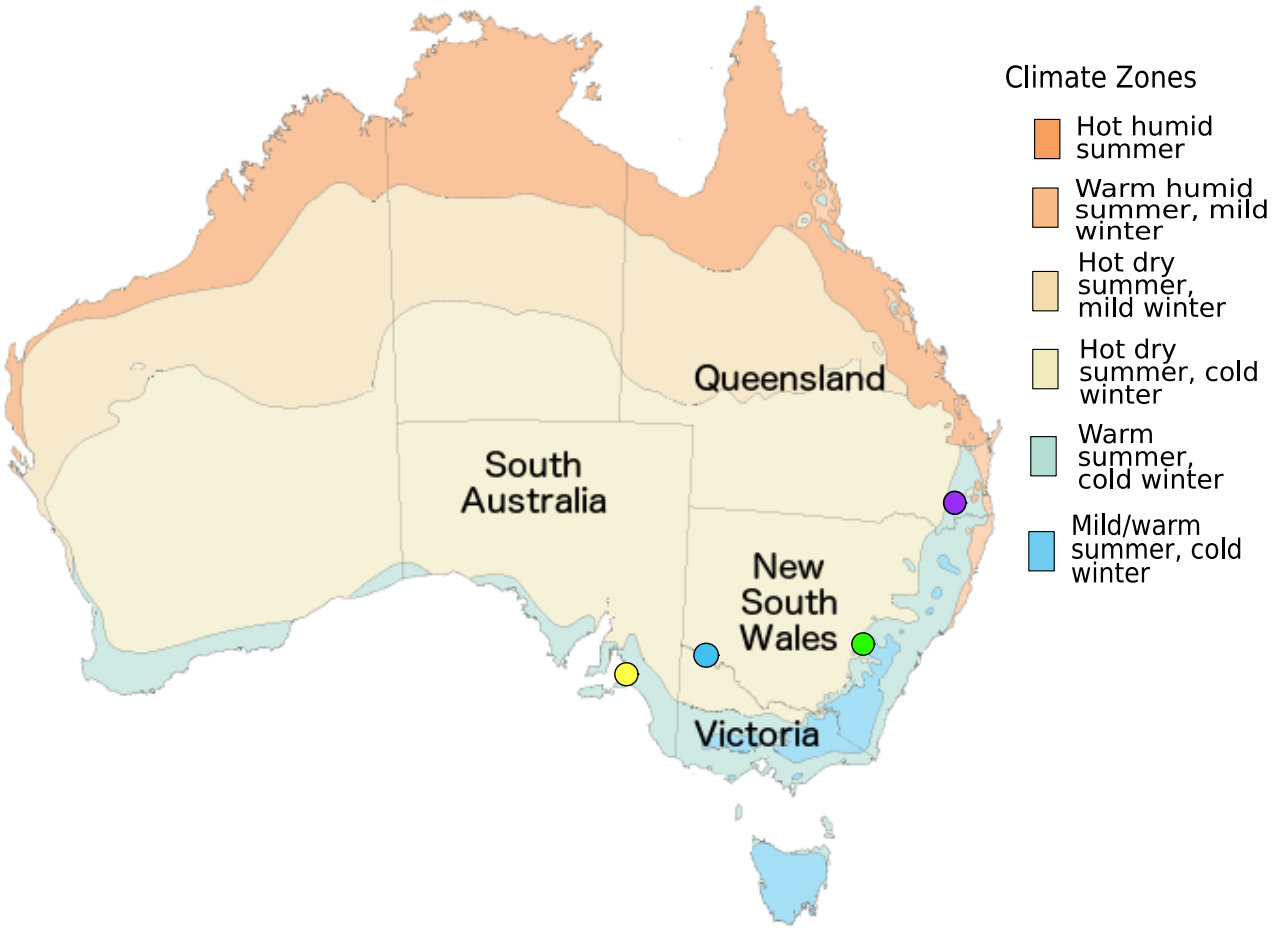


Figure 1. Map of Australia with overlaid temperature and humidity climate zones (Bureau of Meteorology 2012) showing sampling locations. New South Wales, green; Queensland purple; Victoria, blue; South Australia, yellow.

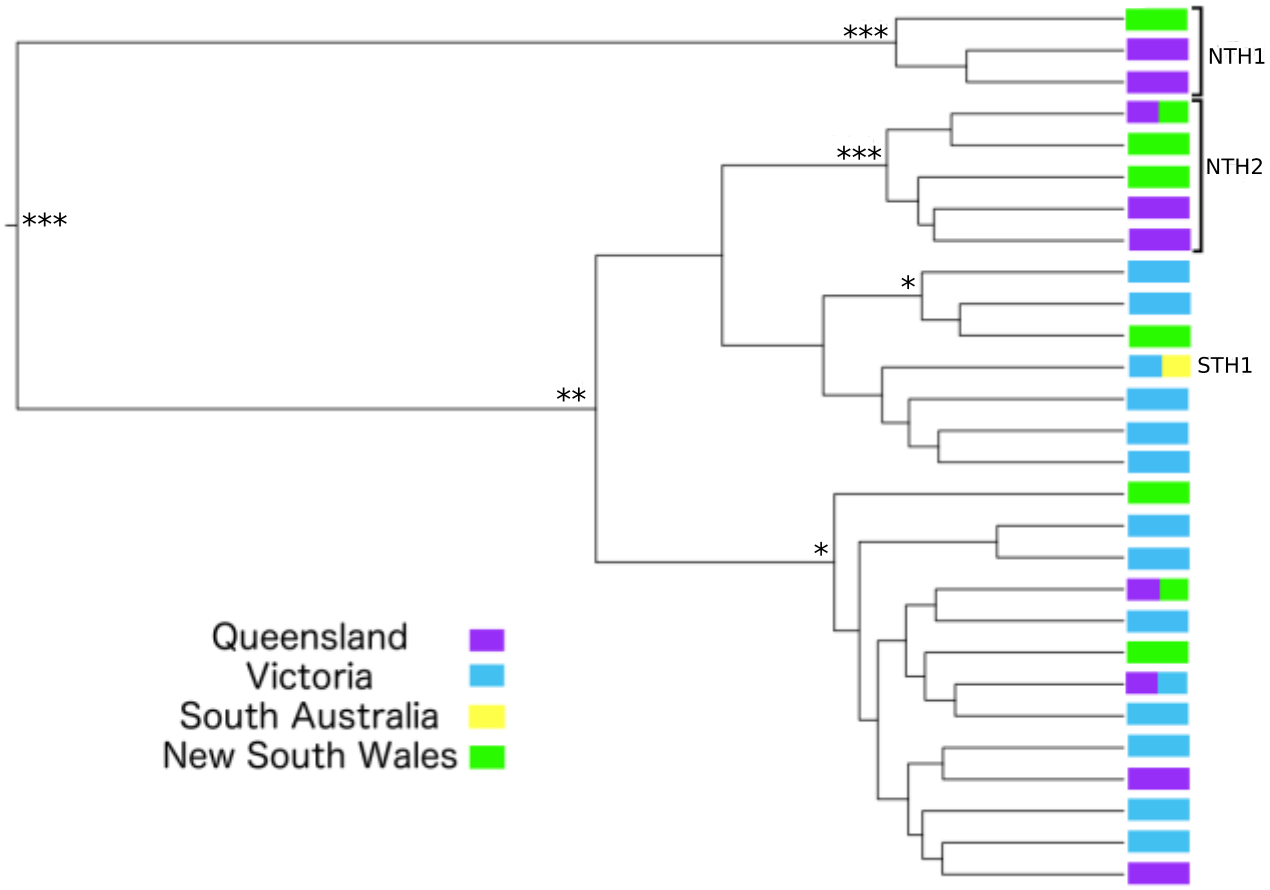


Figure 2. Maximum credibility tree of the *C. australensis* haplotypes from Bayesian BEAST analysis. Clades North 1 (NTH1), North 2 (NTH2) and South 1 (STH1) are indicated. Posterior probability values: *** = 1.0; ** ≥ 95; * ≥ 85.

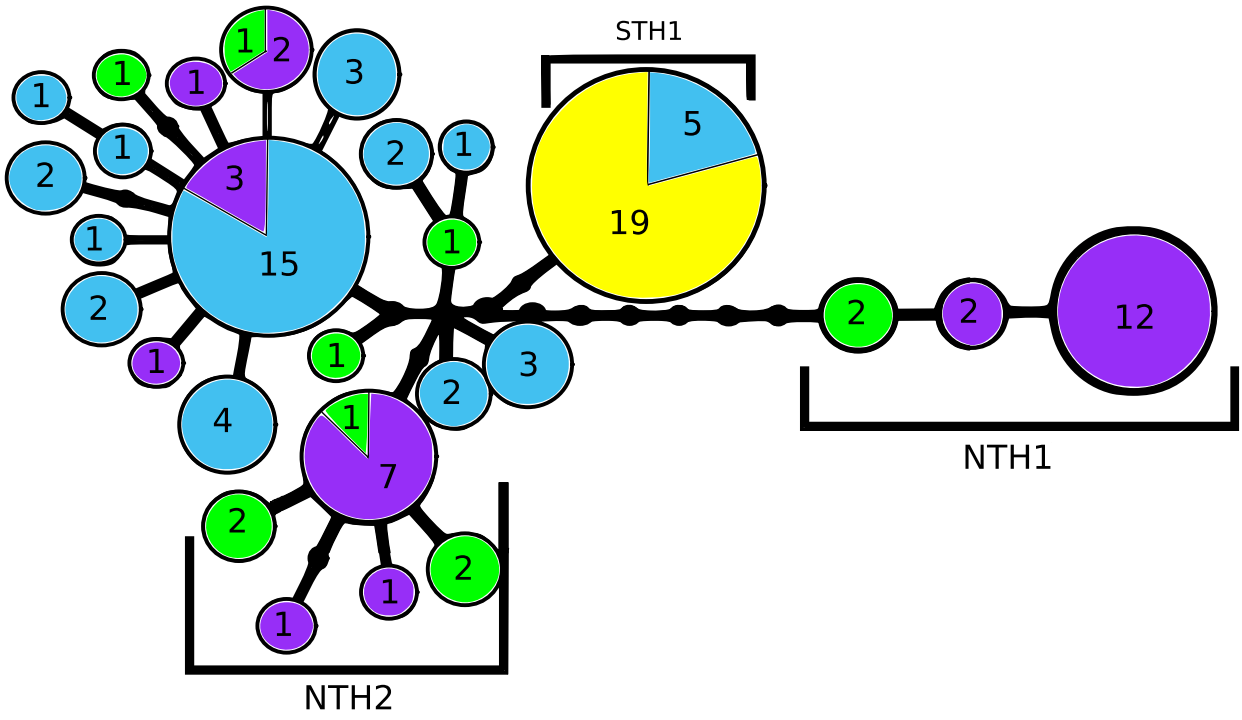


Figure 3. Haplotype network of *C. australensis* populations. Each circle represents a unique haplotype, with the numerals inside indicating the number of individuals sampled of that haplotype. Each step between haplotypes indicates one base pair substitution.

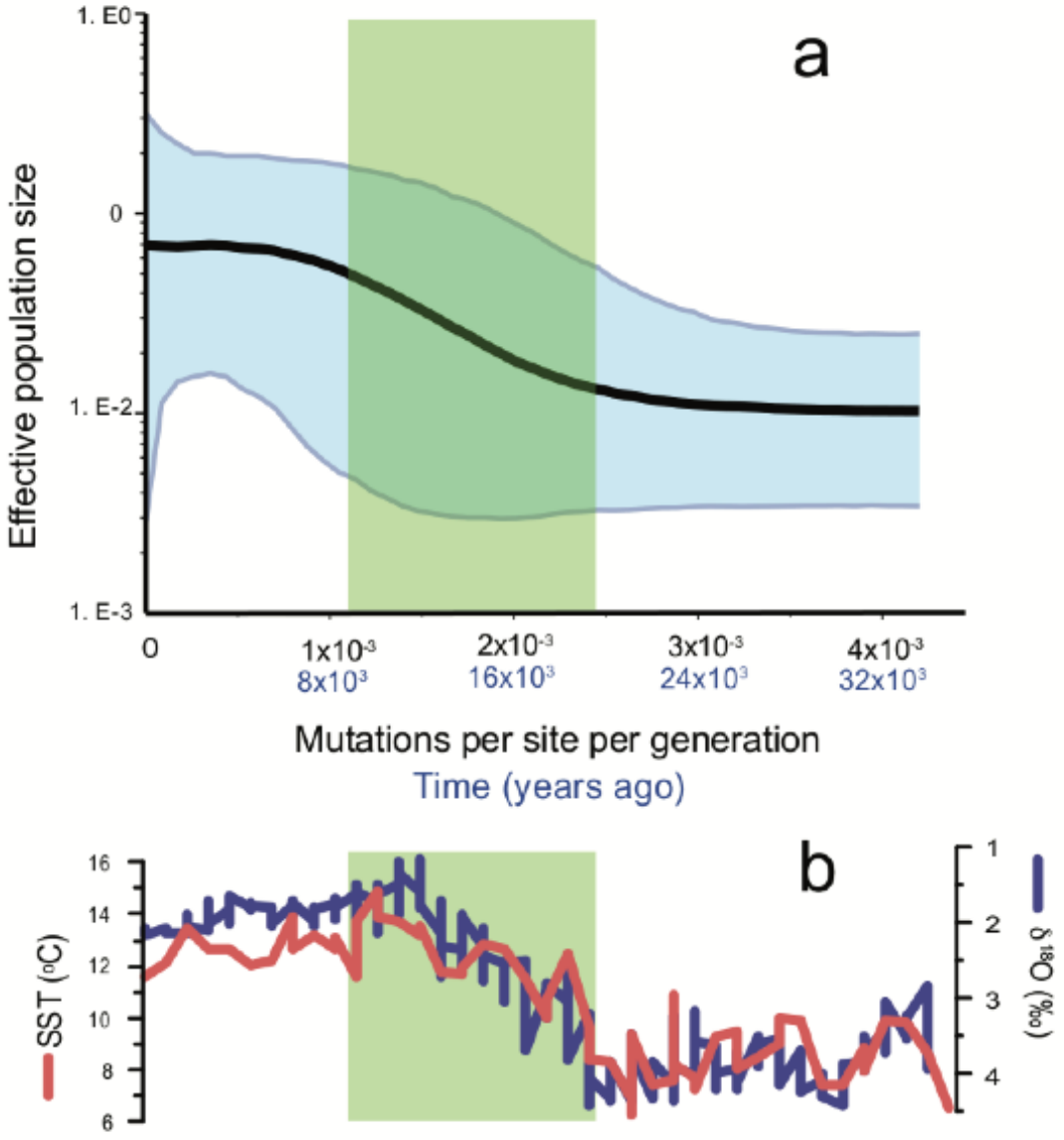


Figure 4. Bayesian skyline plot (a), and (b) graphs of two proxies for historical climate in the southern hemisphere (adapted from Pahnke et al. 2003). These proxies are $\delta^{18}O$ ‰ and sea surface temperate (SST) based on Mg/Ca ratios. The boxes indicate the approximate period of elevated haplotype accumulation.

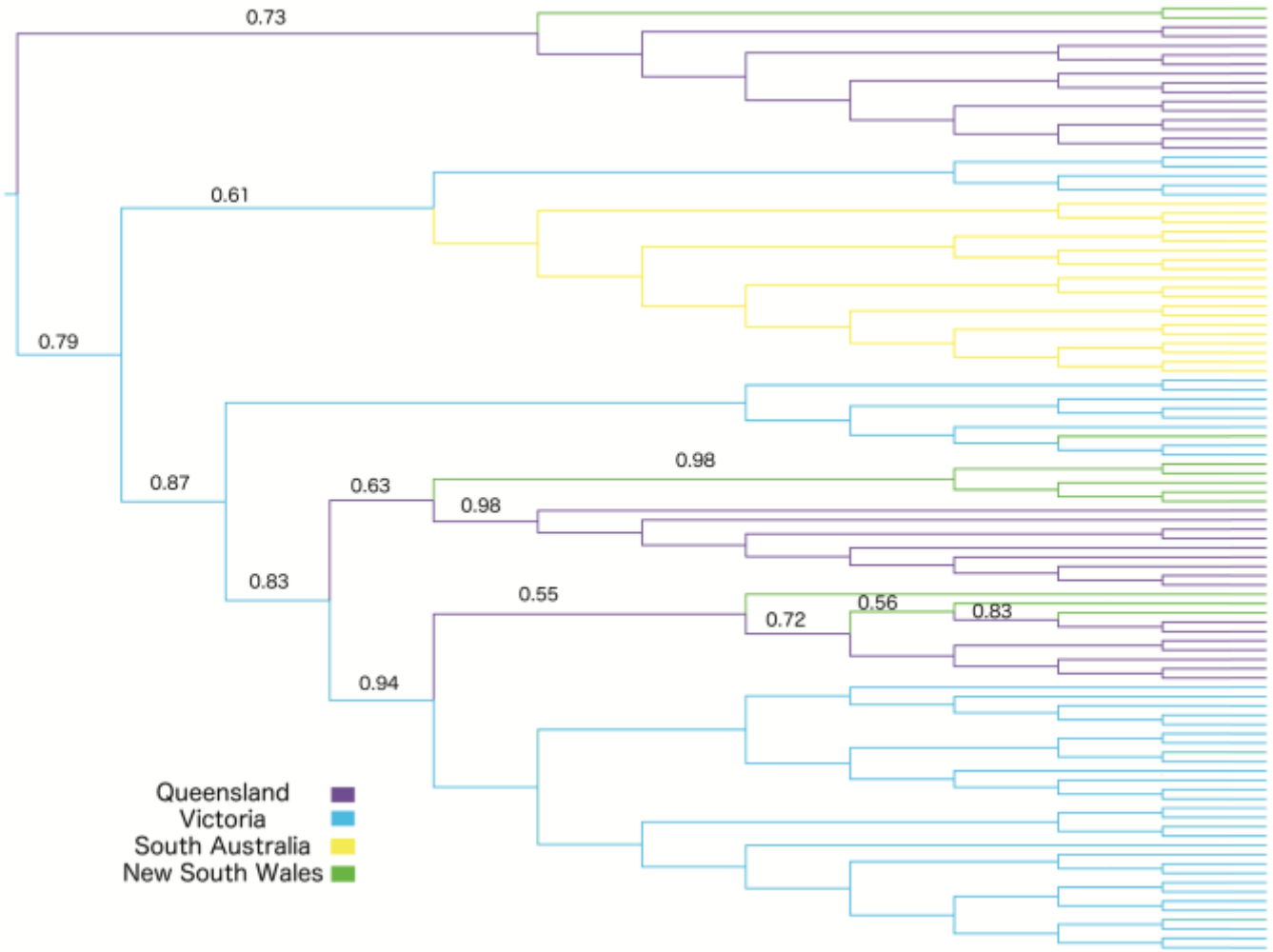
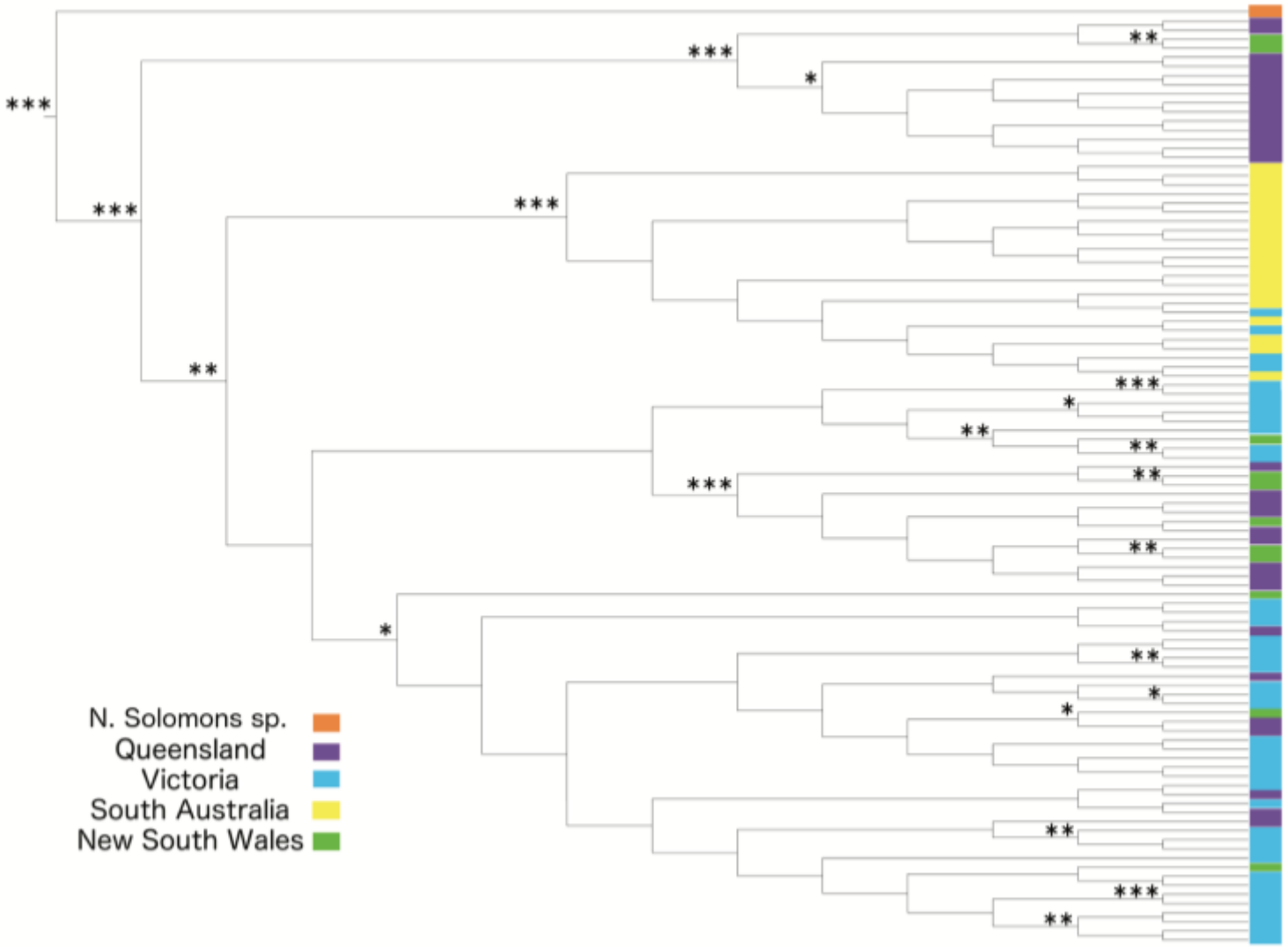


Figure 5. Cladogram showing inferred ancestral ranges of haplotypes from a BEAST traits analysis. Posterior probabilities of location reconstruction are shown only on those branches with support less than 0.99.



Supplementary Figure 1: Cladogram including *Ceratina (Neoceratina)* Solomons sp. as the outgroup to root the tree. Posterior probability values: *** = 1.0; ** ≥ 95; * ≥ 85.

CHAPTER V

Mixed responses of arid-adapted bees to the Last Glacial Maximum: The role of behaviour in responses to climate change

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ABSTRACT

Aim One approach for assessing how species will respond to future climate change is to look at responses to past climate change events. Bees from tropical, subtropical and temperate regions have shown rapid population expansions following the Last Glacial Maximum (LGM). The LGM was a time of colder climates and greater aridification than the present, so taxa adapted to arid climates may have shown different population level responses than species from tropical and temperate habitats. Here we compare the responses of two arid zone species of allodapine bees, *Exoneurella tridentata* and *E. setosa*, and explore differences in these responses.

Location Sampling locations across Australia covering the range of both species.

Methods We sequenced 658bp of the mitochondrial gene cytochrome *c* oxidase subunit 1 for each specimen of the two species. These data were used in haplotype network analyses and Bayesian Skyline Plot analyses.

Results We found that both species show clear geographical genetic structure with low matriline dispersal, possibly due to habitat fragmentation and/or natal philopatry. Bayesian skyline analyses revealed that *E. tridentata* underwent a population expansion likely after the LGM but *E. setosa* has had a highly stable population across the last 100-200ky.

Main Conclusions These results demonstrate that bees can display very different responses to past global warming events. These differences suggest that adaptation to particular climatic conditions is not the only determinant of response to climate change; rather we argue that responses are complex and may also be influenced by differences in social behaviour. Species with more behavioural flexibility may have greater long-term population stability, particularly in light of future climate warming.

Keywords

Allodapine, Biogeography, Ceratinine, Coalescent Analyses, Effective Population Size, Global Warming, Historical Demography, Social Behaviour

INTRODUCTION

Insect-pollinator species around the world are currently declining due to human activities (Potts et al. 2010; Vanbergen et al. 2013). Bees are a major insect pollinator, including for many crop species (Klein et al. 2007). In the face of future rapid climate change it is important to know how pollinators such as bees will respond. Studies using museum collections from the last 50-200 years show that climate change has already resulted in changes to some pollinator distributions, though responses are varied between taxa (Bedford et al .2012; Burkle et al. 2013; Aguirre-Gutiérrez et al. 2016). A study on pollinator species in the Netherlands found that most of the bee species studied had experienced northern range shifts in response to climate change (Aguirre-Gutiérrez et al. 2016), while bumblebees across the northern hemisphere have not shifted their range and are likely to face population declines as a result (Cameron et al. 2011; Kerr et al. 2015). Alterations in angiosperm communities due to changes in contemporary climates have also initiated changes in morphology and social behaviour in different bee species (Miller-Struttmann et al. 2015; Schürch et al. 2016).

Another approach to determining the future impacts of climate change is to look at the influences of past climate change events as inferred from genetic analyses. At the end of the Last Glacial Maximum (LGM), approximately 19kya, there was a significant period of global warming (Clark et al. 2009). Using genetic data, coalescent methods have identified post-LGM population expansions in tropical south west pacific halictine bees (Halictidae: Halictini; Groom et al. 2013, 2014), small carpenter bees *Ceratina* (*Zandontomerus)* in temperate North America (Apidae: Ceratinini; Shell and Rehan 2016), and the Small

Australian Carpenter bee, *Ceratina (Neoceratina) australensis*, which ranges across subtropical, semi-arid and temperate zones (Dew et al. 2016). Various species of orchid bees (Apidae: Euglossini) from equatorial America showed signatures of population expansion during the Pleistocene, with many species likely experiencing severe reductions in niche breadth during the LGM, up to 30-40% in some species (Lopez-Uribe et al. 2014). Similarly, the tropical halictine bee populations decreased during the LGM followed by marked increases, which could reflect restriction of populations in LGM refugia (Groom et al. 2014).

Different climatic adaptations and the presence of refugia may affect the ability of species to respond to climate changes. Arid zones experience minimal precipitation and cover about two thirds of Australia (Martin 2006). The LGM in Australia was a period of extreme aridification (Hesse et al. 2004; Martin 2006). Heightened aridity during the LGM may have had very different effects on arid adapted taxa compared to taxa adapted to other climate zones. Of the bee species previously used to examine historical demography, the only one found in arid areas is *Ceratina australensis,* which has semi-arid populations, but also ranges into subtropical and temperate zones (Dew et al. 2016).

Strict arid zone taxa are known in the bee genus *Exoneurella* (Apidae: Allodapini). *Exoneurella tridentata* and *E. eremophila* are restricted to arid and semi-arid zones, with populations across the arid interior of Australia. The range of *E. tridentata* is further limited by its reliance on two arid to semi-arid hardwood trees, *Acacia papyrocarpa* and *Alectryon oleifolium*, for nesting sites (Hurst 2001). But not all *Exoneurella* are exclusively arid zone species. *Exoneurella setosa* is largely sympatric with the previously studied *Ceratina*

australensis, having populations in riparian semi-arid areas of southeast Australia but also temperate inland and coastal regions and extending to subtropical woodlands.

Here we detail the population genetics and historical demography of *E. tridentata* and *E. setosa*. We take a comparative approach, exploring if adaptations to arid and semi-arid zones may have influenced historical responses to past climate change by comparing the strictly semi-arid to arid zone *E. tridentata* to the wide-ranging *E. setosa*. Our findings are discussed in context to previous work on *C. australensis* and we consider the role of social behaviour in responses to climate change.

METHODS

Sample collection

Samples of *E. tridentata* and *E. setosa* were collected from multiple localities across Australia from November 2012 to November 2015. *Exoneurella tridentata* was sampled from six localities covering the range of this species, from central western Australia to central south-eastern Australia (Table 1). Populations of *E. setosa* are found in diverse climates including subtropical, temperate and semi-arid regions and our seven collections spanned this diversity, ranging from subtropical north-eastern Australia, to the semi-arid central south-east and temperate western Australia (Table 2).

Samples were collected either by sweep netting flowers or whole-nest collection. Sweep-netted samples were placed immediately into 100% ethanol. Nests were stored on ice in a cool box for transport to Flinders University where they were processed. Only one random adult individual from each nest was used for DNA sequencing to avoid relatedness effects.

DNA barcoding

The mitochondrial DNA cytochrome *c* oxidase subunit I (COI) gene, also widely used as a DNA barcoding region, was sequenced for each sample. DNA extraction and PCR amplification was performed at the South Australian Research Facility for Molecular Ecology and Evolution (SARFMEE). One leg of each sample was used for DNA extraction following a glass microfiber vacuum plate method modified from Ivanova et al. (2006). Amplification of

the extract was performed with 25µl PCR reactions containing 10v/v% DNA extract, 20v/v% MRT buffer (constituted of 1x immolase buffer, 0.8mM dNTP, 0.05mg/ml bovine serum albumin), 4v/v% of each primer and 0.4v/v% Immolase DNA polymerase (Bioline) as the active enzyme. The forward primer combines M13/pUC (Messing 1988) and LC01490 (Folmer et al. 1994; 5'-GT TTT CCC AGT CAC GAC CCT TTT ATA ATT GGA GGA TTT GG-3'). The reverse primer comprises the reverse M13/pUC with primer M399 (Schwarz et al. 2004; 5'-CA GGA AAC AGC TAT GAC TCA TCT AAA AAC TTT AAT TCC TG-3'). The PCR reaction ran for an initial 10min at 94°C, followed by 38 cycles of 60s at 94°C, 90s at 50°C and 60s at 72°C. The elongation stage was run for 10min at 72°C and the reaction was terminated with 2min at 25°C.

Products of the PCR reaction were checked with gel electrophoresis, indicating bands of approximately 700bp. The products were washed with 100µl TLE (10mM TRIS, 0.1mM EDTA pH8) on a vacuum filter, and collected off the filter in an additional 30µl TLE. Sequencing of both forward and reverse primers was performed at the Australian Genome Research Facility (AGRF).

Sequence alignment

Consensus sequences were made from forward and reverse reads and these were edited and aligned in GENEIOUS v6.1.6 (Kearse et al. 2012). In order to get meaningful interpretations of haplotype networks and coalescent analyses (e.g. Joly et al. 2007; Grant 2015), it is important that there are no base ambiguities within the DNA alignment. In order to achieve this some samples were removed from the final alignments for each species, as

they had unresolvable ambiguities. Some samples were also trimmed to remove ambiguous sections. Our final alignments contained 114 samples with 597bp sequences for *E. setosa* and 48 samples with 589bp sequences for *E. tridentata*.

Allelic Diversity

Due to the difference in sample sizes for each species, allelic diversity was compared by sample-based rarefaction curves calculated in ESTIMATES (Longino and Colwell 2011; Colwell et al. 2012). Allelic diversity of *E. setosa* from the sampled n=114 individuals was compared to the rarified allelic diversity of *E. tridentata* from a calculated 114 individuals. Further unrarified diversity indices (Fisher's Alpha, Shannon's exponential and Simpson's reciprocal) were compared at n=48 individuals of *E. tridentata* and a randomized selection of 48 *E. setosa* individuals. To obtain an estimate of 'species-wide' allelic richness from our samples we applied the Incidence-Based Coverage Estimator (ICE; Lee and Chao 1994).

Population genetic structure

Minimum spanning trees were generated in NETWORK v.5.0 (Fluxus Engineering 2016). A median-joining analysis was implemented with a 'connection cost' criterion and episilon set at zero (Bendelt et al. 1999). Genetic population structure was calculated in ARLEQUIN v.3.5 (Excoffier et al. 2010). Pairwise differences and pairwise F_{ST} values were calculated within and between populations, with 16,000 permutations. Tajima's D and Fu's Fs were calculated to detect departures from neutral evolution.

Phylogenetic Reconstruction and Historical Demography

To reconstruct historical effective population sizes we used BEAST v.1.8.3 to implement Bayesian Skyline Plot (BSP) analyses (Drummond and Rambaut 2007). The low number of amino acids differences between our samples indicates that purifying selection may be acting on the 1st and 2nd codon positions. BSP analyses assume that selection is neutral (Grant et al. 2015), so we limited our dataset to just the 3rd codon positions (Ho and Shapiro 2011; Dew et al. 2016). All samples, including duplicate haplotypes were included in the analyses (Joly et al. 2007; Grant 2015). Possible nucleotide substitution models were assessed using JMODELTEST via Bayesian Information Criterion (BIC) and indicated HKY+Γ (Posada 2008) as the best-fit model for both species. We applied a strict clock, with mutation rate set to 1.0 and a Bayesian skyline tree model. Setting the clock rate to 1.0 makes it simpler to convert branch lengths into chronological years for varying assumed mutation rates. The analysis was run for 100 million generations, with sample parameters logged every 1,000th iteration. Run stabilization was checked in Tracer v1.5 and the entire analysis was repeated three times to confirm convergence.

Lineage through time plots (LTT) were also generated for each species in BEAST v.1.8.3 to visualise haplotype diversification (Drummond and Rambaut 2007). Duplicate haplotypes were pruned from the alignments before analysis and all codon positions were included. An HKY+Γ nucleotide substitution model was used based on BIC comparison of models in JMODELTEST (Posada 2008). The analysis was run with a Yule process tree model, strict clock

(mutation rate 1.0) and all three codon positions separately partitioned. In total these analyses were run three times for both species, each run proceeding for 50 million iterations, with samples taken every 1,000th generation.

The x-axis of the BSP and LTT plots were converted into estimates of time by assuming a mitochondrial mutation rate and the number of generations per year. In the absence of an estimate of mitochondrial mutation rate for bees we used the mitochondrial mutation rate of *Drosophila melanogaster* Meigen as the best available estimate (6.2 x 10^{-8} mutations per site per generation; Haag-Liautard et al. 2008), which is similar to that of the nematode *Caenorhabditis elegans* Maupas at 9.7 x 10^{-8} mutations per site per generation (Denver et al. 2000). Importantly, these species have mitochondrial AT biases of 82% and 76% respectively, which closely correspond to both *E. tridentata* (76%) and *E. setosa* (75%). The use of *D. melanogaster*'s mutation rate follows previous studies on the historical demography of bees (Groom et al. 2013; Dew et al. 2016; Shell and Rehan 2016). *Exoneurella setosa* has two generations per year in semi-arid regions of central southeast Australia and one in coastal southern Australia (Neville et al. 1998; Dew et al. *in review*). Accurately representing generation time for *E. tridentata* is difficult due to the high level of reproductive skew in colonies. While queens produce brood year-round most of these are non-reproductive workers. The exact rate and timing of queen-destined brood production is unknown but these are uncommon in nests, new nest initiation is rare, and queens are very long lived (potentially up to 10 years – Hurst 2001), indicating that generation times for queens are likely to be greater than 1 year. Date estimates based on one generation per year to one generation every three years are presented on Fig. 5.

RESULTS

From the 48 *E. tridentata* specimens sequenced, 12 haplotypes were identified, while the 114 sequenced *E. setosa* specimens yielded 19 haplotypes. Sample-based rarefaction curves indicated that allelic diversity was equivalent for *E. tridentata* and *E. setosa*, though a greater mean ICE for *E. tridentata* may indicate that it has greater species-wide allelic richness (Fig. 2). Diversity indices were comparable between species (N = 48; Table 3).

Haplotype networks and gene flow

The minimum spanning tree for *E. tridentata* shows an east to west division in haplotypes (Fig. 3). Population central west 1 near Yellowdine was the most westerly *E. tridentata* sample location and was genetically distinct from the south central 1 and 2 and central south east samples sites in the Gawler Ranges, Lake Gilles and Danggali respectively (Table 1). All sample sites except the south central sites at Lake Gilles and the Gawler Ranges contained some unique haplotypes. The three specimens from the Gawler Ranges (south central 2) had a single haplotype that was also common to specimens from Lake Gilles (south central 1). This was reflected in the pairwise comparisons, which indicated no significant genetic divergence between these two sites (Table 4). The central southeast site at Danggali was the most easterly site sampled and was genetically distinct from the south central site at Lake Gilles. The west central sites were separated from each other by a relatively high number of bp changes (4-11bp). This is a large amount of variation given the relative geographic proximity of the western sample sites but no significant genetic

differentiation was found between the west central sites or with the south central and central southeast sites (Table 4).

All populations of *E. setosa* were genetically distinct. The lowest F_{ST} values were between the northeast site in Warwick and central southeast site near Mildura (F_{ST} = 0.24, P < 0.001; Table 1) and between the northeast site and southeast site near Cowra (F_{ST} = 0.18, P < 0.001). Each of these pairs of populations shared a common haplotype (Fig. 4). Notably there is a group of haplotypes from Mildura in the central-southeast and Warwick in the north-east that is widely separated from all other haplotypes, including those from the specimens collected at the same sample sites.

Tests for signatures of departure from neutral evolution within populations found that *E. tridentata* at the west central 1 site had less variation than expected from neutral evolution (Tajima's D = -1.51, P = 0.044; Fu's F_S= -1.92, P = 0.0090). All other populations were indicated as having neutral evolution (D tests, P $\geq$ 0.08, *Fs* tests, P $\geq$ 0.057). Tajima's D and Fu's F_S indicated no significant departures from neutral evolution in any population of *E. setosa* (D tests, P $\geq$ 0.12, *Fs* tests, P $\geq$ 0.18).

Historical Demography

Dates on the Bayesian Skyline Plot (BSP) and Lineage Through Time (LTT) plots reflect calculations based on 1-2 generations per year for *E. setosa* and one generation per year to one generation every three years in *E. tridentata*. The LTT for *E. setosa* indicates a steady increase in lineages across the last 40-81 kya, demonstrating a gradual increase in genetic

diversity (Fig. 5(a)). The BSP displays no evidence of population size changes, but the 95% confidence intervals broaden greatly as they approach the present (Fig. 5(b)). For *E. tridentata* the LTT plot showed very slight lineage accumulation over the past 28-86k years (Fig. 5(c)). This is far less than that seen in *E. setosa,* even if only the last 28 kya are considered. Population sizes for *E. tridentata* show a slight to almost negligible increase, starting between 24 - 73 kya (Fig. 5(d)).

DISCUSSION

Our study revealed different historical patterns in relative effective population size (N_e) between *Exoneurella tridentata* and *E. setosa*. A gradual increase in N_e occurred in *E. tridentata*, starting at ~24-73kya (Fig. 5(d)). In comparison, *E. setosa* seemed to have maintained a relatively stable N_e across the last 100-200 ky (Fig. 5(b)). This stability is surprising given the extreme climatic events that have occurred across that timescale (Hesse et al. 2004; Martin 2006; Byrne et al. 2008), and contrasts to the trend of increasing population size reported for most other bee species studied to date (Dew et al. 2016; Shell and Rehan 2016; Groom et al. 2013, 2014; Lopez-Uribe et al. 2014). The relative stability of historical populations of *E. setosa* compared to *E. tridentata* may relate to its broader geographic distribution and its relatively abundant and diverse nesting substrates.

Exoneurella tridentata and *E. setosa* both demonstrated gradual haplotype diversification corresponding to a birth-death model without large extinctions (Fig. 5(a,c); Nee et al. 1994; Morlon 2014). Haplotype accumulation appeared much lower in *E. tridentata*, as would be expected for a species with longer generation times and more limited nest-sites. N_e is proportional to the diversification of non-neutral mutations (Charlesworth 2009), but neutral mutations can be spread to fixation via genetic sweeps (Bazin et al. 2006). This complicates comparisons between species, meaning that species with a larger N_e may show a larger apparent diversification rate due to fixation of neutral markers (Kimura and Ohta 1971; Galtier et al. 2009). Therefore, the greater comparative diversification of *E. setosa* could be an artefact of greater N_e, and consequently fixation of more neutral mutations via genetic sweeps. This issue has not been addressed in previous studies on bees.

Both *E. tridentata* and *E. setosa* had comparable species-wide allelic diversity and limited maternal gene flow (Fig. 2; Table 3). All populations of *E. setosa* were indicated by F_{ST} values to be genetically distinct but the shared haplotypes between the eastern sites shows that gene flow has occurred between these areas (Table 4). Within the minimum spanning tree there are two divergent groups of eastern site haplotypes, separated by more than 10 base pair mutations (Fig. 4). These two distinct eastern groups are suggestive of two separate matriline dispersals. *Exoneurella tridentata* showed an east to west separation between haplotypes. The most western site (west central 1, Fig. 1) is genetically distinct from all other populations based on F_{ST} values, except for its close neighbour west central 3. Sample sizes for the west central 2 and 3 sites were very low (N = 2 and 1 specimens respectively). While F_{ST} values gave no support for genetic distinction between these sites or with the southern and eastern sites, sample sizes are not adequate to draw conclusions from, especially given the large number of base pair changes between these limited samples (Fig. 3). All three of the south central 1 samples were of a single haplotype, shared with the south central 2 population, indicating strong geographical structure in maternally inherited genes. The Lake Gilles southcentral site showed a significant genetic distance from the most easterly site sampled, central southeast. This suggests that the east-west divergence continues from central to eastern Australia but the low sample size indicates that haplotype diversity may be undersampled at this site. The only population for either species to show a departure from neutral evolution was west central 1 with a negative Tajima D value, which could suggest a recent population expansion or selection removing diversity. These results indicate that in the present day, matriline gene flow across large distances is rare or sporadic, especially for *E. tridentata*. We discuss the implications of this in the face of

contemporary climatic changes and compare findings across the bees studied so far, discussing the role of behaviour with respect to species flexibility to respond to change.

Limitations on matriline dispersal

The east-west divergence observed for *E. tridentata* corresponds to dispersal limitations identified for various other Australian taxa. These include mammals, insects and birds (summarised in Neaves et al. 2012). These divergences have been frequently attributed to the Nullarbor Plain, which forms an extreme arid belt between western Australia and south-central regions (e.g. Toon et al. 2007, Salinas et al. 2009, Crisp and Cook 2007). *Exoneurella tridentata*'s distribution is limited by its reliance on *Acacia papyrocarpa* (Western Myall) and *Alectryon oleifolium* (Bullock Bush) for nesting sites. These plants are present in the Nullarbor Plain but occurrences are much sparser than central and eastern regions (Carroll et al. 2005; Gillieson et al. 1996; Lange and Purdie 1976; Atlas of Living Australia 2016). Within the Nullabor, *Acacia papyrocarpa* is recorded at highest density along the coast, and this may have been a refugial area during times of aridification (Byrne 2008). Bands of *Acacia* also extend north of the Nullabor through the southern Gibson desert in what is referred to as the 'Giles corridor' (Pianka 1972). Both the Nullarbor and Giles corridors have been proposed as dispersal routes for a number of lizard species (Chapple et al. 2004; Pianka 1972). These two vegetation corridors could modulate gene flow between western and south-central populations of *E. tridentata*.

The strong population structure of *E. setosa* is likely a result of natal philopatry rather than habitat patchiness. *Exoneurella setosa* nests in a wide and abundant variety of annual plants

from the genera *Senecio* (Asteracea), *Cakile* (Brassicaceae), *Ferula* (Apiaceae) and *Verbena bonariensis* L. (Verbenaceae) across a diverse range of climates. Having abundant nesting sites may increase the chances of successful long distance female dispersals, but F_{ST} values for *E. setosa* indicate that matrilines are highly population structured. The relative abundance of annually renewed nesting sites for *E. setosa* may mean that females are able to find suitable nest sites in close proximity to the natal nest, and therefore rarely disperse long distances. *Exoneurella setosa* is facultatively social, with social colonies found in up to 36% of nests in some areas (Dew et al. *in review*). While many females do disperse to found new nests, life history studies indicate that social colonies are most likely mother-daughter or sister associations formed by females remaining in the natal nest (Dew et al. *in review*, Neville et al. 1998). This natal philopatry of *E.* setosa may help explain why matrilines are largely geographically restricted for this species.

Role of behaviour in population genetics: past - present

The ceratinine bee *Ceratina* (*Neoceratina*) *australensis*, a member of the sister tribe to the allodapines, is largely sympatric with *E. setosa* and has a remarkably similar population structure (Dew et al. 2016). These two species co-exist in all southern to eastern sites sampled for this study and utilise the same plants for nesting substrates. Their haplotype networks are strikingly similar, both having a divergent eastern haplotype clade (Fig. 4; *c.f.* Dew et al. 2016). This could potentially represent similar dispersal events by both species, facilitated by a mediating factor such as an environmental change or weather events, or alternatively, be the signature of separate rare and sporadic dispersals. While *C. australensis* has strong genetic structure between most areas, the northeast Warwick site and southeast

Cowra site lacked strong genetic differentiation. This species is predominantly solitary and most females disperse from the natal nests, perhaps facilitating higher mitochondrial gene flow (Rehan et al. 2010). Over long periods of time slight differences in maternal gene flow could have large species-wide consequences.

Comparison of the historical demography of *C. australensis* to that of *E. setosa* shows that they have responded very differently to past climatic events; *C. australensis* undergoing a marked post-LGM population expansion (Dew et al. 2016), while *E. setosa* had no major fluctuations in population size in the last 100-200ky. These species share an environmental niche, so climate preadaptation and habitat loss does not seem to explain the large differences in response - but behaviour might. The remarkably stable population size of *E. setosa* may be explained by its facultatively social behaviour combined with progressive rearing, which might give it the flexibility to respond to environmental changes, such as decreased nesting sites or more variable food sources. Social living is advantageous from a brood survival perspective; nestmates can act as guards, assist with brood care, share foraging effort and act as insurance for brood survival if the mother dies (Kukuk et al. 1998; Bull and Schwarz 2001; Hogendoorn and Zammit 2001; Stevens et al. 2007; Lucas and Field 2011). Being casteless, females of *E. setosa* in social groups do not face losses of fitness due to unequal reproductive opportunities. Brood rearing behaviour is also different between these species. *Exoneurella setosa*, like other allodapines, progressively provisions the brood throughout their development (Schwarz et al. 2007). Progressive provisioning allows redistribution of resources as needed, for example, stopping provisions if brood is sick or parasitised (Field and Brace 2004). *Ceratina* are mass-provisioners, they pre-provision brood cells with pollen balls onto which individual eggs are laid (Rehan and Richards 2010). These

pollen balls sustain the brood through to adulthood without any additional food supplies. The strategy employed by *C. australensis* means that brood numbers are determined by resources available at the start of the season, as cells are fully provisioned before oviposition. If brood become sick, resources cannot be redistributed, making *C. australensis* more vulnerable to changes in parasitism, microbial load, and desiccation/humidity. Flexibility in social behaviour and brood resource allotment may be more important to population stability than climate preadaptation during rapid environmental changes.

N_e with reproductive skew

Like *C.* australensis, *E. tridentata* shows an historical increase in effective population size, starting between ~24-73kya, though this increase is very slight. This date range reflects an estimate of one per year to one generation every three years respectively, which seems most likely for this species. As this species is eusocial, generation time is linked to the production of queen-destined brood rather than worker females. Brood is reared year-round in *E. tridentata* colonies (Hurst 2001), so potentially some queen-destined brood could be reared to maturity each year. But it is doubtful that this equates directly to generation time, as these queens are very long lived and successful founding of new nests in an environment with highly limited nesting sites is likely to be low (Dew et al. 2013). There is also an initial delay at colony establishment when only workers are produced, before queen-brood are laid. In many eusocial species new queens disperse at set stages in the colony life-cycle and this is may also be the case for *E. tridentata* with queen morphs commonly observed foraging in early spring (pers. obs. R. Dew). Therefore, we estimate the generation time is most likely constrained to 1 year intervals, and probably less frequently.

An effective generation time of more than one year places the commencement of increasing N_e either during or at the close of the LGM.

An increase in N_e at the close of the LGM matches patterns seen in several other bee species. For some species this may represent expansions out of refugia as climates warmed and became wetter (Groom 2014; Lopez-Uribe 2014). These studies suggest that many bees are highly responsive to climate change, possibly due to their strong evolutionary ties to angiosperms. But the comparatively small to negligible responses of *E. tridentata* and *E. setosa* show that responses to climate change are mixed and complex. Unfortunately given the predicted pace of contemporary climate change many plant species are predicted to struggle to adjust quickly enough (Corlett and Westcott 2013). Future impacts of climate change on pollinator networks will likely include not only global climatic shifts but also the risk of population crashes due to more frequent extreme weather events and increased pathogen transmission, with losses in pollinator biodiversity heightening the probability of epidemics (Brown et al. 2015). Our study indicates bee species can respond quite differently to climate and that responses may vary with social and brood-rearing behaviour, and this has wide implications for global pollination services in light of future climate scenarios.

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Author contributions: R.M.D and MPS conceived the idea; R.M.D, M.P.S and M.I.S performed field collections, R.M.D sequenced the genetic material and performed all data analyses; R.M.D wrote the paper with revisions by all authors.

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Table 1: Collection information for each population of *Exoneurella tridentata* sampled*,* including date, GPS of sample localities, sample size and sampling method (nest collection or floral sweeps).

Populations	Date	General Locality	Latitude/ Longitude	Method	Specimens Barcoded	Accession Numbers
West Central 1	November 2013	Yellowdine	31° 15’ 02.7’’ S / 119° 50’ 58.8’’ E	Sweeps	21	XXXX
West Central 2	November 2013	Bullabulling	30° 59’ 50.2’’ S / 120° 50’ 57.9’’ E	Sweeps	2	XXXX
West Central 3	November 2013	Norseman	32° 08’ 18.8’’ S / 121° 44’ 31.2’’ E	Sweeps	1	XXXX
South Central 1	November 2012	Gawler Ranges	32° 22’ 23.2’’ S / 135° 12’ 05.6’’ E	Sweeps	3	XXXX
South Central 2	March and May 2012	Lake Gilles	32° 56’ 28.5’’ S / 136° 44’ 40.7’’ E	Nests	19	XXXX
Central Southeast	September 2014	Danggali	33° 18’ 47.6’’ S / 140° 35’ 32.8’’ E	Sweeps	2	XXXX

Table 2: Collection information for each population of *Exoneurella setosa* sampled*,* including date, GPS of sample localities, sample size and sampling method (nest collection or floral sweeps). Accession numbers for barcoded specimens listed.

Populations	Date	General Locality	Latitude (S)/ Longitude (E)	Sample Method	Specimens barcoded	Accession Numbers
West	November 2013	Perth	32° 09’ 08.3’’ / 116° 07’ 30.6’’	Sweeps	2	XXXX
South Central 1	November 2012	Gawler Ranges	31° 10’ 19.5’’ / 135° 09’ 06.9’’	Nests	6	XXXX
South	July 2014	West Beach	34° 56’ 28.0’’ / 138° 29’ 59.0’’	Nests	31	XXXX
Central Southeast	June and October 2013	Mildura	34° 09’ 25.0’’ / 142° 09’ 58.0’’	Nests	51	XXXX
Southeast	October 2015	Cowra	33° 52’ 07.8’’ / 148° 45’ 07.3’’	Nests	14	XXXX
Northeast	January 2015	Warwick	28° 12’ 58.0’’ / 152° 02’ 10.0’’	Nests	10	XXXX

Table 3: Comparison of mean diversity indices, based on the rarefaction curve at n = 48 individuals for *E. setosa* and *E. tridentata.*

	Fishers's Alpha	Shannon (Exponential)	Simpson (Reciprocal)
E. setosa	3.48	5.76	4.43
E. tridentata	5.14	5.66	3.78

Table 4: Population genetic differentiation of *E. tridentata*. Diagonal: pairwise differences within population, sample size in brackets; Above diagonal: corrected pairwise differences between populations, P-values in brackets; Below diagonal: pairwise Fst values, P-values in brackets. Significant differences highlighted by darkened squares.

	West 1	West 2	West 3	South Central 1	South Central 2	Central Southeast
West 1	0.19 (21)	0.41 **(0.026)**	0.91 (1.000)	0.91 **(<0.001)**	0.61 **(<0.001)**	1.00 **(0.004)**
West 2	0.73 **(0.026)**	1.0 (2)	0.50 (1.000)	0.50 (0.104)	0.21 (0.051)	0.91 (0.328)
West 3	0.81 (1.000)	0 (1.000)	0 (1)	1.00 (1.000)	0.71 (1.000)	0.50 (1.000)
South Central 1	0.84 **(<0.001)**	0.65 (0.104)	1.00 (1.000)	0 (3)	0.073 0.365	1.00 (0.099)
South Central 2	0.62 **(<0.001)**	0.33 (0.051)	0.41 (1.000)	0 (0.528)	0.59 (19)	0.70 **(0.011)**
Central Southeast	0.83 **(0.004)**	0.50 (0.328)	1.00 (1.000)	1.00 (0.099)	0.51 **(0.011)**	0 (2)

Table 5: Population genetic differentiation of *E. setosa*. Diagonal: pairwise differences within population, sample size in brackets; Above diagonal: corrected pairwise differences between populations, P-values in brackets; Below diagonal: pairwise Fst values, P-values in brackets. Significant differences highlighted by darkened squares.

	West	South	South central	Central Southeast	Southeast	Northeast
West	0 (2)	0.88 **(0.002)**	0.70 **(0.040)**	0.58 **(<0.001)**	0.82 **(0.010)**	0.68 **(0.018)**
South	0.78 **(0.002)**	0.24 (31)	0.58 **(<0.001)**	0.47 **(<0.001)**	0.70 **(<0.001)**	0.56 **(<0.001)**
South Central	0.54 **(0.040)**	0.68 **(<0.001)**	0.60 (6)	0.28 **(<0.001)**	0.52 **(<0.001)**	0.38 **(0.002)**
Central Southeast	0.32 **(<0.001)**	0.43 **(<0.001)**	0.25 **(<0.001)**	0.83 (51)	0.34 **(<0.001)**	0.26 **(<0.001)**
Southeast	0.69 **(0.01)**	0.72 **(<0.001)**	0.55 **(<0.001)**	0.31 **(<0.001)**	0.36 (14)	0.10 **(0.028)**
Northeast	0.48 **(0.018)**	0.63 **(<0.001)**	0.37 **(0.001)**	0.24 **(<0.001)**	0.18 **(<0.001)**	0.65 (10)

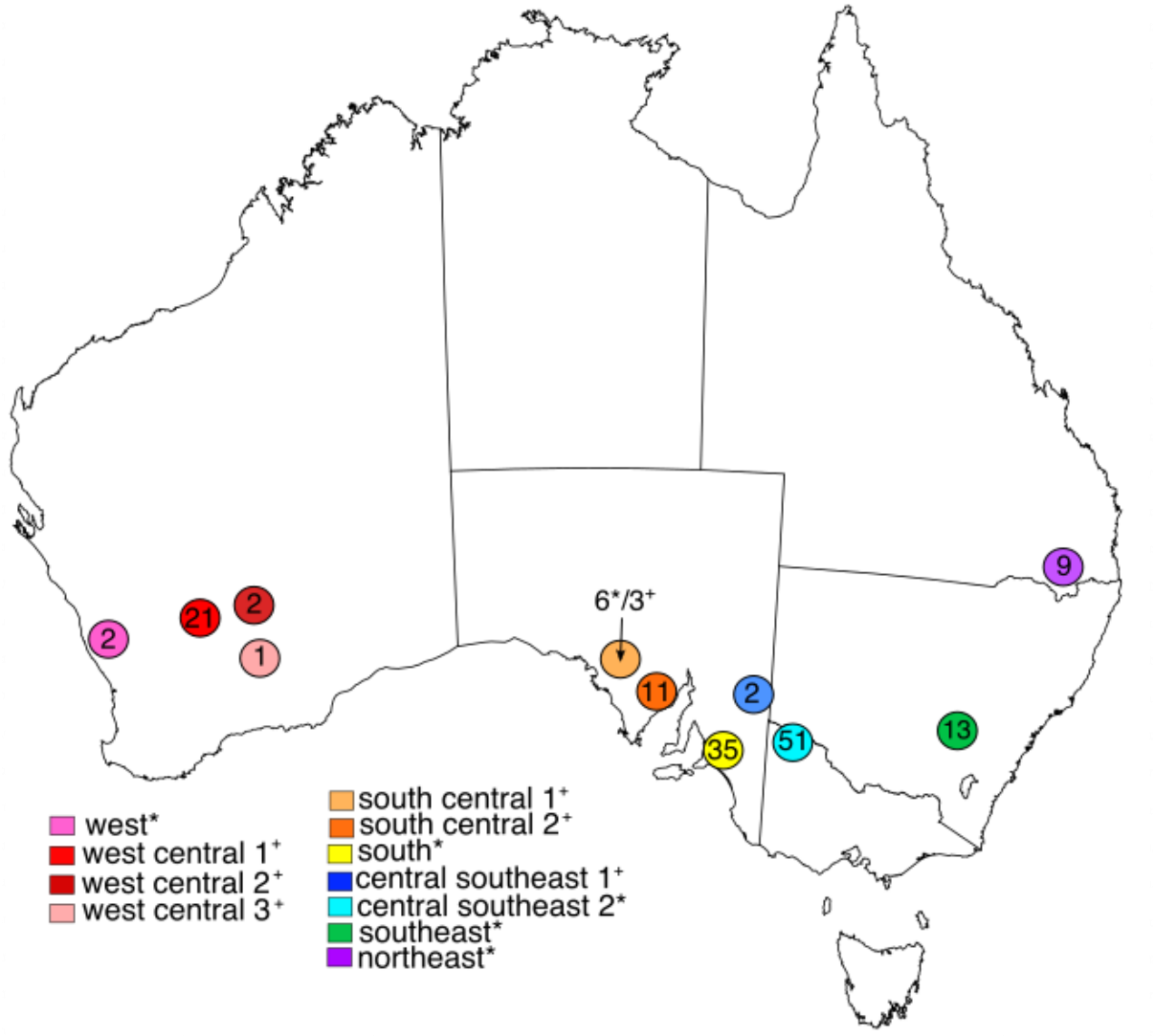

Fig. 1. Sample sites across Australia with sample sizes shown within the circles. Collections of *Exoneurella setosa* = *; *Exoneurella tridentata* = +.

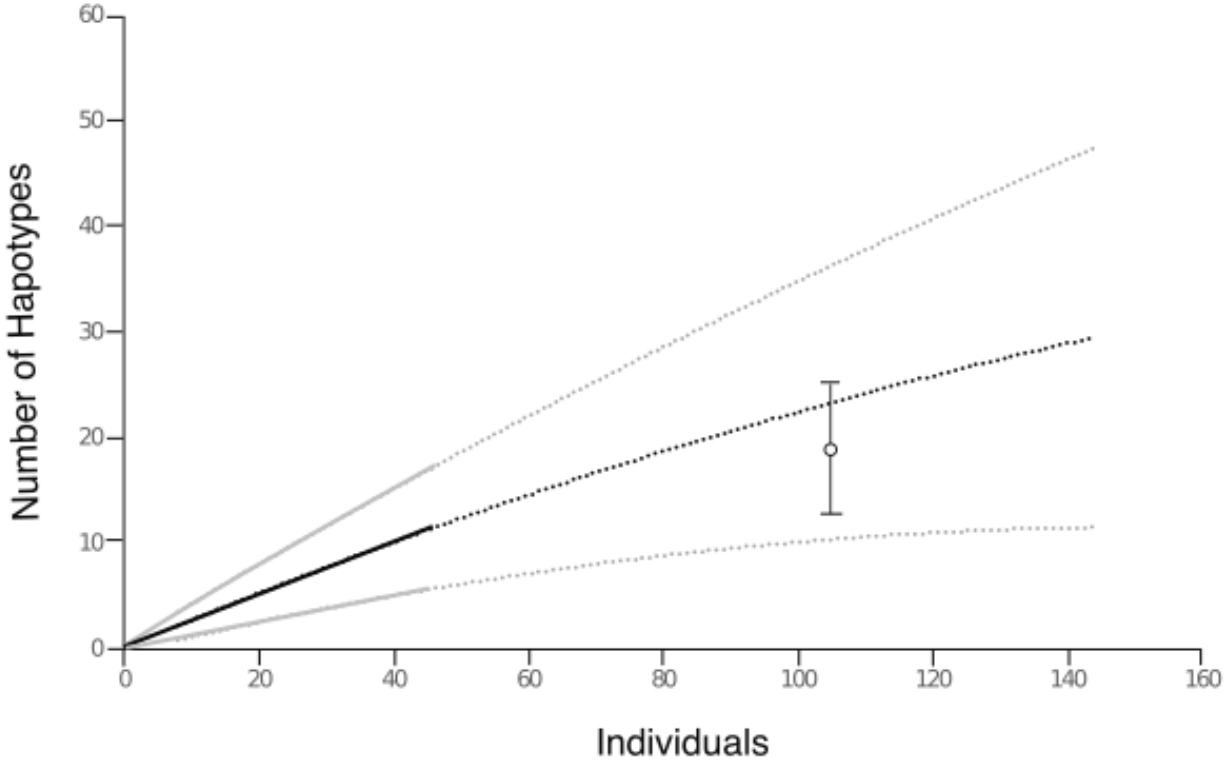

Fig. 2. Sample based rarefaction curve of the number of haplotypes for *Exoneurella tridentata* (black line) with 95% confidence intervals (grey lines). Dashed portion of the black line indicates the calculated portion of rarefaction curve. White circle shows the Incidence Coverage Estimator with 95% confidence intervals of the species-wide allelic diversity for *E. setosa* based on the 114 specimens collected.

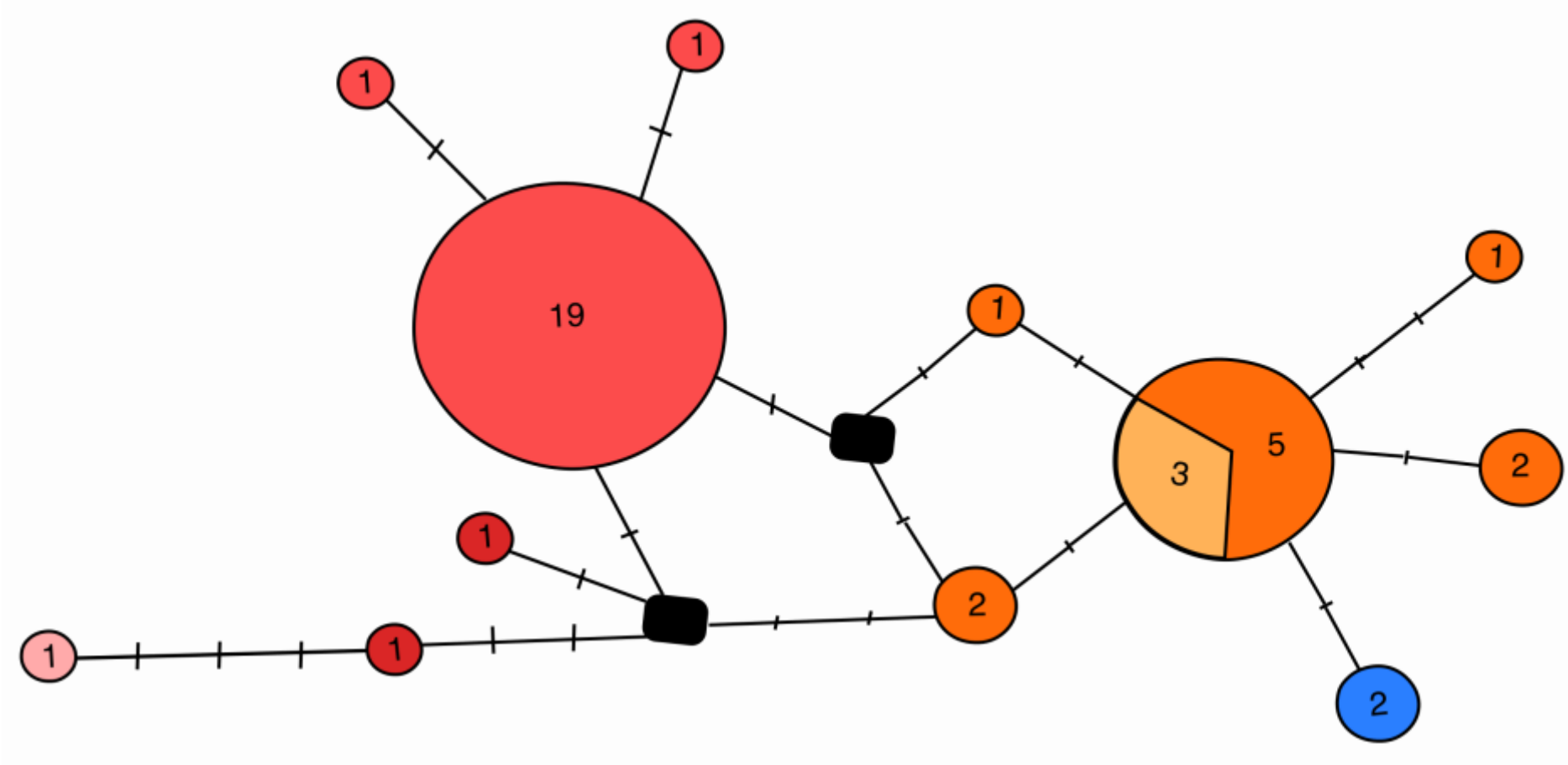

Fig. 3. Minimal haplotype spanning tree for *Exoneurella tridentata*. Numbers indicate the total samples of each haplotype. Colours correspond to populations shown in Fig. 1. Dashes represent base pair changes between haplotypes, black boxes represent shared common haplotypes between branches of the network.

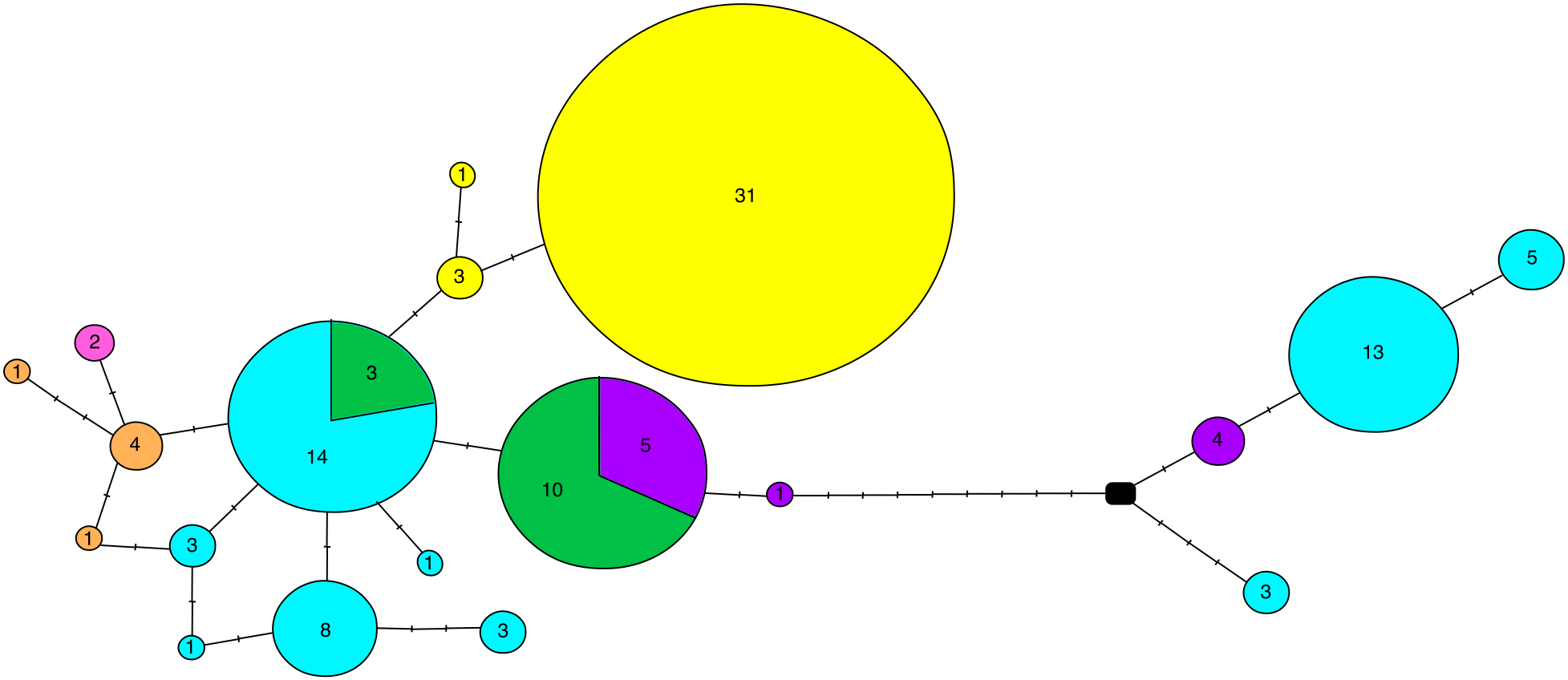

Fig. 4. Minimal haplotype spanning tree for *Exoneurella setosa*. Numbers indicate the total samples of each haplotype. Colours correspond to populations shown in Fig. 1. Dashes represent base pair changes between haplotypes, black boxes represent shared common haplotypes between branches of the network.

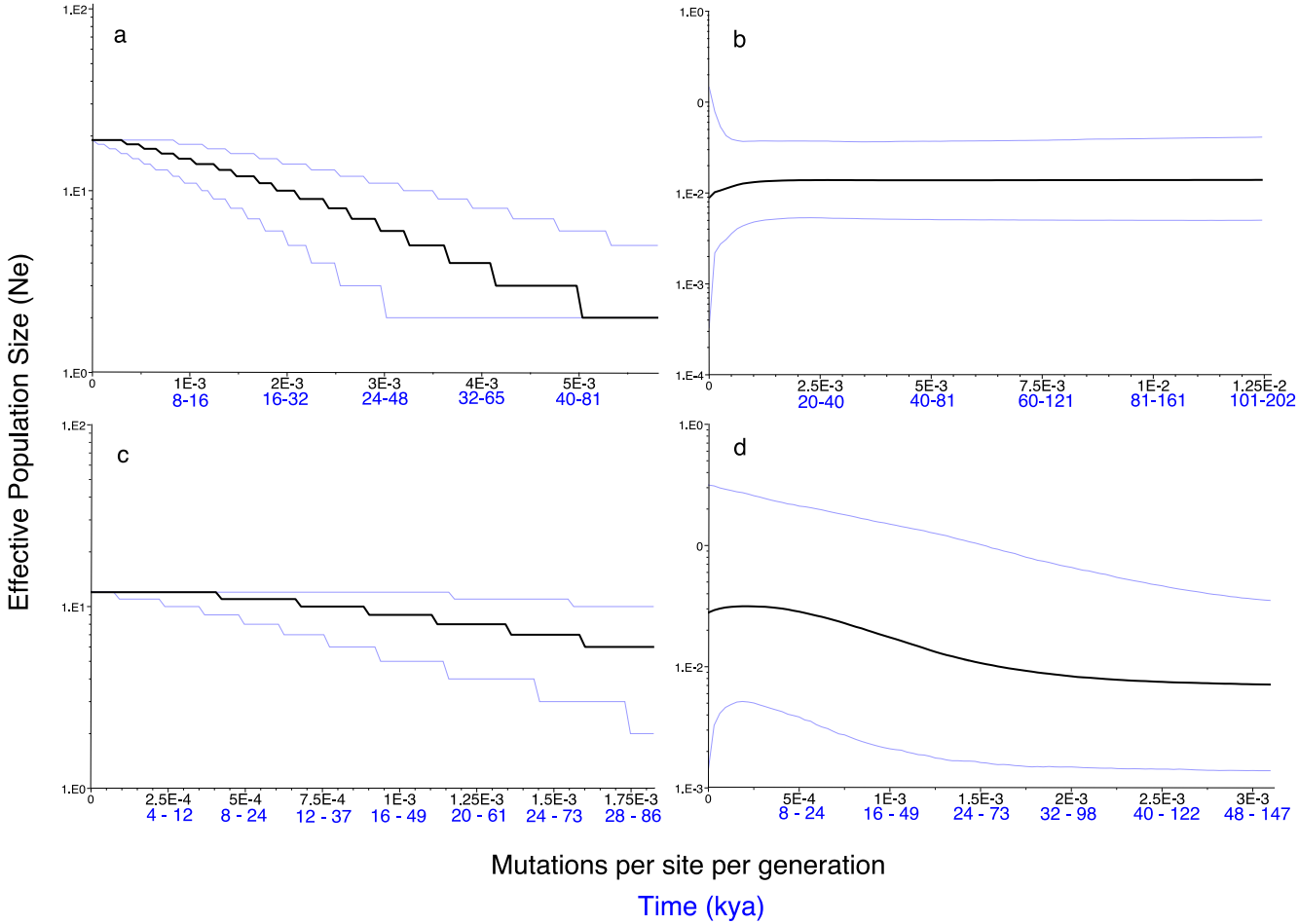

Fig. 5. Lineage Through Time plots for *Exoneurella setosa* (a), *Exoneurella tridentata* (b); and Bayesian skyline plots for *E. setosa* (c), *E. tridentata* (d), showing changes in effective population size. The x-axis shows mutations per site per generation, which have been converted to estimates of time (kya) based on 1-2 generations per year.

CHAPTER VI

Taxonomy and generic status of the Australian allodapine bee genera *Exoneurella* and *Inquilina* (Apidae: Xylocopinae: Allodapini)

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Abstract

The taxonomic status of lineages within the Australian allodapine bees has been unstable over the last six decades, with frequent changes in generic and subgeneric assignments. This is unhelpful given the continuing attention to these bees for understanding social evolution and biogeography. The Australian genus *Exoneurella* has received substantial attention because it contains a highly eusocial species, *E. tridentata*, as well as casteless species, while the parasitic taxon *Inquilina* is important for unraveling issues in the evolution of social parasitism. Here we describe a new *Exoneurella* species, *E. micheneri* sp. n. With the addition of this species, and re-examination of the four other *Exoneurella* species, we redefine the genus and use both genetic and morphological data to explore its phylogenetic relationships to the other Australian allodapine genera. We show that *E. tridentata* is highly unusual, not just in terms of queen/worker morphology, but also in terms of male genitalia. As with other allodapine genera, larval morphology is highly divergent between genera, while female morphology is more conserved. We also review the taxonomic treatment of *Inquilina* and molecular phylogenetic studies on it and its host genus *Exoneura*, and restore its original generic status, bringing its taxonomy in line with that of the other parasitic allodapine genera.

Introduction

Bees in the tribe Allodapini (Apidae: Xylocopinae) have been widely used to explore social evolution, because of their range of social forms, and are most abundant and diverse in sub-Saharan Africa and Australia. The Australian allodapine bees comprise two very distinct clades: *Braunsapis* (which also occurs in the Oriental region, southern parts of the Arabian Peninsula, Africa and Madagascar), and the 'exoneurines' *Exoneura*, *Brevineura*, *Exoneurella* and *Inquilina*. Fuller et al. (2005) showed that the Australian species of *Braunsapis* result from a dispersal from southern Asia sometime in the late Miocene. Using DNA data, Chenoweth and Schwarz (2011) showed that exoneurines comprise a highly supported monophyletic clade that resulted from a single dispersal event from Africa, probably via Antarctica, in the Oligocene (Chenoweth and Schwarz 2011).

Exoneurella was first described as a monospecific genus by Michener (1963), based on re-examination of *Exoneura lawsoni* (Rayment, 1946). Generic status was justified by a number of traits including forewing venation, shape of the sixth tergite with lack of pubescence at its apex, and larval morphology (Michener 1963). Subsequently, Michener (1965) relegated *Exoneurella* to subgeneric status and recognized two genera that we now regard as exoneurines: the parasitic genus *Inquilina* (Michener 1961), and the genus *Exoneura* which contained three subgenera, *Exoneura s. s.*, *Brevineura* and *Exoneurella*. Subsequently, three other *Exoneurella* species, *E. (E.) eremophila, E. (E.) setosa* and *E. (E.) tridentata,* were described by Houston (1976) and diagnostic traits for the subgenus were expanded by including the shape and apex of the 6th tergite, metasomal pigmentation and male eye shape.

Using a cladistic analysis, Reyes (1998) raised *Exoneura s. s.*, *Brevineura* and *Exoneurella* to generic status, whilst retaining *Inquilina* as a subgenus of *Exoneura*, and this convention has been used in all subsequent studies (reviewed in Schwarz et al. 2007) apart from Michener (2001, 2007) who treated *Inquilina* and *Exoneurella* as having generic status, but with *Brevineura* and *Exoneura s. s*. as subgenera of *Exoneura*. Lastly, in a phylogenetic supermatrix treatment of bees Hedtke et al. (2013) stated that *Inquilina* was no longer regarded as having generic status, but did not provide citations to support this contention.

The above considerations reveal a high level of uncertainty regarding the current taxonomic status of the exoneurines. Michener's (2007) treatment is largely based on morphology rather than phylogenetic relationships, whilst Reyes' (1998) treatment is based on cladistic analyses of a small number of morphological characters that are problematic in terms of coding and polarity (Schwarz et al. 2003). Lastly, Chenoweth and Schwarz's (2011) phylogenetic analysis of the exoneurines was based purely on DNA sequence data and did not consider morphological characters or how those could be used to define taxonomic groupings.

The exoneurines have received substantial attention because of their relevance to studies of social evolution (Schwarz et al. 2007; Stevens et al. 2007; Schwarz et al. 2011, Dew et al. 2012) and the evolution of social parasitism (Smith et al. 2007, 2013). For future research to continue it is important to establish a formal taxonomic framework for the exoneurines based on a well-supported phylogeny using an integrative approach (e.g. Stevens et al. 2011; Stevens & D'Hease 2016).

Chenoweth and Schwarz (2011) included an undescribed allodapine bee from S.W. Western Australia in their molecular phylogenetic analyses and this species was recovered as a basal lineage in the *Exoneurella* clade. However, they did not describe the morphology of this species or consider how it may inform the taxonomic treatment of the exoneurines. Here we formally describe this new species and use DNA sequence and morphological data to explore deep phylogenetic relationships among the exoneurines. Here we formally raise *Exoneurella* and *Inquilina* to generic status and provide revised generic descriptions.

Methods

Bee samples were collected November 2013 from south-west Western Australia, including both nest samples and specimens from floral sweeps (Table 1). Nests, found in dead stems of Kangaroo Paw (*Anigozanthos* sp.), were collected during early morning or late evening when all adults would be in the nest. The adults and brood present in each nest were recorded and preserved in 100% ethanol. Images were obtained using a Nikon® D5100 digital camera mounted on a Nikon® SMZ1000 stereo microscope, and manually-focused to obtain 6 to 12 images using the software Camera Control Pro2 ver. 2.22.0 (Nikon®) to produce a compiled montaged image using Helicon Focus ver. 6.4.1. (Helicon Soft Ltd). An additional three females and one male were also viewed for the description. Collection details of these specimens and accession numbers for genetic material of the then undescribed *Exoneurella* Western Australia D are available in Chenoweth and Schwarz (2011).

DNA barcoding

We performed DNA barcoding on two *Exoneurella* specimens from separate nests in order to confirm that these were indeed samples of the same undescribed species that was included in Chenoweth and Schwarz (2011). DNA was extracted using a micro-fiber vacuum plate method (Ivanova et al. 2006). Extracted DNA was eluted into 50µl TLE (10mM TRIS, 0.1mM EDTA pH8). PCR reactions of 25µl were used for DNA amplification of a 612bp region of COI. Reactions contained 0.1µl immolase as the active enzyme, 5µl of MRT Buffer, 15.4 µl water, 2.5µl DNA and 1µl each of the forward and reverse primers. The forward primer is a

combination of M13/pUC (Messing 1988) and LC01490 (Folmer et al. 1994; 5'-GTTTTCCCAGTCACGACCCTTTTATAATTGGAGGATTTGG -3'). The reverse primer combines the reverse M13/pUC and primer M399 designed by S. Cooper (Schwarz et al. 2004; 5'-CAGGAAACAGCTATGACTCATCTAAAAACTTTAATTCCTG-3'). The PCR cycle began with 10min of 94°C. The annealing stage had 5 cycles consisting of 60s at 94°C, 90s at 45°C and 90s at 72°C followed by 35 cycles of 60s at 94°C, 90s at 50°C and 60s at 72°C. Elongation was 10min at 72°C with a final 2min at 25°C. Raw PCR products were cleaned by a vacuum plate wash with 100µl TLE before storage in 30µl TLE. The clean PCR product was sent to the Australian Genome Research Facility for sequencing. The sequences have been submitted to GenBank, accession numbers (KY292349, KY292350, HQ268578).

Phylogenetic Reconstruction

We used both morphological and molecular data to place *E. micheneri* sp. n. within the exoneurine phylogeny. We used a subset of the molecular data from Chenoweth and Schwarz (2011), including 16 allodapine species from 14 genera, covering a wide range of the non-parasitic allodapines. The data consisted of three gene regions: mitochondrial cytochrome *c* oxidase 1 (COI), and nuclear Elongation Factor 1-α (EF1-α) and Elongation Factor 2-α (EF2-α).

Ceratina speculifrons (Apidae: Ceratinini) was used as the outgroup. Of the allodapine taxa, ten samples were from African allodapine genera, to help root the exoneurines, and secondly to allow more informative ancestral reconstruction of morphological traits.

Within the allodapines, adult female morphology is often blurred between genera (Michener 1976). Larval traits, however, show clear generic discontinuities. This is particularly important for *E. micheneri* sp. n. which shows distinct *Exoneurella* larval morphology, but adult female morphology that falls between *Brevineura* and *Exoneurella* (see below). To capture these morphological patterns in our phylogeny we assembled data on 16 morphological traits, using those identified by Reyes (1998) as being most informative. Morphology data were obtained from various papers describing the Allodapini and their larvae (Michener 1975a,b,c; Michener 1976; Michener 2007; Chenoweth et al. 2008; Kayaalp et al. 2011). A full list of the traits used (Table S1) and coding for each species (Table S2) is available in the supplementary material.

The phylogenetic analysis was run in BEAST, using *BEAST ancestral reconstruction for all morphological characters (Table S1). Each gene (COI, EF1-α, EF2-α) and codon position were partitioned separately. A Yule process tree prior with a GTR+ I+ Γ substitution model was employed and run with an uncorrelated log normal relaxed clock model. The analysis ran for $1x10^8$ generations, with trees and model parameters logged every 500 generations. Stabilisation of the posterior was examined in Tracer ver. 1.6.1 (Rambaut et al. 2014) and a maximum credibility tree was generated using a burnin of $5x10^6$ iterations. The analysis was run a total of three times.

Results

Phylogenetic Reconstruction

The maximum credibility tree gives very high support for the placement of *E. micheneri* sp. n. within *Exoneurella* (PP = 1.0; Fig. 1). It was recovered at the root of the other *Exoneurella species.* This phylogenetic position may help explain the similarities in adult morphology to *Brevineura* species. *Inquilina* and *Exoneura* are strongly supported as sister clades (PP = 1.0). The placement of *Brevineura* was not resolved, with a PP value of 0.62 grouping it with *Exoneura* + *Inquilina*. Chenoweth and Schwarz (2011) obtained moderately strong support (PP = 0.93) for this same topology using a larger taxon set, suggesting that *Brevineura* is closely united with these two genera.

Morphological ancestral trait reconstruction

The BEAST traits analysis gives strong support for origins of the larval bilobed head (PP = 0.97), bilobed labrum (PP = 0.96) and bent body shape (PP = 0.99), in the most recent common ancestor (MRCA) of the *Exoneurella* (Fig. S1-S3). The larval morphology of *E. micheneri* is strongly similar to that of *Exoneurella* species, for all traits with the singular exception that it is relatively hairless compared to all other *Exoneurella* larvae, instead displaying pubescence similar to the less hairy *Exoneura* and *Inquilina* (Fig. S4). All *Exoneurella* lack tubercles, unlike *Brevineura* and particularly *Exoneura* where these are greatly elaborated (Fig. S5). A lack of larval tubercles is also seen in *Macrogalea* (basal to the other allodapine genera) and in the outgroup *Ceratina.* Our tree was unable to resolve

whether the lack of larval appendages was due to retention of the ancestral condition or secondary losses, either at the MCRA of *Macrogalea* (PP = 0.711), MCRA of the other allodapine genera (PP = 0.55) or the MCRA of the exoneurines (PP = 0.50). Notably the male genital morphology of *E. tridentata* does not follow that of the other exoneurines. Rather it has a straightened and relatively elongated valve like those of *Macrogalea*, *Allodapula* and *Eucondylops*.

Taxonomic diagnosis - *Exoneurella*

Family APIDAE

Subfamily XYLOCOPINAE

Tribe ALLODAPINI

Exoneurella

Our analyses firmly place *E. micheneri* sp. n. in a monophyletic clade with the four described *Exoneurella.* We re-define the generic description of *Exoneurella* here so that it incorporates all five species.

Female. Costal margin of the second submarginal cell is shorter than the 1st transcubital vein (except *E. tridentata* where the costal margin of the second submarginal cell is slightly longer than the 1st transcubital vein; Fig. 7). T6 is concave and upturned, sometimes strongly so (as in *E. tridentata* and *E. eremophila*). Margin of T6 often has lateral flanges (except *E. micheneri* and *E. lawsoni*; Fig. 5). Apex of T6 not obscured by pubescence.

Remarks. The adult female morphology of *Exoneurella*, *Brevineura* and some *Exoneura* are difficult to distinguish. But these species are clearly separated based on larval morphology and genetic data.

Male. Wing venation as for female. Eyes bulbous. Ocelli larger than antennal sockets. Cream to yellow clypeal marks.

Remarks. Males genitalic traits may provide useful traits for generic determination, as they provide clear species level delineation within *Exoneurella* (Figs 7; 8). The comparative variation among and within other exoneurine genera is not currently known but may prove useful for future systematics studies of this group.

Larvae. (Fig. 10). Body bent at an approximately 90 degree angle at the 5-6th abdominal segment, forming an angulate shape; antennae tapering to a sharp point; lateral areas of head with bulging lobes; body lacks tubercles and appendages.

Remarks. This generic description does not mention the long tapering hairs on the ventro-lateral lobes of the head, referred to in the previous generic description by Michener (2000). These hairs are absent in *E. micheneri* sp. n. and are relatively short and blunt in *E. tridentata*. This variability means it is no longer a defining trait of the genus.

***Exoneurella micheneri* sp. n.**

Zoobank registration - urn:lsid:zoobank.org:pub:5979B6BC-B1F9-4188-AD4B-8DA8910FDF89

Holotype. ♀**,** AUSTRALIA: Western Australia, Gardner State Forest, 34° 46' 36.6" S, 116° 10' 59.1" E, 20. xi. 2013 (leg. *M. Stevens*), (WAM E-95910).

Allotype. ♂, AUSTRALIA: Western Australia, Gardner State Forest, 34° 46' 36.6" S, 116° 10' 59.1" E, 20. xi. 2013 (leg. *M. Stevens*), (WAM E-95909).

Paratypes. 1♀, AUSTRALIA: Western Australia, Northcliffe, 34° 35' 24.8" S, 116° 4' 39.7" E, 16. xi. 2013, (leg. *R. Dew and M. Stevens*), in dead and broken stems of kangaroo paw (*Anigozanthos* sp.), (SAMA 32-035315; Genbank accession KY292349); 1♀, AUSTRALIA: Western Australia, Northcliffe, 34° 35' 24.8" S, 116° 4' 39.7" E, 16. xi. 2013, (leg. *R. Dew and M. Stevens*), in dead and broken stems of kangaroo paw (*Anigozanthos* sp.), (WAM 95913, Genbank accession KY292350); 1♂, 1♀, AUSTRALIA: Western Australia, Northcliffe, 34° 35' 24.8" S, 116° 4' 39.7" E, 16. xi. 2013, (leg. *R. Dew and M. Stevens*), in dead and broken stems of kangaroo paw (*Anigozanthos* sp.), (♂: SAMA 32-035316, ♀: SAMA 32-035317); 1♂,AUSTRALIA: Western Australia, Gardner State Forest, 34° 46' 36.6" S, 116° 10 '59.1" E, 20. xi. 2013 (leg. *M. Stevens*), (SAMA 32-035318); 1♂,1♀,AUSTRALIA: Western Australia, Northcliffe, 34° 35' 24.8" S, 116°, 4' 39.7" E, 16. xi. 2013, (leg. *R. Dew and M. Stevens*), in dead and broken stems of kangaroo paw (*Anigozanthos* sp.), (♂: WAM E-95911, ♀:95912).

Additional Specimens Examined. 2♀, AUSTRALIA: Western Australia, Northcliffe, xi. 2013, (leg. *M. Stevens*).

Etymology

Named in tribute to the late C.D. (Mich) Michener who did so much, and inspired so many about the wonders of bees. Mich provided the first revision of Australian allodapines, he was the first to recognize social parasitism in this group, and the first to realize their utility for understanding social evolution. His magnum opus, Bees of the World, stands as a truly monumental resource to all researchers whose work involves bees.

Description

Female (Fig. 2A-D)

Dimensions. Body length ~5 mm; wing length ~2.5 mm; head width ~1.20mm; relative head measurements: width 50, length 48, lower interocular distance 24, upper interocular distance 32, interantennal distance 8, antennocular distance 15, interocellar distance 9, ocellocular distance 9.

Colouration. Live specimen integument glossy dark brown to black; variable pale yellow clypeal marking from stripe to T-shape; Antenna dark brown at base, with gradient to light brown at tip of flagellum; wing venation and stigma dark brown (N.B. Metasoma, leg, antenna and wing colouration lightens to a yellowy brown over time in ethanol storage).

Pubescence. Long white feathered hairs form thick ring at the margin between the scutum and pronotum, and thinly layer ventral side of thorax; short sparse hairs on scutum and scutellum; isolated long feathered hairs on metanotum and upper propodeum; short setae on vertex of head and clypeal area; short sparse golden setae on T1-T3 of metasoma, with longer setae on lateral edges; Longer and more closely spaced setae to T4-T6, but not so as to obscure the integument or apex of T6; Thick bands of setae at margins of S3-S5; Feathered white hairs on femur of the front leg; Tibia and basitarsus of hind and middle legs and barsitarsus of front legs with long thick golden scopa.

Form. Head circular; clypeus extends almost to the antennal sockets; mandible tridentate; Strongly raised frontal line; Forewing with two submarginal cells; costal margin of second submarginal cell half as long as the first transcubital vein; shortened Cu1 vein and missing the 2nd recurrent vein; Hindwing with five hamli; Basitibial plate present; Inner hind tibial spur simple, with very fine serration; Graduli strongly indicated on T2-4 with lateral gradular carinae; viewed laterally T5-6 slope steeply down to the apex, where there is a slight upturn; viewed dorsally margin of T6 curves smoothly to apex; ventral side of apex is slightly notched.

Male (Fig. 3A-C)

Dimensions. Wing length ~2.2 mm; head width ~1.2mm; relative head measurements: width 50, length 49, clypeal length 22, lower interocular distance 18, upper interocular distance 30, interantennal distance 9, antennocular distance 11, interocellar distance 9, ocellocular distance 7.

Colouration. As for female, except clypeal mark covers the entire clypeus plus the area where the compound eye inner orbit converges on the clypeus.

Pubescence. As for female but lacking scopa, reduced and minimal hair on the metasoma except for long setae on T6 and T7.

Form. As for female but larger compound eyes and extended antennae typical of males; Front tibial spur forks towards the end into two even points; Genitalia with transparent flap-like ventro-apical plate curved upwards to ¾ the length of the penis valve; minute ventral gonostylus with brush-like apex reaching ½ the length of the penis valve; Penis valve broadened halfway along length to form a triangulated structure; Multiple thick setae, posteriorly angled on lateral edges of the penis valve.

Larvae (Fig. 4A-C)

Dimensions: Body width = 0.85mm; Body length = 3.0mm; Head width = 0.48mm; Head length = 0.56mm; Antenna length = 0.19mm.

Pubescence. Head with very short fine hairs on dorsal surface; Lateral lobes of head each with one blunt setae, about 2/3 antennal length; fine hairs 2/3 antennal length at back of head and dorsal surface of 1st segment; shorter slightly thicker hairs on distal edges of segments; no hairs on lateral or ventral surfaces.

Form. Body bent at an approximately 90 degree angle at the 5th – 6th abdominal segments forming an angulate shape; head with pronounced ventrolateral lobes; antennae thin and

tapering to acute point; strong intersegmental lines; body lacking tubercles or appendages; labrum does not extend beyond margin of head or over mandibles; mandibles simple, slender apically.

Diagnosis

Adult females of *E. micheneri* sp. n. can be distinguished from other *Exoneurella* by the presence of the basitibial plate and the margins of T6 curve smoothly to a simple apex, without additional dentation or flanges (Fig. 5B). This is similar to *E. lawsoni* (Fig. 5F) but the dorsal and ventral sides of the apex are the same length. Males are easily identified by the clypeal mark, which covers the entire clypeus plus the area where the compound eye inner orbit converges on the clypeus (Fig. 6A). The gonostylus is characterised by transparent flap-like ventro-apical plates curved upwards to ¾ the length of the penis valve; minute ventral gonostyli with brush-like apices reaching ½ the length of the penis valve (Figs 5B; 6B). Larvae with short hairs on the distal edges of segments, lacking the large spines of some *Exoneurella* larvae (Fig. 4A); a single blunt seta on each lateral lobe of the head (Fig. 4B); dorsal surface of segments 2 and 3 raised into wave shaped structures (Fig. 4C); hair not present on ventral surface of body.

Species Keys

Females

1. (1) Margins of T6 with lateral projections or flanges (Fig. 5C-E).
…… 2

- (2) Margins of T6 without lateral projections or flanges (Fig. 5B,F).
…… 4

2. (1) Costal margin of the second submarginal cell is slightly longer than the 1st transcubital vein (Fig. 7C); apex of T6 simple, ventral surface extends beyond the dorsal (Fig. 5C). …….. *E. tridentata*

- (2) Costal margin of the second submarginal cell is shorter than the 1st transcubital vein (Figs 7B, D-F); apex of T6 tridentate or emarginated (Fig. 5D,E). …… 3

3. (1) Metasoma mottled yellow to brown with large irregularly shaped cream stripes towards distal edges of terga; T6 with pointed lateral flanges (Fig. 5D).
…… *E. eremophila*

- (2) Metasoma predominantly dark brown to black, with thin (often faint) cream bands at distal edges of terga; T6 with blunted lateral flanges (Fig. 5E).
…… *E. setosa*

4. (1) Apex T6 simple, dorsal surface extends beyond ventral (Fig. 5F); basitibial plate not indicated. …… *E. lawsoni*

- (2) Apex of metasomal tergum of female simple, dorsal and ventral surface of equal length (Fig. 5B); basitibial plate indicated.
…….. *E. micheneri* sp. n.

Males

1. (1) Compound eyes greatly enlarged, bulging inwards to outer margin of antennal sockets; Ocelli also enlarged, greater than antenna socket; cream to light yellow T-shaped clypeal mark (Fig. 6B); straightened, scoop-like penis valve, sides parallel, slighter wider towards the apices (Figs 8B; 9B).

   …….. *E. tridentata*

- (2) Compound eyes not greatly enlarged, at least an antenna width between compound eye and margin of antennal socket; ocelli approximately the size of antennal socket; entire clypeus filled with cream to light yellow mark (Fig. 6A, C-E); penis valves broadened halfway along length to form a triangulated structure (Fig. 8A, C-E). …….. 2

2. (1) Face mark does not extend into area where the compound eye inner orbit converges on the clypeus. …… 3

- (2) Face mark extends into area where the compound eye inner orbit converges on the clypeus. …… 4

3. (1) Metasoma with large cream blotches on lateral faces of terga, extending onto sides of dorsal surface; Face mark covering entire clypeus and the space between the clypeus and compound eye; gonostyli with short, posteriorly projecting setae (dorsal view, Fig. 9C); laterally raised ventro-apical plate; no membrane present between the penis valve (Fig. 8C).

   …… *E. eremophila*

- (2) Metasoma with thin (often faint) cream bands at distal edges of terga; Face mark covering entire clypeus with additional dots on either side of the clypeus in the paraocular lobes; gonostyli lacking setae; flat ventro-apical

plate (Fig. 8D); transparent membrane present on inner edges of penis valve (Fig. 9D). ……..... *E. setosa*

4. (1) Face mark extending into both the paraocular lobes and the area where the compound eye inner orbit converges on the clypeus; flat ventro-apical plate; setae proceed almost entire length of penis valve flanks (viewed ventrally, Fig. 8E). …… *E. lawsoni*

\- (2) Face mark extending into the area where the compound eye inner orbit converges on the clypeus but not the paraocular lobes; transparent flap-like ventro-apical plate curved upwards to ¾ the length of the penis valve (Fig. 9A); setae proceed at most halfway down penis valve flanks (viewed ventrally, Fig. 8A). ..... *E. micheneri* sp. n.

Larvae (4th instar)

1. (1) Head with a single thick blunt seta on each lateral lobes; Dorsal surface of segments 2 and 3 raised into wave shaped structures; hair not present on ventral surface of body (Fig. 10B). ..... *E. micheneri* sp. n.

- (2) Head with multiple long hairs on lateral lobes; Dorsal surface of segments 2 and 3 unmodified; hair present on ventral surface of body. 2

2. (1). Large obtusely pointed ridges on dorsal surface of abdominal segments 5 and 6 (at bend in body); Lateral lobes of head with thick blunt setae; Antennae short, less than ¼ the height of the head (from dorsal to ventral surface, Fig. 10C). ..... *E. tridentata*

- (2) Dorsal surface of segment 6 unmodified; Lateral lobes of head with long hairs, tapering to an acute point; Antennae long, ¼ head height or more. 3

3. (1) Dorsal surface of segment 5 bulging (Fig. 10E). ..... *E. setosa*

- (2) Dorsal surface of segment 5 unmodified. 4

4. (1) Very long straight antennae, more than ½ head height; Head with very long hairs on lateral lobes, about ½ head height; slight bulges at defining segments ventro-laterally (Fig. 10F). ..... *E. lawsoni*

- (2) Antennae about ¼ head height, slightly hooked at apex; Lateral lobes of head with hair just longer than antennae; no bulges ventro-laterally (Fig. 10D). …….. *E. eremophila*

Taxonomic diagnosis - *Inquilina*

Family APIDAE

Subfamily XYLOCOPINAE

Tribe ALLODAPINI

Inquilina **stat. r.**

Inquilina Michener 1961

Exoneura (*Inquilina*) Michener 2000

Type species: *Inquilina excavata* (Cockerell 1922)

Features same as for *Exoneura* except for the following (females only): Face broadly concave and lacking cream or yellow markings, but often with dark red to orange coloration of clypeus, frons and lower paraocular areas; mandible with ventral tooth reduced to a convexity and therefore mandible bidentate instead of tridentate; glossa and labium greatly reduced compared to *Exoneura*; femora of fore and hind legs robust with hind femur approximately twice as long as broad; hind leg scopae greatly reduced and comprising stout and shortened setae, largely restricted to dorsal surface of basitarsis; setae on femora and basitarsi greatly reduced and largely restricted to ventral surfaces; hind tibial spurs robust and apically curved.

All *Inquilina* species described to date are obligate social parasites of *Exoneura* spp. Males have been rarely collected, and only identified from samples based on colony contents.

Discussion

The placement of *E. micheneri* sp. n. in *Exoneurella* is confirmed by both phylogenetic reconstruction and morphological considerations. The most informative morphological characters were the larval traits, including the bent body shape, bilobed head and lack of tubercles associated with *Exoneurella*. The phylogenetic position of *Brevineura* is still unresolved, but *Exoneura* and *Inquilina* stat. r. resolve as two separate, non-nested clades. As part of our taxonomic treatment of the exoneurines we reinstate *Inquilina* to full generic status, to align with the taxonomic nomenclature of other allodapine genera (Michener 2000, 2007).

Systematics of *Exoneurella* Michener 1963

Brevineura and *Exoneurella* are difficult to discriminate from each other based on adult morphology alone. Both have oscillated between generic and subgeneric status in successive taxonomic treatments but their distinctive larval morphologies clearly distinguish them. The description of *Exoneurella micheneri* sp. n. and re-examination of the other *Exoneurella* species further blurs the morphological distinctiveness of adult female morphology between the two genera. *Exoneurella micheneri* sp. n. females lack the lateral flanges or apical teeth on the metosomal T6 found in other *Exoneurella* species, though it has the same concave and upturned dorsal surface. It also has a basitibial plate, a feature of *Brevineura* not present in any other *Exoneurella*. This may be associated with the basal position of *E. micheneri* sp. n. in the phylogeny of *Exoneurella*, such that it has retained

some features present in the common ancestor to *Brevineura* and *Exoneurella*, now absent in the more distal *Exoneurella* lineages.

Taxonomic status of *Inquilina* stat. r.

Inquilina contains seven described species which are obligate social parasites of *Exoneura* (Smith and Schwarz 2009). Females have very striking morphologies, including concave clypeus, enlarged hind femora, greatly reduced scopae, and greatly shortened glossae.

Molecular phylogenetic studies have consistently recovered *Exoneura* and *Inquilina* as sister clades (e.g. Bull et al. 2003; Schwarz et al. 2003; Smith et al. 2007). *Inquilina* was erected as a genus by Michener (1961) but then relegated to a subgenus of *Exoneura* by Michener (2000, 2001, 2007). In 2000 Michener noted that 'For allodapines, rather arbitrarily I consider *Inquilina* a subgenus of *Exoneura*, but I recognize *Effractapis*, *Nasutapis*, and *Eucondylops* as genera' (Michener 2007, p. 623), where *Effractapis* and *Nasutapis* are social parasites of *Braunsapis* hosts, and *Eucondylops* are social parasites of *Allodapula*. Interestingly, molecular data indicate that *Nasutapis* is firmly nested with *Braunsapis* and *Eucondylops* is nested within *Allodapula*, making the host genera paraphyletic (Smith et al. 2013), and although molecular data are lacking for the monospecific Malagasy genus *Effractapis*, morphological data (Michener 1976) indicate that it too is nested within *Braunsapis*. The recent phylogenetic reconstruction by Hedtke et al. (2013) regards *Inquilina* as a subgenus, but to reflect the taxonomy of the other allodapines this would require that all parasitic genera are relegated to subgeneric status. As it stands the generic status of

Effractapis, *Nasutapis*, and *Eucondylops* is not consistent with the contemporary systematic convention for monophyly of genera (Ward et al. 2015, 2016; but see Seifert et al. 2016).

Michener (2000, 2007) noted that assigning generic status to *Effractapis*, *Nasutapis*, and *Eucondylops,* rendered *Braunsapis* and *Allodapula* paraphyletic but indicated that the morphological peculiarities of those parasitic clades warranted generic status. However, the situation is different for *Inquilina*, as we now outline. No molecular phylogenetic studies of *Inquilina* and *Exoneura* have recovered *Inquilina* as nested with *Exoneura*. Most importantly, Smith et al. (2013) included all seven known *Inquilina* species and 14 *Exoneura* species and recovered these two groups as reciprocally monophyletic with posterior probability support for each group of PP = 1.0. Although that study did not include all known *Exoneura* species, it included species drawn from the complete geographical range of this clade, covering Queensland, New South Wales, Victoria, South Australia and Western Australia, as well as including *Exoneura angophorae* which represents the most morphologically divergent clade within *Exoneura* (Michener 1965).

Given the striking morphological and life-history differences between *Exoneura* and *Inquilina* and their reciprocal monophyly, we raise *Inquilina* to its former generic status erected by Michener (1961) and do not propose any changes to the original description of that genus.

Morphological adaptations of *Exoneurella*

One of the most marked features of *Exoneurella* is their lack of larval tubercles. Both *Brevineura, Inquilina* and particularly *Exoneura* have highly elaborate tubercles, to the point of becoming appendages (Michener 2007). The only other allodapine genus to lack tubercles is *Macrogalea*, which shares the most recent common ancestor to the also tubercle free larvae of the Xylocopinae. It is unclear from our analysis if *Exoneurella* has simply retained the ancestral condition or lost tubercles that are otherwise present in all other exoneurines (Fig. S5). An increased sampling of African taxa in the phylogeny would likely be able to resolve this.

This paper presents the first comparison of male *Exoneurella* genitalia. All *Exoneurella* species show variation in genitalic structure but the morphology of the penis valve in *E. tridentata* is strikingly unique from not only other *Exoneurella* but all other exoneurines (Figs 8B; 9B). The penis valve of *E. tridentata* is straightened with scoop-like sides, not forming the triangulated shape of the other exoneurines. While unique to the Australian taxa, this shape is seen in *Macrogalea*, *Allodapula* and *Eucondylops*.

The dramatically different genitalic morphology of *E. tridentata* is suggestive of strong selective pressure. *Exoneurella tridentata* is the only highly eusocial exoneurine, colonies consisting of only one to two reproductives (queens) per colony, the other being non-reproductive workers (Hurst 2001). One possible explanation is that the high competition among males for mating success drove rapid morphological evolution. Another possibility is that the relatively long and straight valves in *E. tridentata* are associated with the highly

unusual metasomal morphology of queens, where the terminal external tergite is greatly extended both dorsally and laterally, and where internal musculature of the metasoma is extremely well developed.

Implications for social evolution research

The recognition of *E. micheneri* sp. n. as a member of *Exoneurella* has important implications for future studies of social behaviour. This species is the most basal member of the *Exoneurella*. While *E. tridentata* is eusocial, the three previously known species of *Exoneurella* are facultatively social, with social groups that are casteless (Michener 1964; Hurst 2001; Dew et al. 2016; Dew et al. *in review*). Determining the social behaviour of *E. micheneri* sp. n. will provide critical data on the evolution of sociality across the genus. Understanding how morphological traits have moved with or facilitated changes in social behaviour between species is a key part of this process. The resurrection of *Inquilina* to a full genus will also facilitate studies of social parasitism, bringing the taxonomy of this group in line with current systematic practices.

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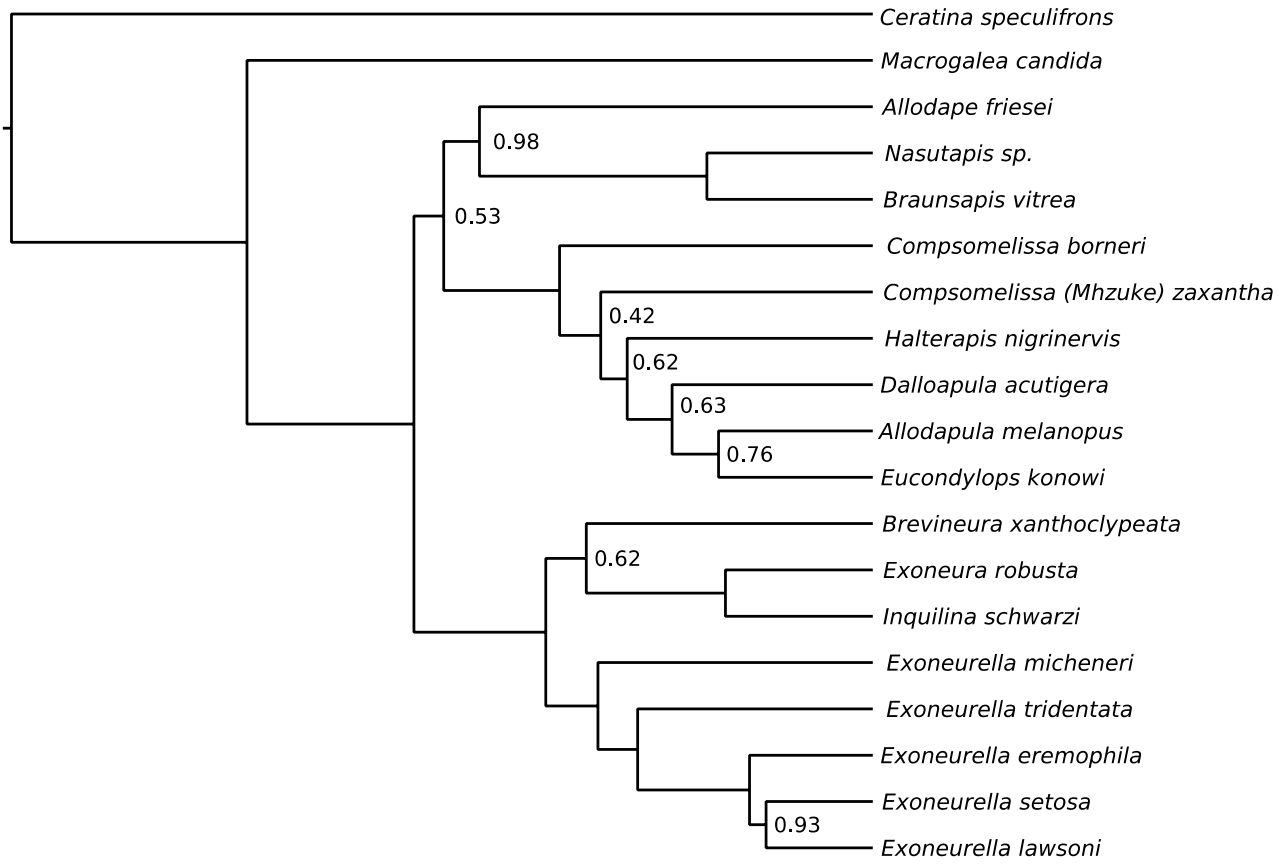

Fig. 1. Maximum credibility tree based on molecular and morphological data. *Ceratina speculifrons* was constrained as the outgroup. Posterior probability indicated if less than 0.99.

Fig. 2. Female of *E. micheneri* sp. n. A, lateral view; B, head; C, dorsal view of head and propodeum; D, female and larvae in nest (credit: Cyrille D'Haese).

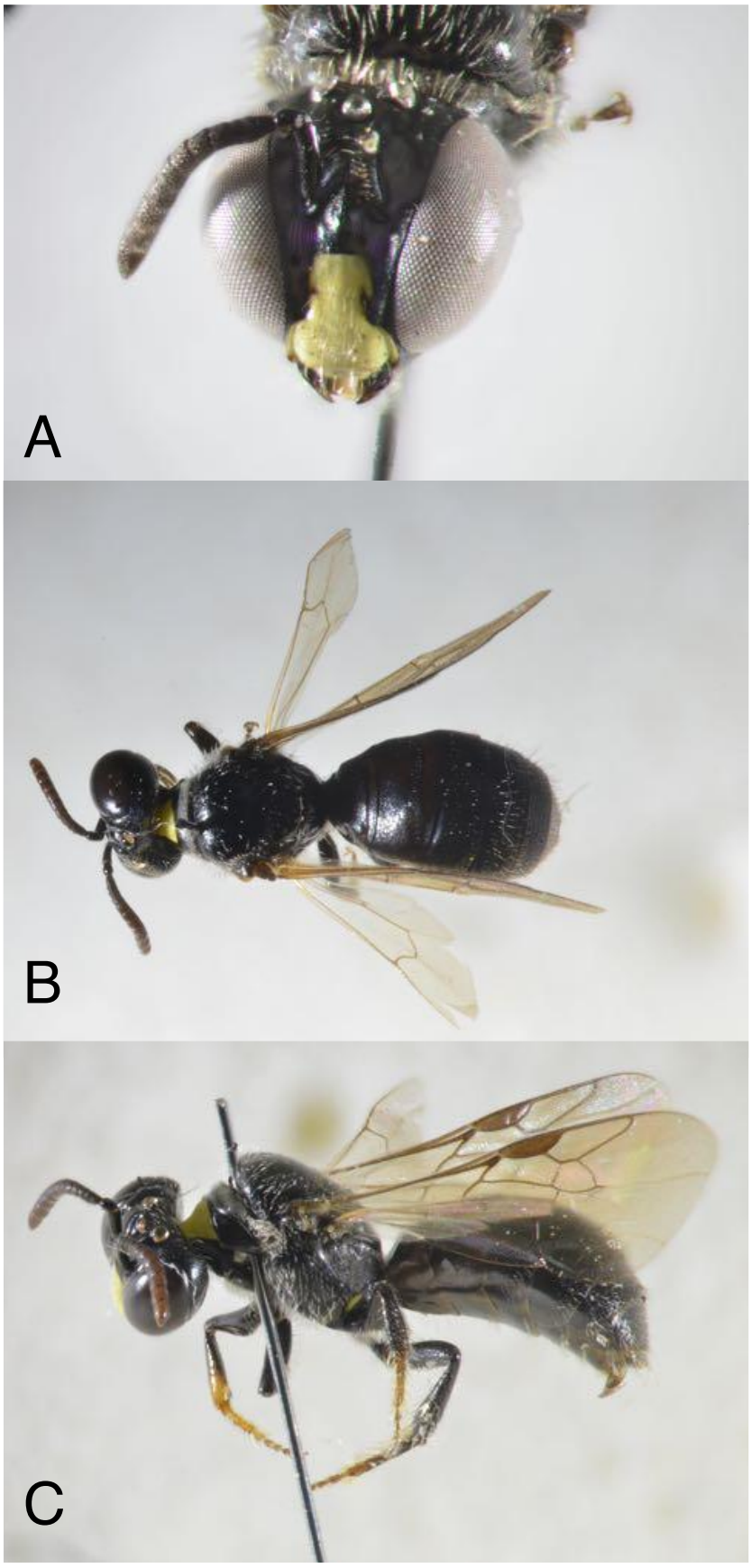

Fig. 3. Male of *E. micheneri* sp. n. A, head; B, dorsal view; C, lateral view.

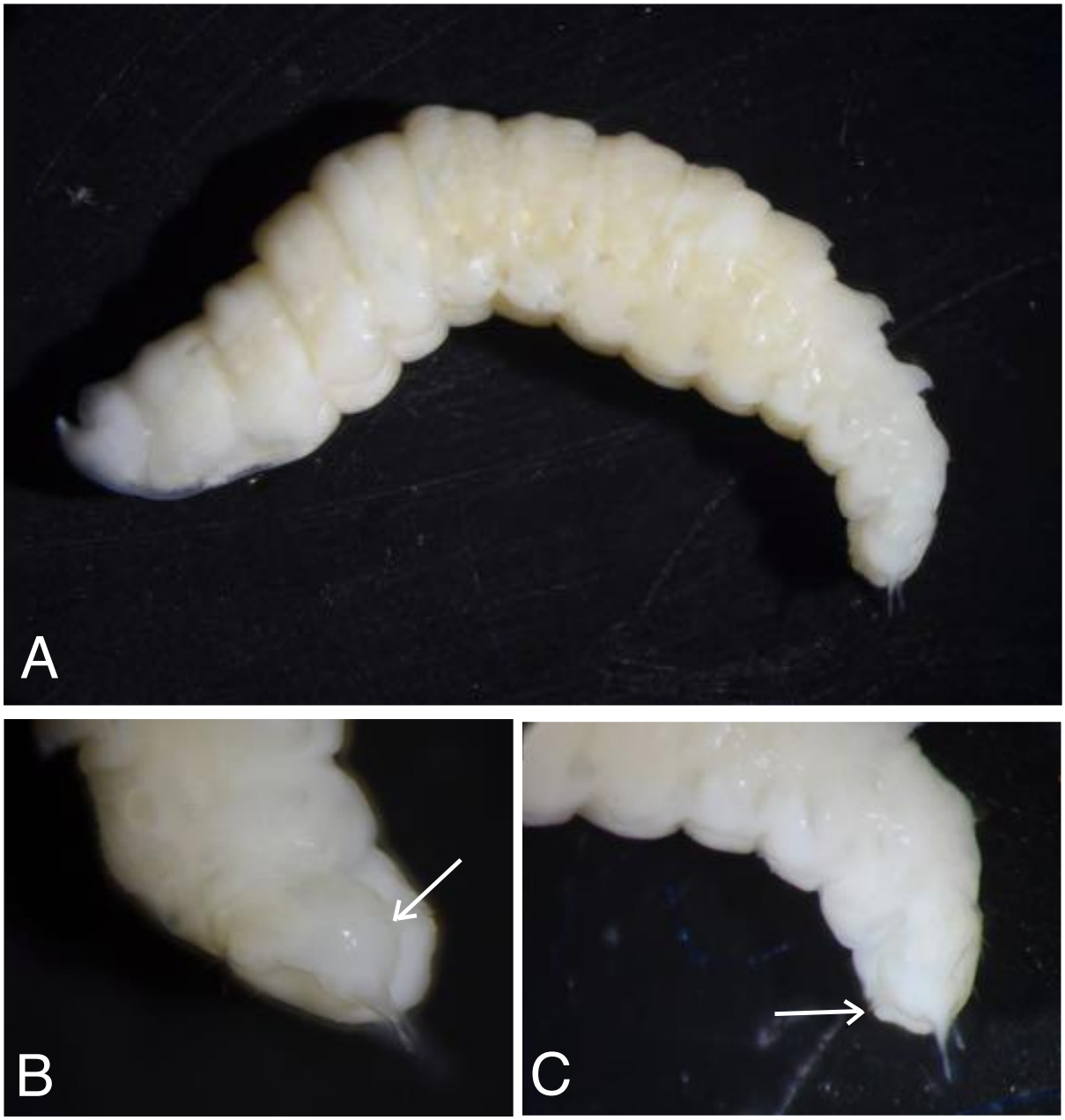

Fig. 4. Larvae of *E. micheneri* sp. n. A, lateral view; B, head, showing lobe seta; C, head, showing mandible. Details indicated by arrows.

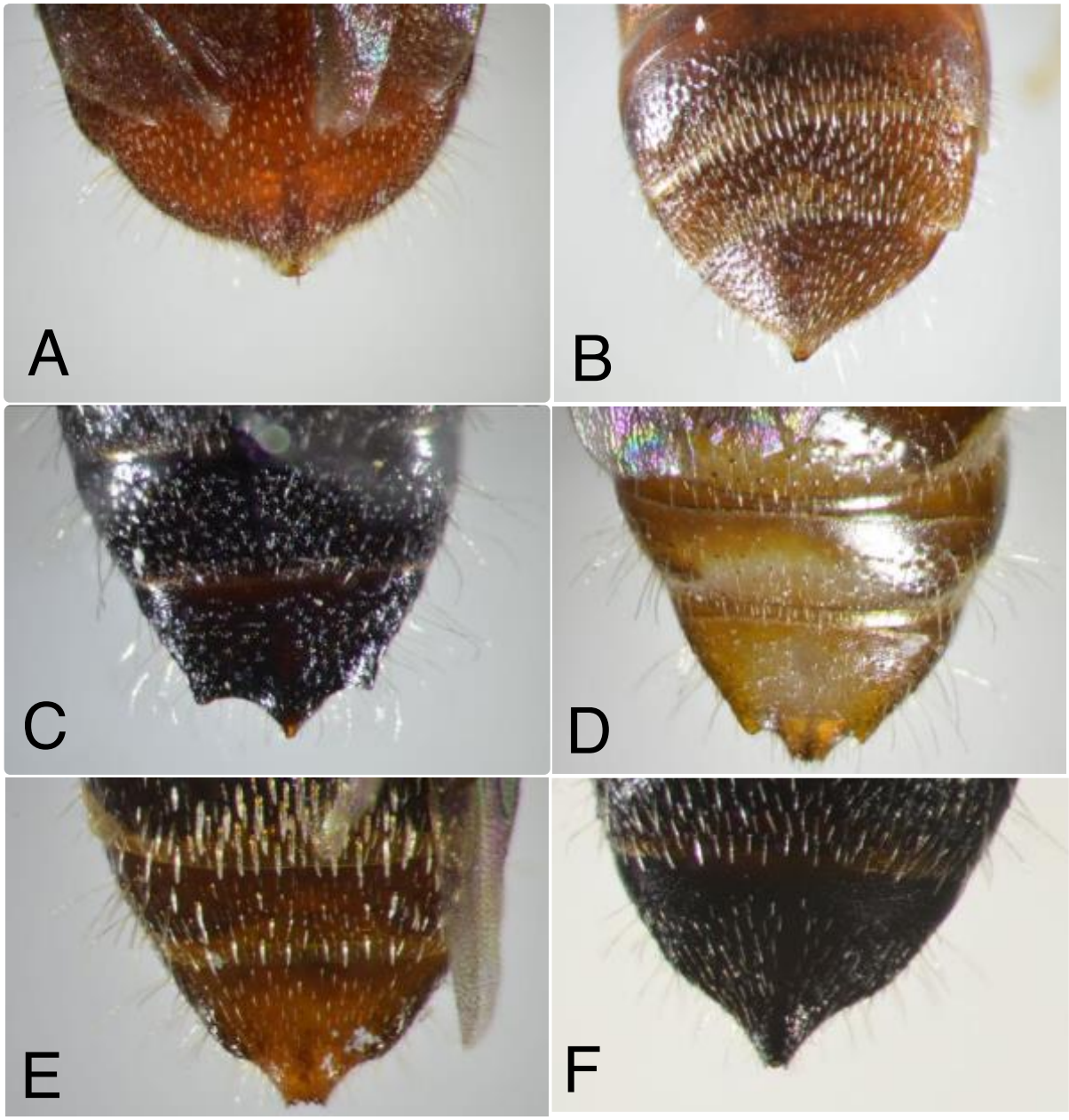

Fig. 5. Dorsal view of T6. A, *Brevineura elongata*; B, *Exoneurella micheneri* sp. n.*;* C, *Exoneurella tridentata*; D, *Exoneurella eremophila*; E, *Exoneurella setosa*; F, *Exoneurella lawsoni*.

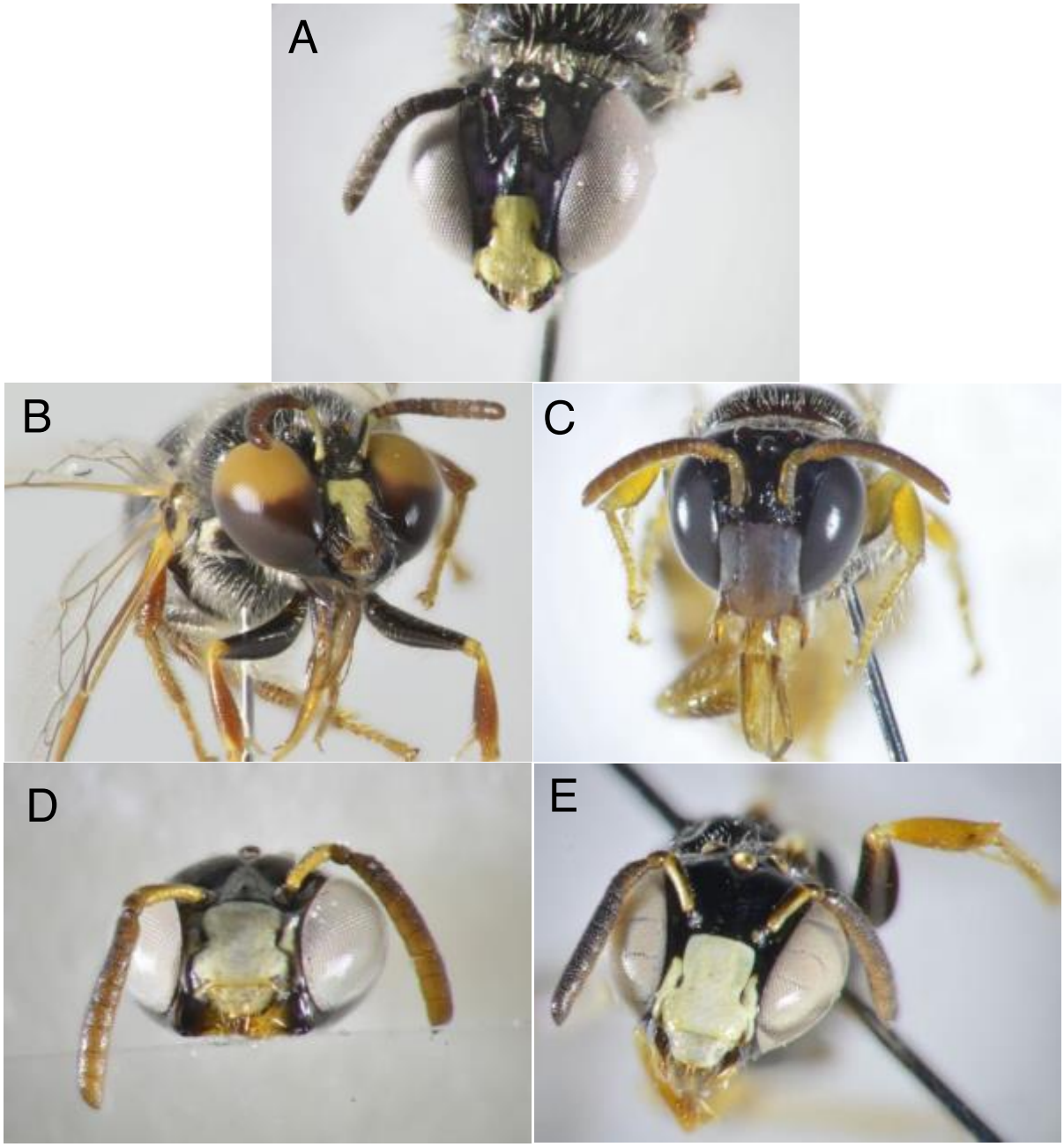

Fig. 6. Male heads. A, *Exoneurella micheneri* sp. n.; B, *Exoneurella tridentata*; C, *Exoneurella eremophila*; D, *Exoneurella setosa;* E, *Exoneurella lawsoni*.

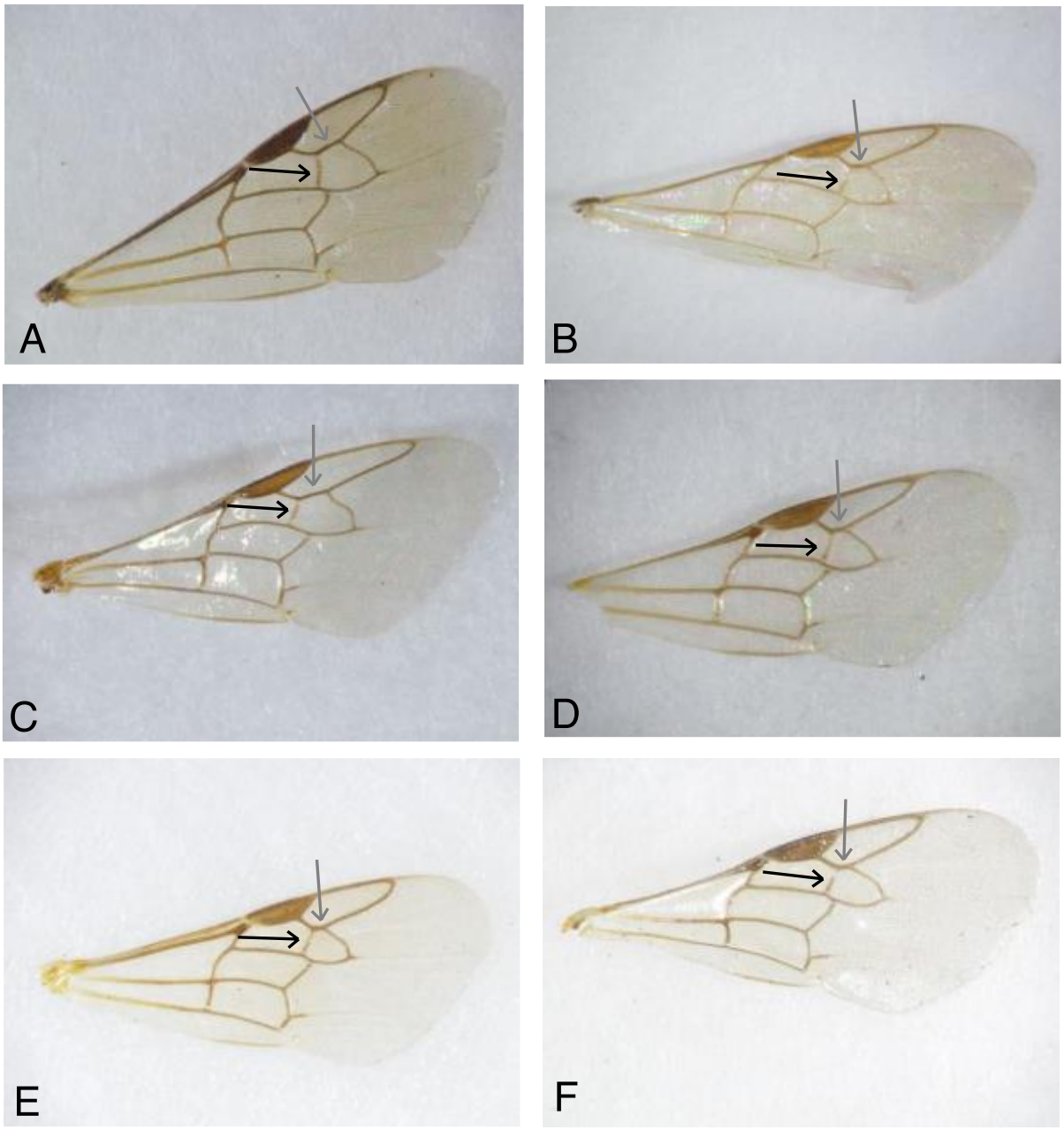

Fig. 7. Forewings. A, *Brevineura elongata*; B, *Exoneurella micheneri* sp. n.*;* C, *Exoneurella tridentata*; D, *Exoneurella eremophila*; E, *Exoneurella setosa*; F, *Exoneurella lawsoni*. Grey arrows: Costal margins of the second submarginal cell, Black arrows: 1st transcubital veins.

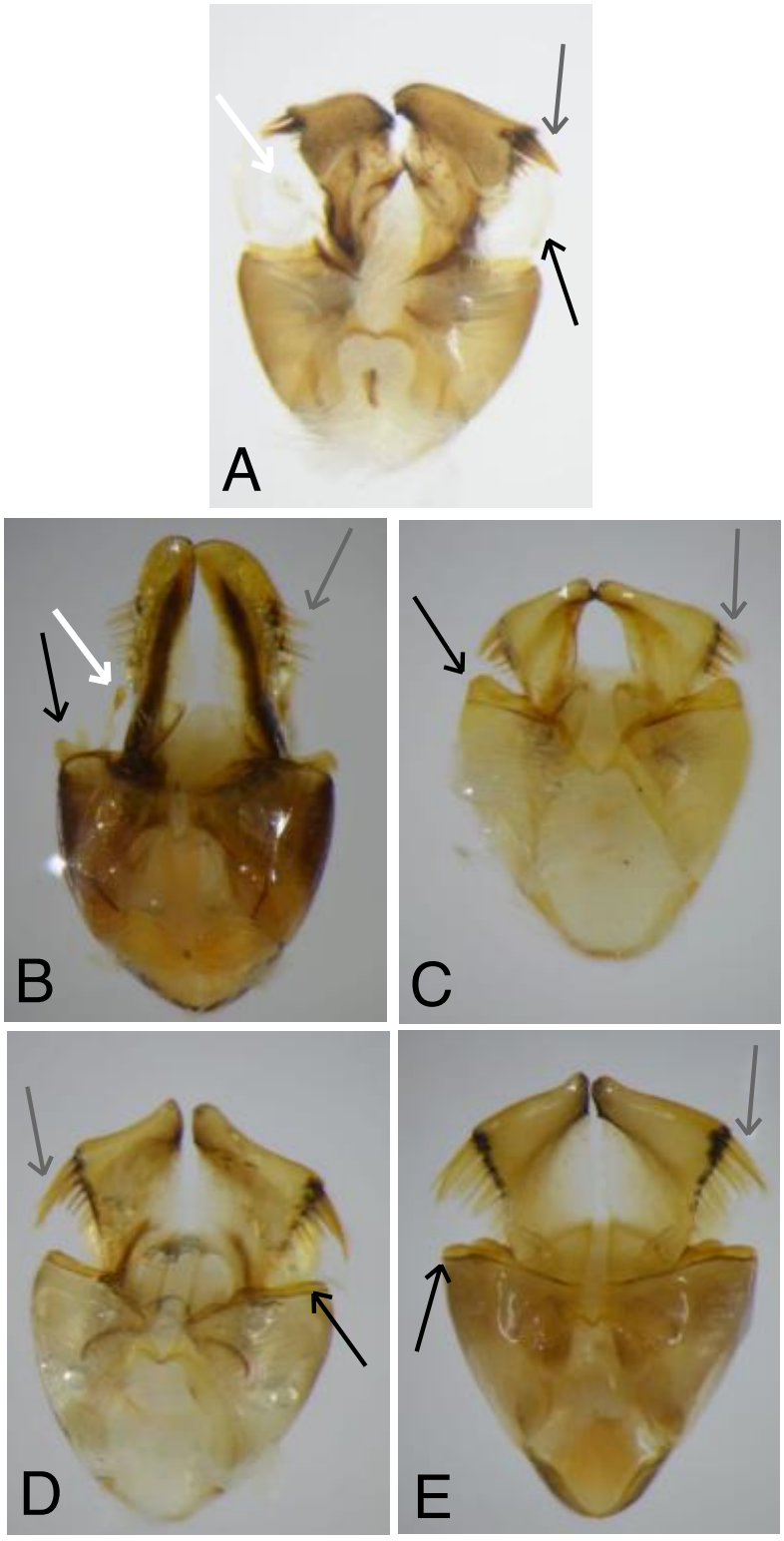

Fig. 8. Ventral view of male genitalia. A, *Exoneurella micheneri* sp. n.*;* B, *Exoneurella tridentata*; C, *Exoneurella eremophila*; D, *Exoneurella setosa*; E, *Exoneurella lawsoni*. Black arrows: ventral-apical plates, Grey arrows: Penis valve spines, White arrows: dorsal gonostyli.

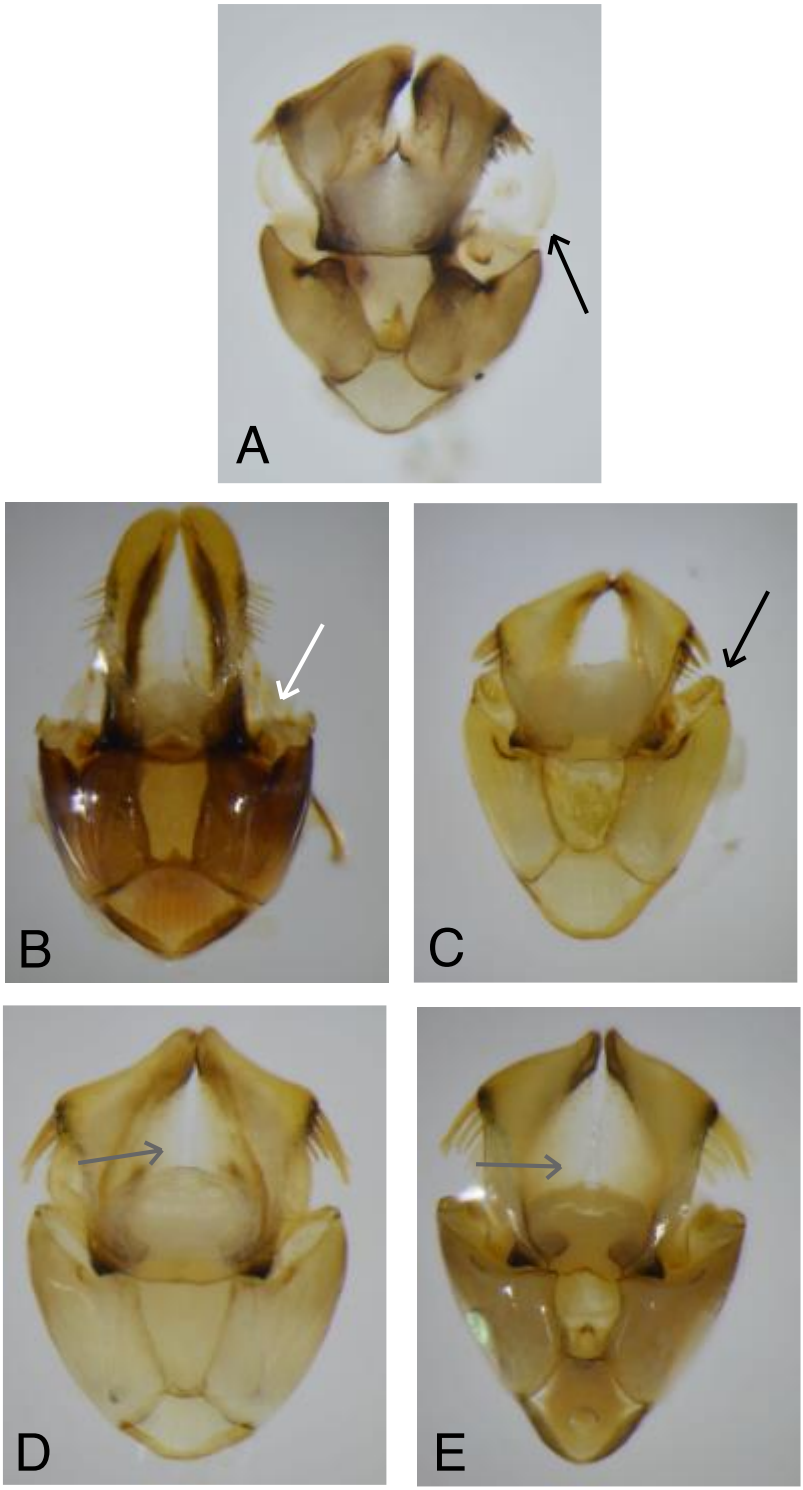

Fig. 9. Dorsal view of male genitalia. A, *Exoneurella micheneri* sp. n.*;* B, *Exoneurella tridentata*; C, *Exoneurella eremophila*; D, *Exoneurella setosa*; E, *Exoneurella lawsoni*. Black arrows: ventral-apical plates, White arrows: dorsal gonostyli, Grey arrows: penis valve membranes.

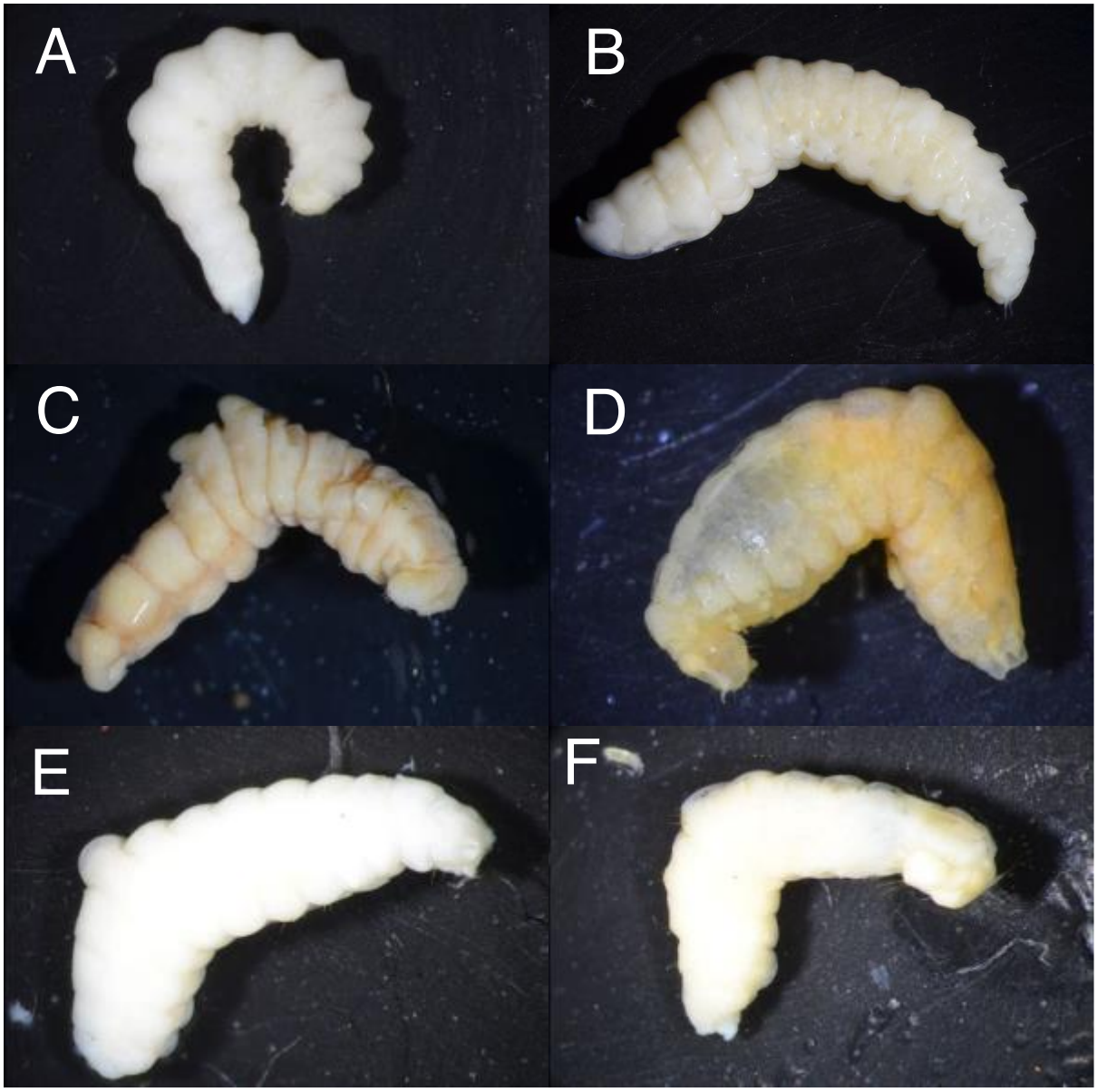

Fig. 10. Comparison of larvae. A, *Brevineura elongata*; B, *Exoneurella micheneri* sp. n.; C, *Exoneurella tridentata*; D, *Exoneurella eremophila*; E, *Exoneurella setosa*; F, *Exoneurella lawsoni*.

Table S1: List of morphological characters used in the phylogenetic reconstruction (adapted from Reyes (1998)). Code numbers of characters do not imply directionality.

	Character	Description
Larvae	1.Head Shape (Frontal view)	[0] Spherical [1] Triangular [2] Bilobed
	2.Antennae	[0] Very short, rounded nodule [1] From ½ to just under the clypeal length [2] From 2 – 4 times the clypeal length [3] From ¼ to under a ½ the clypeal length
	3.Maxillae (and associated palpi)	[0] Large and extend laterally, with protruding palpi [1] Small, follow outline of head (without extending laterally), palpi don't protrude [2] Large and extend laterally, palpi don't protrude [3] Follow outline of head (without extending laterally), with protruding palpi
	4.Labium (and associated palpi)	[0] convex, with protruding palpi [1] convex, with shortened palpi [2] concave, with protruding palpi [3] projection in the middle of the labium

	5.Labrum	[0] Ventral edge completely or partially pushed inwards [1] Ventral edge pushed inwards and/or margin is not smooth [2] Bilobed [3] Shortened and hairy
	6.Head Hairs (Dorsal surface)	[0] Hairless, or nearly hairless with very fine short hairs [1] Thicker straight hairs, from ¼ to just over antennal length [2] Thick long hairs, 2 to 4 times antennal length [3] Thick long sinuous hairs; 2 times dorsal to ventral head height
	7.Body Hairs (excluding head hairs)	[0] Hairless, or almost hairless with very small hairs on dorsal side of 1st segment only [1] Hairs in single rows dorsally on multiple body segments, rows may extend laterally; hairs also present on ventral surface [2] Hairs in single rows on multiple body segments, not extending laterally, absent from ventral surface [3] Hair short hooked, covering entire body [4] Hairs on ventral surface only

	8.Body Tubercles	[0] Absent [1] Present on lateral edges 2nd and 3rd segment [2] Tubercle on 2nd segment elaborated into appendage [3] Two longitudinal rows of ventral tubercles; Sometimes elaborated tubercle on 2nd or 3rd segment [4] Multiple longitudinal rows of tubercles (ventral, lateral and/or dorsal)
	9.Body Shape	[0] Smoothly curved [1] Almost straight, slight curve at head [2] Bent at the 5th abdominal segment
Adult (Both sexes)	10.2nd Recurrent vein	[0] Absent [1] Present
	11.Cu1 Vein	[0] Long, extending towards wing edge [1] Short, shorter than 2nd transcubital vein
	12.Maxilliary Palpi	[0] Six segments [1] Five segments [2] Four segments [3] Three segments; 1st longer than others [4] Three segments; 1st and 3rd longer than 2nd
	13.Penis Valve	[0] Each side reflexed at the apex; sides taper gradually to an acute point

Adult Male		[1] Valve is broadened below the apex, measuring width from the centerline at the apex to the widest point, each side is under ¼ the width of the gonobase, structured may be slightly triangulated [2] Margin slopes downward from apex, broadening into a strongly triangulated structure; Each side is over ¼ to ½ the width of the gonobase [3] almost straight, scoop-like, rounded at the apex
	14.Penis Valve Hairs	[0] Almost hairless to light hairs covering the surface [1] Short, thick spines present, less than 1/8 the width of the penis valve [2] Longer spines present, up to ¼ the width of the penis valve [3] Very long spines, longer than ¼ the width of the penis valve, spines curve down towards the gonobase

	15.Gonostylus[a]	[0] true gonostylus, thicker and taller than penis valve [1] gonostylus reduced into ventro-apical plate and/or ventral gonostylus; reduced component is from ¾ to the length of the penis valve [2] further reduced; components reach ½ or less than the length of the penis valve
Nesting biology	16.Provisioning	[0] mass provisioning [1] mass provisions without internal cells [2] progressive provisioning [3] one common food mass

Table S2: Matrix of codes used for each species in the *BEAST ancestral reconstruction.

Character numbers and codes for each species refer to traits and descriptions in Table S1.

Species	Coded Characters															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Ceratina speculifrons	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	0
Halterapis nigrinervis	0	1	1	1	0	0	0	1	0	1	0	0	2	2	1	0
Compsomelissa (Mhzuke) zaxantha	0	1	1	1	0	2	4	4	1	1	0	0	1	1	1	1
Compsomelissa borneri	0	1	1	1	0	0	0	1	0	0	1	0	1	2	1	1
Dalloapula acutigera	0	1	1	3	0	0	0	4	1	1	0	0	1	2	1	3
Allodapula melanopus	0	1	0	2	1	0	0	4	1	1	0	0	3	0	1	3
Eucondylops konowi	0	1	0	2	1	0	0	4	1	0	0	2	3	0	2	3
Macrogalea candida	0	4	3	1	3	1	3	0	0	1	0	3	3	1	2	2
Exoneurella eremophila	2	2	1	1	2	1	1	0	2	0	1	1	2	3	2	2
Brevineura xanthoclypeata	0	2	1	1	1	1	1	2	0	0	1	1	2	3	2	2
Exoneura robusta	0	2	1	1	1	1	2	3	0	0	0	1	2	3	2	2
Inquilina schwarzi	0	2	1	1	1	1	2	3	0	0	0	1	2	3	2	2
Braunsapis vitrea	1	3	1	1	0	3	1	3	0	1	0	0	1	2	1	2
Nasutapis sp.	1	3	1	1	0	3	1	3	0	1	0	4	0	1	1	2
Allodape friesei	0	3	1	1	0	2	1	3	0	1	0	0	1	2	1	2
Exoneurella micheneri	2	2	1	1	2	1	2	0	2	0	1	1	2	3	2	2
Exoneurella tridentata	2	2	1	1	2	1	1	0	2	0	1	1	3	2	2	2
Exoneurella lawsoni	2	2	1	1	2	1	1	0	2	0	1	1	2	3	2	2
Exoneurella setosa	2	2	1	1	2	1	1	0	2	0	1	1	2	3	2	2

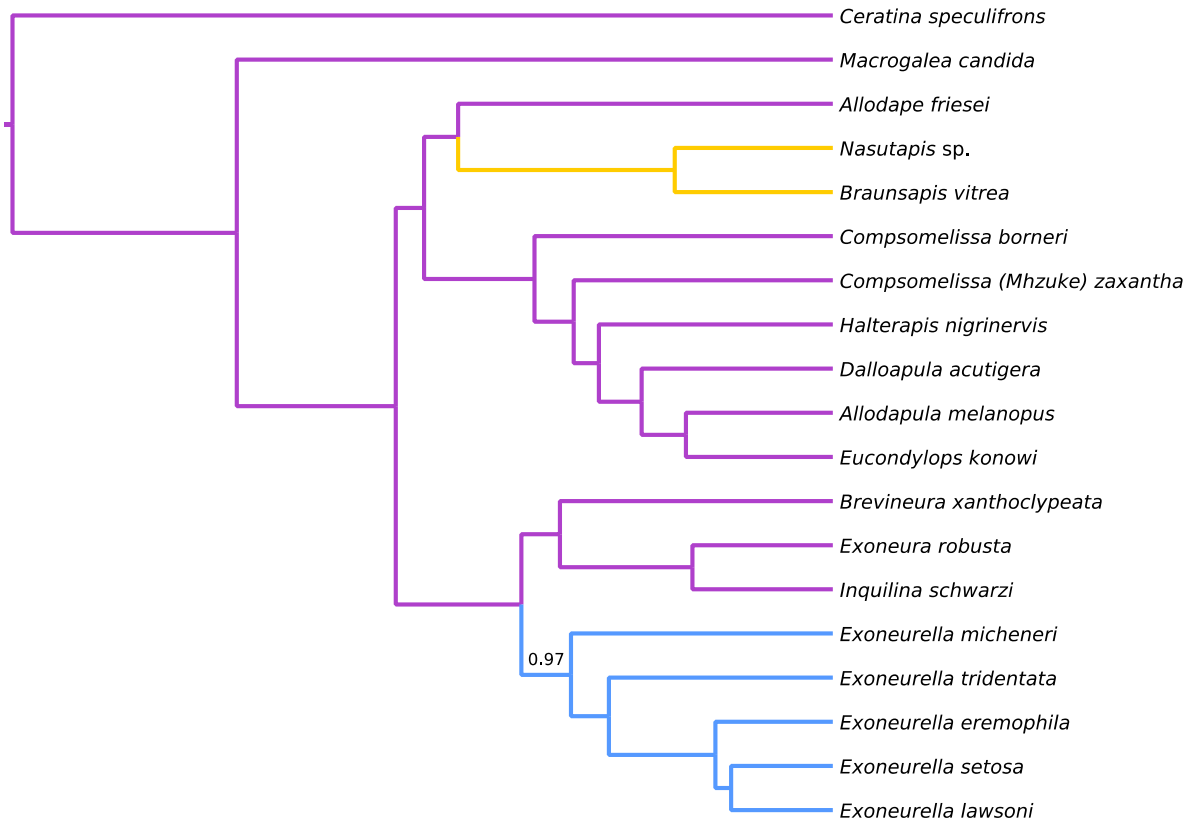

Fig. S1: Maximum credibility tree showing ancestral reconstruction of larval head shape. Branches show posterior probability if less than 0.99. Purple: spherical, Yellow: triangular, Blue: bilobed.

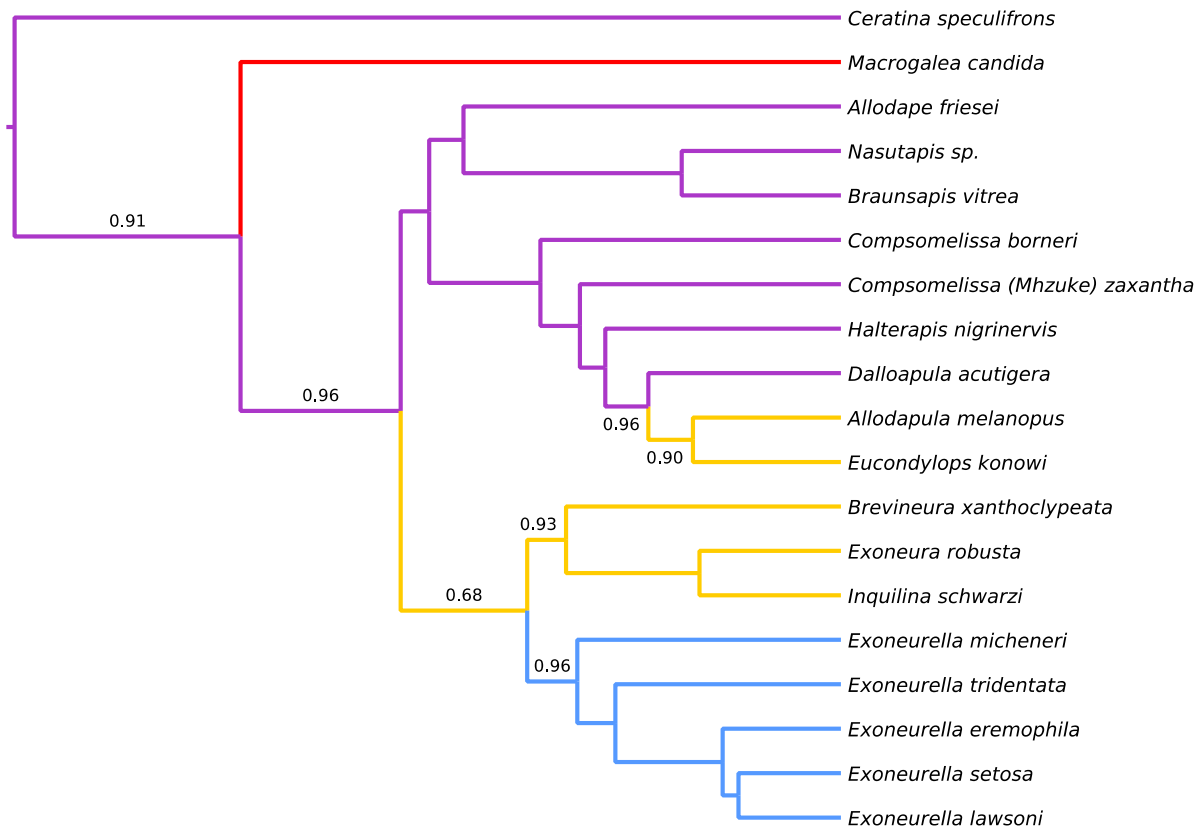

Fig. S2: Maximum credibility tree showing ancestral reconstruction of larval labrum morphology. Branches show posterior probability if less than 0.99. Purple: smooth margin, Yellow: margin not smooth, Blue: bilobed, Red: larbrum short and hairy.

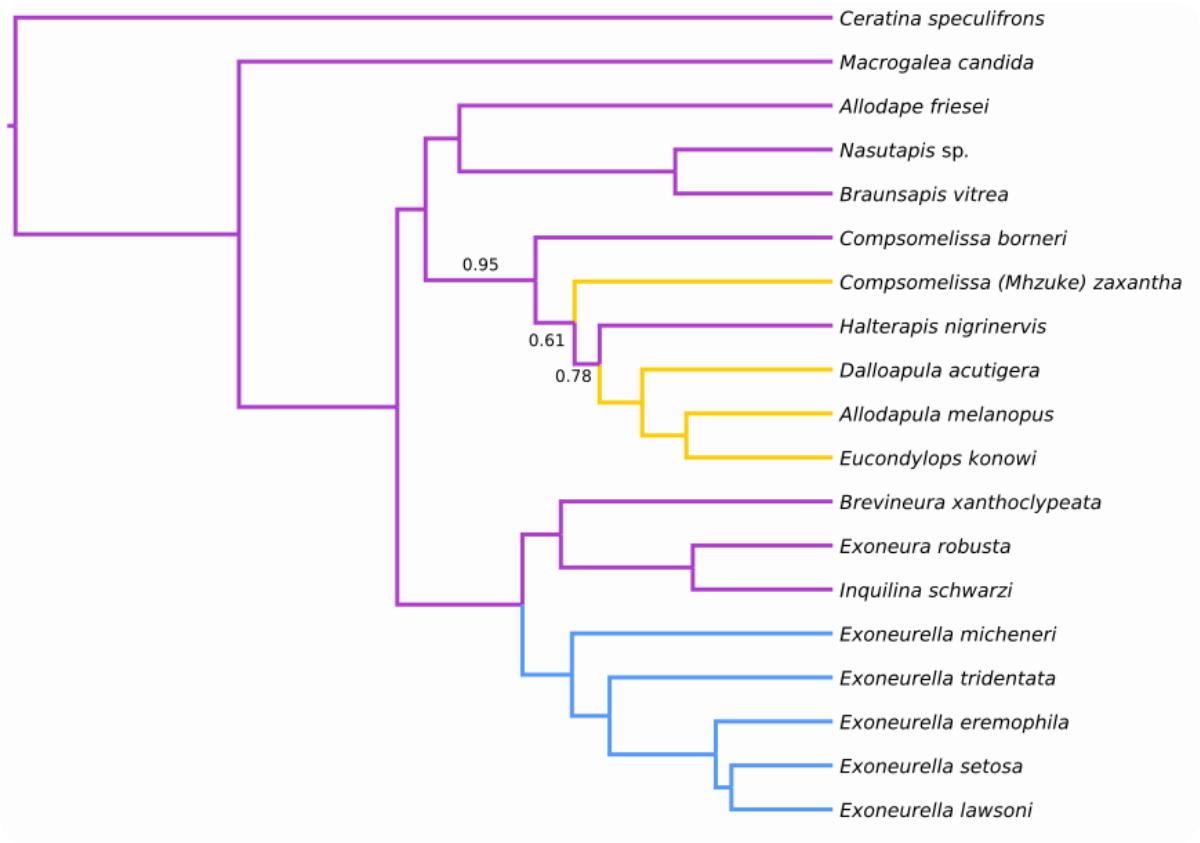

Fig. S3: Maximum credibility tree showing ancestral reconstruction of larval body shape. Branches show posterior probability if less than 0.99. Purple: smoothly curved, Yellow: almost straight, Blue: bent.

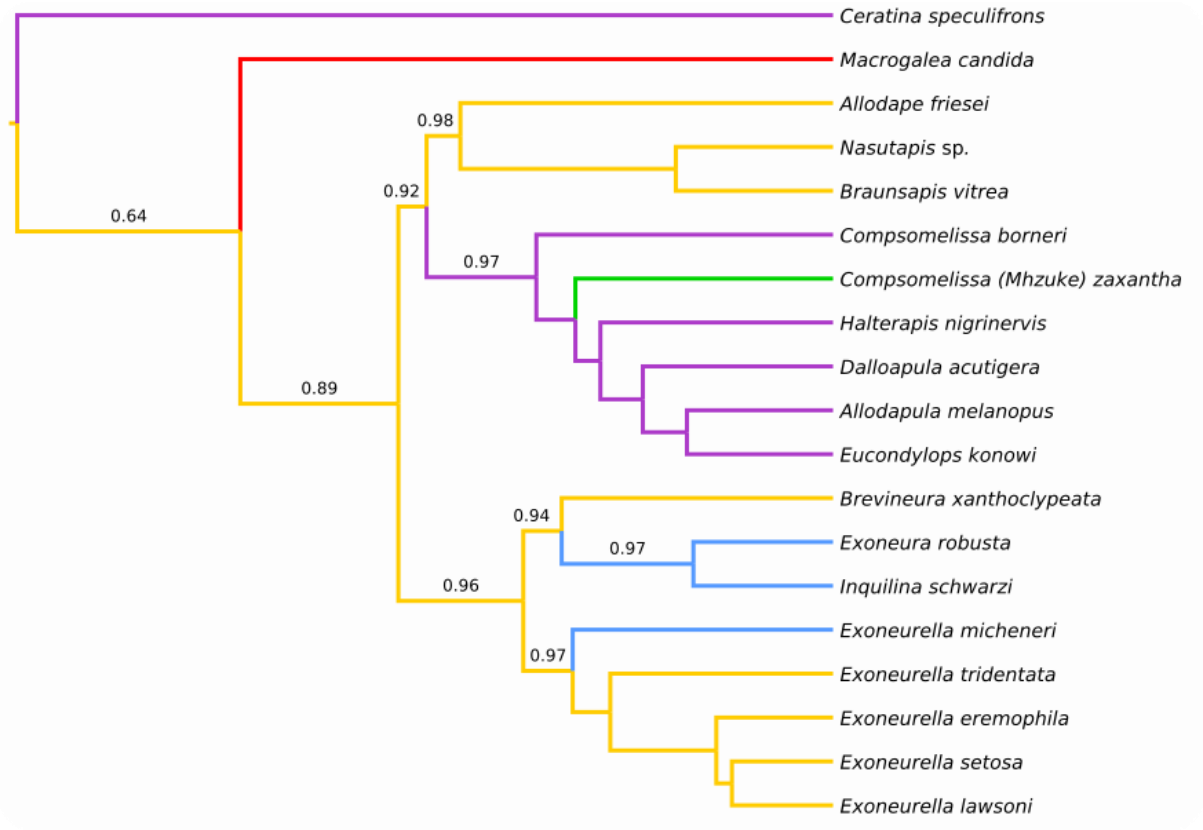

Fig. S4: Maximum credibility tree showing ancestral reconstruction of larval body hairs. Branches show posterior probability if less than 0.99. Purple: almost hairless, Yellow: hairs dorsal and ventral, Blue: hairs absent ventrally, Red: short hooked hairs, Green: hairs absent dorsally.

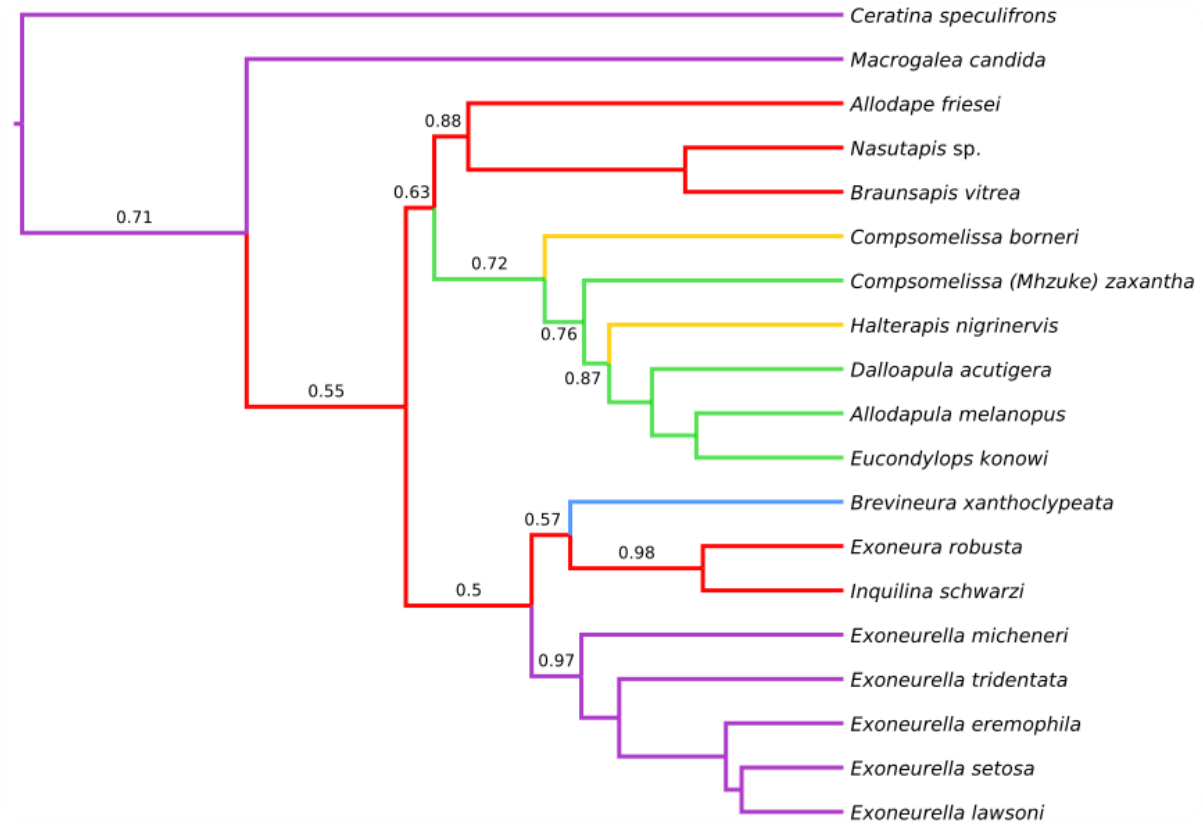

Fig. S5: Maximum credibility tree showing ancestral reconstruction of larval tubercles. Branches show posterior probability if less than 0.99. Purple: absent, Yellow: present lateral edges of 2nd and 3rd segments, Blue: tubercles elaborated into appendages on 2nd segment, Red: longitudinal rows ventrally, elaborate on segments 2 and 3, Green: longitudinal rows on ventral, dorsal and lateral surfaces.

GENERAL CONCLUSION

This thesis highlights the importance of studying the behaviour and ecology of bee species at diverse levels of sociality, and the complex role behaviour may play for pollinator persistence in the face of future climate change.

The importance of behavioural studies, in particular of non-eusocial taxa, is reviewed in Chapter I. Insect social behaviour terminology is revised and the term 'casteless' is proposed to describe species completely lacking hierarchies. This compliments the terms 'communal' and 'quasisocial', which imply a lack of castes but have strong connotations regarding nest architecture. The use of the term 'casteless' empowers a 'bottom-up' approach to understanding social evolution (Crespi 2009). It broadens our comparative power between species, allowing phylogenetic independent comparisons, and the inclusion of non-traditional social behaviour models, such as parasitic wasps (Hu et al. 2012). Chapter I puts forward methods of best practice for identifying casteless taxa and highlights major knowledge gaps in our understanding of social evolution and social insect behaviour more broadly.

The bees of the Xylocopinae display a diverse range of behaviours, making them ideal candidates for comparative studies on social evolution. Additionally, this group has one true origin of eusociality in the allodapine bee *Exoneurella tridentata* (Hurst 2001). Chapter II exemplifies the value of identifying casteless species, finding that the facultatively social *Exoneurella setosa* is casteless, despite being a congener to the eusocial *E. tridentata*. Social colonies of *E. setosa* were found to have minimal benefits over solitary nesting, and nest

sites appear abundant. This led to the hypothesis that small barriers to social living, coupled with low barriers to dispersal may facilitate casteless behaviour. The opposite, where high dispersal risk with limited nestsites may have facilitated the evolution of high reproductive skew, has been previously suggested for *E. tridentata* (Dew et al. 2012), and corresponds to theories on social evolution in eusocial molerats (Sichilima et al. 2008; Jarvis et al. 2005), as well as theoretical models of social evolution (Avila and Fromhage 2015; Fu et al. 2015).

Chapter III continued to explore the social evolution of *Exoneurella,* finding that *E. eremophila* also has casteless social groups. The occurrence of two casteless species in this genus suggests that casteless behaviour is a persistent and successful social strategy. *E. eremophila* joins a growing number of identified casteless species including *Amphylaeus morosus* (Colletidae: Hylaeinae; Spessa et al. 2000), *Euglossa hyacintha* (Apidae: Euglossini; Soucy et al. 2003) and the allodapine bees *Braunsapis puangensis* (da Silva et al. 2016) and the genus *Macrogalea* (Schwarz et al. *in review*). *Macrogalea* form the sister group to all other allodapine bees, so their casteless behaviour raises the possibility that castelessness is an ancestral social behaviour for the tribe as a whole. While conclusions regarding the ancestral social state cannot currently be drawn, the prevalence of casteless behaviour in the Allodapini indicates that social evolution in this tribe has not followed a strict pathway towards increasing hierarchical complexity.

Increasing knowledge of bee social behaviour and ecology can inform studies looking at risks to current bee populations. This is particularly relevant with climate change already linked to population declines in bumblebees (Kerr et al. 2015; Cameron et al. 2011). Chapter IV uses mitochondrial CO1 sequences to reconstruct the historical demography of the well-

studied *Ceratina (Neoceratina) australensis* (Xylocopinae: Ceratinini). This study reveals that this species likely underwent a rapid population expansion at the close of the Last Glacial Maximum, matching the timing of population expansions of halictine bees in Fiji (Groom et al. 2013) and *Ceratina* of the subgenus *Zadontomerus* in temperate North America (Shell and Rehan 2016). *Ceratina australensis* occupies a wide range of climates, from semi-arid to temperate to subtropical, making it an ideal species by which to establish a baseline response to historical global warming.

The Last Glacial Maximum was a period of increased aridification (Hesse et al. 2004), so arid-zone taxa could be expected to have had different responses to climate change during this period, compared to tropical species. Both *E. tridentata* and *E. setosa* live in semi-arid to arid zones, though *E. setosa* ranges more widely and is also found in temperate and subtropical regions. This chapter compares the historical demography of these species, finding a population expansion in *E. tridentata*. This was timed to the close of the Last Glacial Maximum though potential errors with this date estimate due to the difficulties in assessing generation time for eusocial species are discussed. Surprisingly, *E. setosa* does not show any population size changes over a period of 50-100kya. However, *E. setosa* and *C. australensis,* who are commonly sympatric, have strikingly similar population genetic structure. These results suggest that responses to climate change are complex and rely on a matrix of climate pre-adaptation, behaviour and ecology.

The final chapter of this thesis described a new species, *Exoneurella micheneri,* and raised *Inquilina* **stat. r.** to a full genus, rather than a subgenus of *Exoneura*. Phylogenetic reconstruction using molecular and morphological data placed *E. micheneri* as basal to all

the other *Exoneurella*. The description of this species will facilitate studies on social evolution. Likewise, the reinstatement of *Inquilina* to generic level has important implications for studies of social parasitism. This classification reflects the reciprocal monophyly of *Inquilina* and *Exoneura* (Bull et al. 2003; Schwarz et al. 2003; Smith et al. 2007), and brings the taxonomy of these genera in-line with other socially parasitic allodapine genera. Monophyly of parasite and host genera is an important consideration for studies of social parasitism, and this change will assist future research.

Together, the chapters of this thesis provide insights into social behaviour and its evolution. The identification of casteless behaviour will likely allow valuable comparisons for taxa outside the scope of this thesis including other Xylocopinae but also the behaviorally diverse halictines and euglossine bees. Insect pollinators are taxonomically widespread, with a variety of behaviours and understanding how they will respond to future climate change is a complex task. This thesis supports that notion that more generalist behaviour, with flexibility in strategies may promote population stability (Packer et al. 2005) but the interaction of behaviour, ecology and phylogenetics is complex. This will prove a challenge for the future.

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