Population genetic and behavioral aspects of male mating monopolies in *Cardiocondyla venustula*

DISSERTATION ZUR ERLANGUNG DES DOKTORGRADES
DER NATURWISSENSCHAFTEN (DR. RER. NAT)
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Der Promotionsgesuch wurde eingereicht am: 12.06.2020

Die Arbeit wurde angeleitet von: Prof. Dr. Jürgen Heinze

Unterschrift:

Manuscripts that contributed to this thesis and author's contributions

Chapter 2:

Jacobs, S. and Schrempf, A. (2017). **Male-male competition in ants (Hymenoptera: Formicidae).** *Myrmecological News 24: 31-42*
Author's contribution: SJ and AS performed literature research and wrote the manuscript. All authors authors read, reviewed and accepted the final version of the manuscript.

Chapter 3:

Jacobs, S. and Heinze, J. (2017). **Between fighting and tolerance: reproductive biology of wingless males in the ant *Cardiocondyla venustula.*** *Insect Science 24(5): 818-828*
Author's contribution: SJ and JH designed the study. SJ performed the experiments and analyzed the data. SJ and JH wrote the manuscript. All authors authors read, reviewed and accepted the final version of the manuscript.

Chapter 4:

Jacobs, S. and Heinze, J. (2019). **Population and colony structure of an ant with territorial males, *Cardiocondyla venustula.*** *BMC evolutionary biology 10: 115*
Author's contribution: SJ and JH conceived and designed the experiments. SJ conducted molecular experiments and analyzed the data, SJ and JH wrote the manuscript. All authors read and approved the final manuscript.

Contents

1 General Introduction

1.1 Sexual selection in social insects

When Darwin first established his theory of evolution by natural selection, he faced phenomena that appeared to be completely contradictory to his groundbreaking ideas from "On the origin of species" (Darwin, 1859): extravagant, seemingly non-adaptive traits in males of many species, with the often cited peacock's tail as the best-known example, and sterile castes in eusocial insects. The two concepts – sexual selection and inclusive fitness theory – still are some of the most fascinating and intriguing of the evolution of species.

On the one hand, sexual selection has led to the evolution of some of the most stunning features of animals – from the peacock's tail mentioned above to impressive weapons like the stag's antlers to a range of seemingly odd courtship behaviors (e.g., Cronin, 1991). Over the course of the last decades, numerous contributions, both theoretically and experimentally – with the help of modern genetic and genomic techniques – have deepened our understanding of sexual selection theory (e.g., Andersson, 1994; Andersson and Simmons, 2006; Kuijper et al., 2012). On the other hand, the evolution of eusociality brought forth a fascinating range of extraordinarily successful species with colonies that can consist of thousands and thousands of individuals acting as one "superorganism" (e.g., Wilson and Hölldobler, 2009). Eusociality evolved independently at least nine times in Hymenopteran lineages (Hughes et al., 2008a). With more than 12,000 species known so far, ants account for a considerable number of the approximately 19,000 eusocial Hymenopteran species. They inhabit all continents except Antarctica and can be key players in or dominate entire ecosystems (Hölldobler and Wilson, 1990; Baer, 2014). Similarly to the field of sexual selection, theoretical, mathematical and experimental studies have led to a literally overwhelming body of knowledge on the evolution of eusociality and kin selection theory (e.g., Hughes et al., 2008a; Boomsma, 2013; Liao et al., 2015).

One could conclude that the combination of these two intriguing evolutionary concepts was even more fascinating, however, for a long period of time, researchers usually concentrated on either sexual selection or eusociality, with little to no overlap between these research areas (e.g., Boomsma et al., 2005; Boomsma, 2007; Baer, 2014). Mating systems and paternity distribution in social Hymenoptera were mainly focused on their consequences for kin selection and sexual conflict (e.g., Boomsma and Ratnieks, 1996; Boomsma et al., 2005). With a lack of obvious extravagant secondary structures that readily identify sexual selection (Baer, 2014) and a very short mating period early in life, often hard to observe and impossible to manipulate, the topic remained largely unexplored (Boomsma, 2007), with the exception of a few behavioral studies in species with noticeable size differences within males, such as in harvester ants (Davidson, 1982; Abell et al., 1999). Concordant with kin selection theory and Hamilton's rule (Hamilton, 1964a,b) that predict high relatedness between colony members as a crucial precondition for eusociality, all eusocial Hymenopteran lineages evolved from solitary ancestors with strict lifetime monogamy (Hughes et al., 2008a; Boomsma, 2009). Thus, the evolution of sexually selected traits is constrained due to the exclusion of promiscuity as one of the major drivers of sexual selection (Boomsma, 2007). Furthermore, the high mortality associated with nuptial flights seen in most eusocial Hymenopterans renders lengthy pre-copulatory procedures of mate choice unlikely to evolve (Helft et al., 2015). In large mating swarms with scramble competition between males, flight abilities and overall vigor are presumably sufficient for the assessment of male quality (Boomsma, 2013). Similarly, in species exhibiting the "female calling syndrome" where females attract males by pheromones (Hölldobler and Bartz, 1985), the abilities necessary for detecting and reaching the female sexual may be adequate to select for male quality. However, as mating only occurs within a short time frame, queens of social insects rely on the sperm received during this period, without any chance to modify partner choice in later life (Baer, 2016). Mate choice could thus be expected to be particularly important for social insect queens (Helft et al., 2015).

The "standard" ant species with mating swarms and a single, singly-mated queen (thus a monogynous and monandrous colony) indeed leaves minute possibilities for sexual selection to work. But exceptions from this "standard" have been known for a long time and are presumably more than common (Heinze and Tsuji, 1995; Boomsma et al., 2005). One way to increased levels of sexual selection arises from the evolution of polyandry. Polyandry has evolved in a range of species (Baer, 2016), facilitating the evolution of pre- but particularly post-mating sexual selection: cryptic female choice or sperm competition. The adaptive significance of polyandry for social insect colonies has been intensely debated over the last decades (Boomsma and Ratnieks, 1996; Arnqvist and Nilsson, 2000; Crozier and Fjerdingstad, 2001; Kellner et al., 2007; Kronauer et al., 2007). Nevertheless, the apparent discrepancy between the number of observed matings and the paternity distribution in the queen's offspring and/or paternity skew implies a key role for sexual selection and sexual conflict (Jaffé et al., 2012). A growing number of studies reveal mechanisms of sexual selection facilitated by multiple mating of queens: sperm competition (Aron et al., 2016; Liberti et al., 2018) or mating plugs (Mikheyev, 2003), but so far no direct evidence of cryptic female choice. A factor which is highly influential on the potential for sexual selection is the mating location. In large aerial swarms, males may not be able to monopolize virgin queens during and, as described above, lengthy choice processes are likely to be selected against because of high mortality risks. Several ant species diverge from these "standard" mating procedures: mating takes place on the ground as in *Cataglyphis cursor* (Helft et al., 2016) or even within the ants' nest as in several *Hypoponera* and *Cardiocondyla* species (Foitzik et al., 2002; Heinze et al., 1998). The genus dealt with in this thesis, *Cardiocondyla*, described in more detail below, thus represents an ideal model for the observation of sexual selection at play.

The idiosyncrasies of social insects give rise to yet another fascinating phenomenon: inclusive sexual selection – the involvement of workers in mate choice in species, that mate in or close to their colonies. In the polyandrous ant *Cataglyphis cursor*, Helft et al. (2015) found evidence of worker aggression that directly influenced different males' access to virgin queens, while virgin queens themselves do not seem to actively choose their mates (Helft et al., 2016). Studies on sexual selection in these species with mating strategies deviating from the "standard model" did not only reveal fascinating new insights into life history of ants species in general, they also led to the inclusion of formerly neglected members of ant colonies: the males. Chapter 2 reviews the current knowledge of male competitive strategies in ants.

1.2 The ant genus *Cardiocondyla*

Although the genus *Cardiocondyla* is widespread across several continents and some of the species live in close proximity to human settlements, their presence often remains unnoticed by the casual observer, and even field entomologists (Seifert, 2003). Individuals are tiny and colonies comprise only a few dozen to a few hundred individuals, depending on the species. They are inconspicuous with only few workers visible at a time and, contrary to many other ant species, cause little to no nuisance, and may thus often be overlooked. Several species are cosmopolitan tramp ants, presumably spread by human transport (Heinze et al., 2006), but in contrast to infamous invasive ant species like *Solenopsis invicta* or *Wasmannia auropunctata* (e.g., Wittman, 2014), species of the genus *Cardiocondyla* have little, if any, detrimental effect on native ecosystems (Heinze, 2017).
Only a deeper look into colonies of *Cardiocondyla* species reveals the fascinating and extraordinary features of their behavior, their life history and their ecology. The most striking may be the unusual reproductive ecology with the existence of wingless, “ergatoid” (i.e. worker-like) males instead of or in addition to winged males (Kugler, 1983). Contrary to most other ant species, males mate inside the nest, have lifelong spermatogenesis and are thus able to monopolize matings. However, the genus *Cardiocondyla* is not only fascinating regarding its males. With a distribution across large parts of the Old World, inhabiting diverse habitats from tropical rain forests to dry savannas or temperate regions, species vary in life-history traits like colony structure, mating behavior and dispersal strategy (Heinze, 2017). Thus, the genus offers a great opportunity to study the evolution of reproductive strategies. In addition, several of the *Cardiocondyla* species have features that make them a convenient study object: unlike many other ant species they are easy to maintain and breed in a laboratory environment, have short generation times and intranidal matings are easily observable. Colonies can be manipulated or sexuals can be deliberately bred to each other to address specific ecologic, evolutionary or genetic questions.

To date, the phylogenetic origin of the genus *Cardiocondyla* within the myrmicine ants remains unclear with long and unstable branches in phylogenetic analyses (Ward et al., 2015). Several studies suggest that the geographic origin of the genus is the Southeast Asian ecozone, from where it spread to tropical and subtropical Asia and

Africa, as well as the Mediterranean. Polygyny and a distinct male diphenism with wingless fighter males along with peaceful winged males are ancestral within the genus (Oettler and Heinze, 2010). From this ancestral state, during the radiation of the genus numerous combinations of modifications in life-history traits arose: secondary monogyny (convergent in the monophyletic Palearctic clade as well as in some species of the *"C. argentea"* group), loss of winged males (convergent at least twice), mutually peaceful wingless males (in the Palearctic clade) (Oettler and Heinze, 2010) or even clonal reproduction with androgenesis in *C. kagutsuchi* (Okita and Tsuchida, 2016). It has been proposed that factors like density, stability and availability of suitable nesting sites as well as climatic conditions led to the evolution of different life-history strategies (Heinze, 2017). However, with several environmental variables and life-history traits (colony size, number of queens, presence or absence of winged males, male behavior) co-varying between the species, bringing light into the interrelation between these factors may well be impossible. Growing knowledge on a variety of *Cardiocondyla* species as well as life history of social insects in general allows for conclusions regarding possible evolutionary pathways of some of these traits. Similarly to the loss of winged males in fig wasp species with wingless fighter males (Hamilton, 1979), the loss of winged males in *Cardiocondyla* is supposed to be associated with the likelihood of nests being without resident males (Oettler and Heinze, 2010). In *Cardiocondyla*, this likelihood can both be influenced by an increased longevity in males (as in species of the *"C. argentea"* group like *C. "argyrotricha"*) and by larger brood sizes and seasonal production of sexuals (as in species of the Palearctic clade): Although living in the same ecozone under similar climatic conditions with a year-round production of low quantities of sexuals, *C. obscurior* and *C. wroughtonii* produce both winged and wingless males, while species of the *"C. argentea"* group only produce wingless males (Oettler and Heinze, 2010). The former use ephemeral nest sites like leaves or plant gall, whereas the latter nest in more stable environments like cavities in rocks, soil or behind tree bark. This may have led to the evolution of long-lived males in the *"C. argentea"* group, resulting in a low probability of nests being without a resident male and thus providing few mating chances for winged disperser males. On the other hand, loss of winged males in the Palearctic clade, *C. venustula* and *C. mauritanica* may have been a consequence of larger brood sizes and a more seasonal production of sexuals compared to related species like *C. emeryi*, *C. minutior* or *C. tjibodana* (but see

Heinze and Delabie (2005) on seasonal production of female sexuals in *C. obscurior*), increasing the probability of nests being without males and thus the chance of winged males finding unmated queens in the latter.

Unlike many of these traits, which evolved convergently in different species, the difference in mandible shape of wingless males is strongly correlated with phylogeny: Wingless males from the *"C. wroughtonii"* and the *"C. argentea"* group possess sickle-shaped mandibles ("clade A" in Oettler and Heinze, 2010), while those of the other clade possess shear-shaped mandibles ("clade B" in Oettler and Heinze, 2010) (Figure 1.1). However, as none of the related genera possess wingless males, it remains futile to speculate on the question which shape is ancestral and which environmental factors led to the evolution of one or the other.

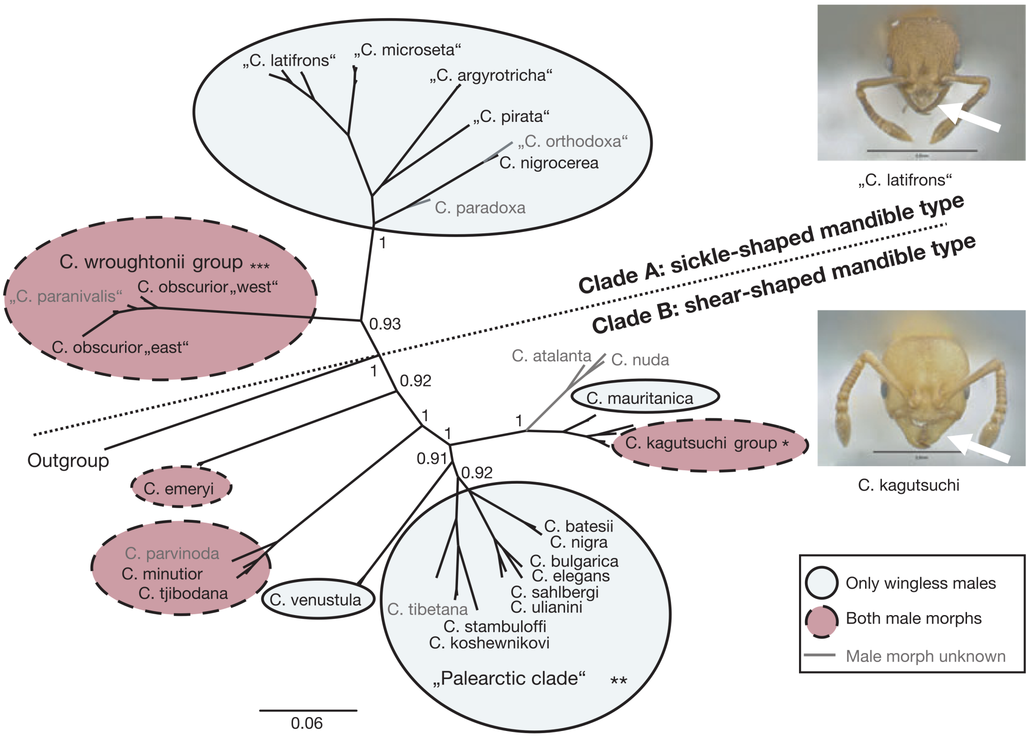


Figure 1.1: Phylogenetic tree of *Cardiocondyla* species highlighting male morph and male mandible shape (Oettler and Heinze, 2010)

Difference in mandible shape influences male agonistic behavior (Schmidt and Heinze, 2018). Sickle-shaped mandibles allow the males to pierce the still soft cuticle of callows and enable them to hold down other adult males. Males of *C. obscurior* and *C. wroughtonii* engage in long-lasting fights (up to 60 h (e.g., Cremer et al., 2012)): they grasp and hold down their opponent and try to besmear it with a substance that elicits aggressive behavior by workers, regularly leading to the loser's death. Males of several species with shear-shaped mandibles crush the cuticle of freshly eclosed callows, while fighting between adult males is rare (Schmidt and Heinze, 2018). In the Palearctic clade, male fighting has been lost completely. In these species typically living in xerothermic environments, colonies may contain several ergatoid males coexisting peacefully (e.g., Schrempf et al., 2005b; Boomsma et al., 2005). Species from the Palearctic clade exhibit a seasonal production of sexuals (Heinze, 2017). With high numbers of promiscuous female sexuals emerging at the same time, a single male may not be capable of inseminating all of them, and monopolizing young queens by eliminating nestmate males in the comparatively large and complex colonies would be fighting a losing battle (Oettler and Heinze, 2010). Moreover, due to local mate competition, mother queens do not produce excess males (Schrempf et al., 2005b). Thus, the temporal availability of female sexuals as well as sex ratio presumably influence the evolution of male behavior.

The study species of this thesis, *Cardiocondyla venustula*, occupies an intermediate phylogenetic position between the Palearctic clade with mutually peaceful males and the other species in clade B with fighting males (Oettler and Heinze, 2010). *C. venustula* was first described from Puerto Rico (Wheeler, 1908), but is probably of African origin, where it has been collected in several countries such as South Africa, Zimbabwe or Ethiopia (Seifert, 2003; Frohschammer and Heinze, 2009). Similarly to other *Cardiocondyla* species, *C. venustula* has spread to formerly uninhabited areas like the Caribbean and other parts of the world as a tramp ant, facilitated by human transport. While *C. venustula* is widespread in anthropogenically disturbed, open habitats such as parking lots, roadsides or gardens, the natural habitat can only be assumed to be naturally disturbed areas with open soil e.g., close to rivers or lakes or sparsely vegetated steppes (Seifert, 2003). Colonies of *C. venustula* inhabit multi-chambered nests in the soil, often in open, sparsely vegetated areas (Figure 1.2). Some of the colonies decorate their nest entrances with small plant material or body parts of other ants (Figure 1.3). The cause and function of this "decoration" is yet unknown.

Similarly to several other species from the genus, winged males have been lost in *C. venustula*. However, in addition to the typical wingless *Cardiocondyla* males,

Figure 1.2: Nesting site of *Cardiocondyla venustula* colonies

some *C. venustula* colonies from South Africa were found to produce “intermorphic” males that combine features of wingless males – big heads with strong, shear-shaped mandibles – with those of winged males – pronounced thoracic sutures, ocelli and vestigial wings (Heinze et al., 2013). “Intermorphic” males are usually larger than typical wingless males, but variability between the males considering body size, wing size and number of ocelli is high (own observation, Heinze et al., 2013). The occurrence of “intermorphic” males in *C. venustula* as well as in some lineages of the *C. kagutsuchi* species complex (Yamauchi et al., 2005) and the convergent loss of winged males in several species show the high phenotypic plasticity in *Cardiocondyla* males, facilitated by a novel developmental switch (Oettler et al., 2019). What makes *C. venustula* even more interesting and fascinating is the fact that the intermediate phylogenetic position between species with fighting males and the Palearctic clade with peaceful males is mirrored in the males’ behavior: in a pilot study, Frohschammer and Heinze (2009) discovered that *C. venustula* males occupy small territories in their natal nests.

Chapter 3 of this thesis describes this fascinating behavior and the factors that might influence male success in obtaining and defending territories in more detail.

Figure 1.3: Decorated nest entrance

1.3 Population genetics in social insects

The genetic structure of social insect populations is shaped by both species-specific and environmental factors. Population size, dispersal ability and inbreeding interact with external influences like climate and availability of suitable habitats (Foitzik et al., 2011). Molecular population genetics can thus serve as a valuable tool for the research of social insect colonies and populations, shedding light on otherwise hard to resolve questions like paternity (or maternity) skew and the interrelation between genetic structure and social organization (Pamilo et al., 1997; Ross, 2001).

As described above, *Cardiocondyla* species display a wide variety of life-history strategies. They differ in the number of queens per colony (polygyny or monogyny), in the number of males mating with one female (polyandry vs. monandry), the dispersal ability of sexuals (winged and wingless male and female sexuals) and colony size. These mostly species-specific factors interact with external factors, such as different environmental conditions (from humid to xerothermic), different climatic conditions (temperate climate with seasonal changes vs. tropic climate), distribution of habitats (clumped, patchy or nearly ubiquitous) or stability of habitats. These factors are expected to influence population genetic structure of the species. With mating inside the nest being the rule for *Cardiocondyla* species, many exhibit high levels of inbreeding (e.g., Lenoir et al., 2005, 2007; Heinze et al., 2014; Schmidt et al., 2016). While inbreeding has the potential of adverse affects on colonies and individuals, tolerance to high levels of inbreeding may be crucial for tramp species (Eyer et al., 2018). With only few founders transported to a new, previously uninhabited environment, genetic resources are small and inbreeding levels of the introduced population will be high. However, tolerance to inbreeding may be a beneficial preadaptation as well as a consequence of introduction events (Tsutsui and Suarez, 2003). In the former case, both native and introduced populations are expected to show high levels of inbreeding resulting in low genetic diversity, in the latter, genetic diversity is expected to be lower in introduced populations . Several species of the genus *Cardiocondyla* have also evolved strategies to increase their colonies' genetic variability despite the loss of winged males as dispersers in many of them. Most of the tropical species are usually polygynous, while the monogynous palearctic species are polyandrous. Some of the latter have even evolved more fascinating strategies. Besides accepting alien young queens in their nests, they "trade" sexuals by transporting pupae into foreign nests (Lenoir et al., 2007). In the case of *C. venustula*, I was interested in how the peculiar mating system affects colony structure and population genetics. Furthermore, with the species' intermediate phylogenetic position between the Palearctic and the tropical clade, resolving colony structure of *C. venustula* might provide further insights into life-history evolution of the genus. Chapter 4 of this thesis addresses the topic of population genetics in the species *C. venustula*.

Aims of the thesis

The genus *Cardiocondyla* offers a unique opportunity to study reproductive strategies and sexual selection in social insects. With an intermediate phylogenetic position between species with fighting and species with mutually peaceful males, *C. venustula* represents an interesting model for the study of the evolution of these strategies. Apart from some more intensively researched genera such as *Cardiocondyla* (e.g., Heinze et al., 2005; Cremer et al., 2011; Oettler and Heinze, 2010) or *Hypoponera* (e.g., Yamauchi et al., 2001; Foitzik et al., 2010; Kureck et al., 2011), male mating behavior and male competition has only sporadically been in the focus of social insect studies. In Chapter 2 of this thesis, I thus review the knowledge on male competition in ants and place the competitive behavior of *Cardiocondyla* males into a bigger context. In the following Chapter 3, the fascinating behavior of territorial males in the species *C. venustula*, that had first been described by Frohschammer and Heinze (2009), is studied in greater detail. Since mating strategies, including variation in mating location and dispersal, influence population genetic parameters and vice versa, I investigated molecular population genetic parameters of *C. venustula* in Chapter 4 of this thesis. Population genetic tools also allow for conclusions on different life-history traits such as the number of matings or the mode of colony founding. This thesis thus investigates the intriguing strategy of *C. venustula* males from several different perspectives.

2 Male-male competition in ants (Hymenoptera: Formicidae)

Susanne Jacobs & Alexandra Schrempf

Institut für Zoologie, Universität Regensburg, Germany

2.1 Abstract

Aspects of male mating behavior in ants have been largely ignored in research until recently. Mating in ants is usually a short episode at the beginning of their life, often in large, anonymous swarms; therefore it has been argued that the potential for male-male competition is limited. Despite of this, several earlier studies on mating in ants described that males heavily compete for female sexuals, indicating pre-copulatory male competition. In the last few years, more and more studies investigating post-copulatory competition have been conducted, and they revealed fascinating examples of male traits to gain fertilization advantages over other males. Ant species exhibiting intranidal mating have been researched thoroughly, providing new insights also into pre-copulatory conflicts. We review what is known so far on male-male competition in ants. Further studies may uncover additional unknown male competitive tactics and provide a better understanding of sexual selection in ant males, and we believe these studies offer an ideal system to compare male competition in social and non-social insects.

Key words: male competition, male traits, mating, pre-copulatory, post-copulatory, fighting, review

2.2 Introduction

The mating biology of ants

While the fascinating cooperative behavior of social insects in general and ants in particular has been studied intensively for the last decades, mating biology has been largely neglected (e.g., Baer, 2014). Research on conflict in ant societies has rather been focused on fitness optimizing strategies and reproductive conflict of female individuals than on competitive behavior between males (e.g., Boomsma et al., 2005). This has several reasons. In most social hymenoptera, mating is a short event early in life, with males serving only as “flying sperm missiles” (Shik et al., 2013) and queens never remate after starting reproduction, resulting in a lifelong partner commitment (e.g., Boomsma et al., 2005). Exceptions have been discussed to occur in army ants (e.g., Raignier and Boven, 1955; Rettenmeyer, 1963; Denny et al., 2004; but see Kronauer and Boomsma, 2007) and found recently in some species of

Cardiocondyla ants, where virgin queens start reproducing by laying haploid eggs and mate only afterwards with their own sons (Schmidt et al., 2014, N. Moske, J. Heinze & A. Schrempf unpubl.). This short time frame and the often inconspicuous mating make observation of nuptial flight in social hymenoptera difficult. Even if researchers are in the right spot at the right time to witness a mating, it is often difficult to observe copulations in detail due to mating locations and the number of participating individuals. The evolution of male traits in social insects in general has been reviewed by Boomsma et al. (2005) and male mating behavior of bees has been reviewed by Paxton (2005), but a review on male mating behavior, especially male - male competition in ants does not exist so far.

Hölldobler and Bartz (1985) distinguished between two main mating syndromes in ants: the “male aggregation” and the “female calling” syndrome. In case of the male aggregation syndrome, males take off in large aggregates and attract female sexuals chemically, which enter the mating swarm and mate with one or several males. These swarms can consist of thousands or even millions of individuals (e.g.: “24 to 60 millions of ants were involved in this event” described by Carlton and Goldman (1984) for *Lasius alienus*), and individuals may fly and mate up to 250 m above ground or even higher (Hölldobler and Wilson, 1990), making it complicated if not impossible to track individual behavior. Swarming is usually triggered by environmental cues to synchronize the timing of taking off for flight, often after rain, as it makes it easier for queens to dig in humid soil. In such swarms, males frequently outnumber female sexuals, and some authors describe that males struggle for access to female sexuals. For example, in (Wheeler, 1916), observations of W.W. Froggatt of mating in bulldog ants are described: “As soon as a male (and there were hundreds of males to every female) captured a female on a bush, other males surrounded the couple till there was a struggling mass of ants forming a ball as large as one’s fist”. Brown (1955) described mating in *Notoncus ectatommoides*:“in a few seconds, the female was surrounded by a dense swarm of males in the form of a ball” (c.f. Hölldobler and Wilson, 1990). Generally, species that exhibit the male aggregation syndrome are often characterized by large colony sizes and a seasonal production of large numbers of reproductives (Hölldobler and Wilson, 1990; Grueter and Keller, 2016). Obviously, such mating swarms are usually not inducible in the laboratory due to confined time, space and number of colonies. Furthermore, the exact conditions needed to elicit the swarming behavior may be difficult to simulate under laboratory conditions (but see e.g., breeding experiments with different *Formica* species by Gösswald and Schmidt, 1960).

In the case of the "female calling syndrome", usually fewer individuals are involved and copulations happen near or on the ground. Virgin queens disperse close to the nest and use pheromones to attract males searching for mating partners. Colonies of female calling species are often small at maturity and produce relatively few reproductives (Hölldobler and Wilson, 1990; Peeters and Ito, 2001). This system has been described for several myrmicine, ponerine and also formicine ants (Hölldobler and Haskins, 1977; Buschinger and Alloway, 1979; Haskins, 1978; Lenoir et al., 1988; Villet, 1999). However, variations of these two mating strategies and intermediate stages are manifold, and can impact e.g., dispersal distance from the nest, dispersing sex, aerial or ground mating and the number and sex ratio of participating individuals (Hölldobler and Wilson, 1990; Ayasse et al., 2001; Peeters and Ito, 2001; Peeters and Molet, 2010). Similarly, species within a genus can differ in mating syndrome. In the genus *Formica*, the male aggregation syndrome has been described for some species (Marikovsky, 1961; Talbot, 1972; O'Neill, 1994; Mori et al., 2001), whereas other species exhibit female calling (for instance Kannowski and Johnson, 1969; Halverson et al., 1976; Henderson and Jeanne, 1992; Cherix et al., 1993). Moreover, the range of dispersal during flights can vary dramatically: from "several tens of meters" in *Cataglyphis cursor* males (Lenoir et al., 1988) or "a few hundred meter" for *Solenopsis invicta* gynes (Goodisman et al., 2000) to "up to ten miles" for *Solenopsis saevissima richteri* (Markin et al., 1971). However, data of most studies are calculated on genetic markers and do not distinguish between pre- and postmating dispersal. A recent study estimated dispersal distance based on queen range from the natal colony and suggested a median distance of 60 m for *Formica exsecta* gynes (Vitikainen et al., 2015). The female calling syndrome with small numbers of sexuals mating close to their nest makes matings easier to observe and offers the opportunity to research male strategies and reproductive success. In addition, there are several detailed descriptions of mating in *Pogonomyrmex* harvester ants, in which mating is unique and shows "strong parallels to vertebrate lek systems" with male aggregations (Hölldobler, 1976; Davidson, 1982). Mating takes place on the ground or on bushes and trees, and males strongly compete for queens (Hölldobler, 1976).

In a much smaller number of species, mating takes place within the nest: this is for example the case for some social parasites (e.g., *Epimyrma* sp.: Winter and

Buschinger, 1983, Buschinger and Winter, 1982, Buschinger, 1989; *Rhoptromyrmex*: Bolton, 1986; *Plagiolepis xene*: Trontti et al., 2006), a few unicolonial species (e.g., *Monomorium pharaoni*: Peacock et al., 1950, *Lasius sakagamii*: Yamauchi et al., 1981, *Linepithema humile*: Keller and Passera, 1992) and in the genera *Hypoponera* (e.g., Foitzik et al., 2002) and *Cardiocondyla* (e.g., Heinze and Hölldobler, 1993). In several of these, neither males nor female sexuals disperse before mating and thus mate with their nestmates, often resulting in high inbreeding levels (e.g., Schrempf et al., 2005b; Lenoir et al., 2007). Some species produce dispersing sexuals in addition to or alternating with dealate sexuals (e.g. in *Cardiocondyla* sp.: Heinze et al., 2004, *Hypoponera* sp.: Foitzik et al., 2010, *Plagiolepis xene*: Kutter, 1952, Passera, 1964, Trontti et al., 2006). In other species, males still fly for mating with apterous female sexuals inside or in the surrounding of the colony (several doryline species, e.g. *Eciton* sp.: Rettenmeyer, 1963, Franks and Hölldobler, 1987, Peeters, 1991 and *Harpagoxenus sublaevis*: Buschinger and Alloway 1979). Finally, there are even cases where both sexes are flightless: apterous queens together with apterous males have been described in *Hypoponera opacior* (Foitzik et al., 2002), and short winged queens together with wingless males in *Cardiocondyla batesii* (Schrempf and Heinze, 2007). In case of apterous males, local mate competition will influence the sex ratio of the colony and result in a female bias, as for example shown in *Cardiocondyla obscurior* (Cremer and Heinze, 2002). Species with intranidal mating often also mate under laboratory conditions and thus make perfect subjects for studying mating in ants.

Male traits

As already mentioned above, the evolution of male traits in social insects including ants has been reviewed in detail by Boomsma et al. (2005). Similarly, there are two excellent reviews on the copulation biology of ants as well as on sexual selection in social insects by Baer (2011, 2014), and we thus describe the male phenotype briefly. As in all haplodiploid species, male ants arise from unfertilized eggs. In the social environment, males are probably the "least social" individuals, as they usually only stay within the colony for a short period of time before leaving to mate with female sexuals after which they die. Thereby, males are not engaging in social activities with "...their repertory almost always limited to grooming themselves and receiving food from the workers" (Hölldobler and Wilson, 1990). Exceptions are found in *Hypoponera* sp., where ergatoid males engage in stomodeal trophallaxis (mouth-to-mouth transfer of food) with queens (Hashimoto et al., 1995) and in *Camponotus* sp., where stomodeal trophallaxis between males and other members of the colony has been observed (Hölldobler, 1964, 1966, c.f. Hölldobler and Wilson, 1990). With a few exceptions (e.g., *Cardiocondyla* sp., Boomsma et al., 2005), ant males are short-lived and their testes degenerate after eclosion, therefore they can often mate only once or a few times (up to 10 times, see Boomsma et al., 2005) during a short period of time (Keller and Passera, 1992).

The characteristics of male ants' traits and even male longevity depend on the mating syndrome of the species (Shik and Kaspari, 2009; Shik et al., 2013). Hölldobler and Wilson (1990) already mentioned that males are often larger and "more robust" in case of female calling species, while they are smaller in species exhibiting the male aggregation syndrome. Usually, ant males have a bulky thorax and are winged. For orientation during mating flights, either in large swarms or in search of a calling female, most ant males have large compound eyes and ocelli while species mating on the ground close to the nest may have smaller compound eyes (Shik et al., 2013). In species mating in the nest, wingless male types have evolved, known from the genera *Cardiocondyla*, *Hypoponera* and *Formicoxenus* (see Kinomura and Yamauchi, 1987). These males resemble workers, usually possess small compound eyes and have reduced ocelli or completely lack them (e.g., in *Cardiocondyla*, see Kugler, 1983). Even in case males stay inside of the colony for a longer period, they do not seem to contribute to social life. Furthermore, in these wingless males, male behavior is usually limited to auto-grooming, receiving food from the workers and patrolling the nest in search of female sexuals or rival males (but see Heinze et al., 1993 for males carrying brood).

If and how male competition can arise depends on several factors – e.g., mating system, mating site or colony structure. While swarm mating bears few chances for competition before and during mating and flying ability might be the only factor for queens to assess male quality, mating in confined spaces – in territories, leks, on the ground close to the nest or in the nest give the opportunity to monopolize female sexuals or to gain fitness by being more "attractive" to them. Only in case females mate multiply, i.e. under polyandry, competition after copulation might occur and result in the evolution of postcopulatory male traits to gain fertilization (see below). If queens mate with several males, males might try to prevent rivals from mating as multiple insemination can reduce a male's fitness by lowering its contribution to the queen's offspring(Allard et al., 2002). Especially in polygynous colonies, where competition between queens occur, queens may have greater interest in influencing the number and timing of male production (e.g., local mate competition, Cremer and Heinze 2002, de Menten et al., 2005, Foitzik et al., 2010) and workers might influence male mating success (Sunamura et al., 2011; Helft et al., 2015) or worker and male interests may be conflicting (Bourke and Franks, 1995; Schrempf et al., 2007).

In many solitary species, male-male competition may have detrimental effects on the females, for instance if males try to force females into investing more resources into offspring they sire even if this reduces the female's lifespan (e.g., Johnstone and Keller, 2000). In social insects however, sperm storage results in a lifelong partner commitment and queens have to establish sufficiently large colonies consisting of sterile workers before they can start to produce sexual offspring. Thus, reducing a female's lifespan will also entail a reduction in male fitness (Bourke and Franks, 1995; Schrempf et al., 2005a). Even if intersexual conflict seems to be reduced or even reversed to cooperation in social insects, male competition may be contrary to the females' interests. Due to haplodiploidy, males only profit from female sexual offspring, thus their interest lay in influencing the queens towards a more female-biased offspring. Contrary, queens share their alleles with both their male and female offspring and are expected to profit from a less biased sex-ratio (Boomsma, 1996). If sperm from one male is sufficient to establish a colony and raise sexual offspring, it would still be in the interest of males to monopolize paternity e.g., by sperm competition. For the queens, however, sperm competition may be beneficial in terms of selection for the fittest sperm, but in the long term, they will profit from having as much sexual offspring as possible. Thus, queens are expected to eventually terminate male-male competition, e.g., by stopping or relaxing sperm competition (den Boer et al., 2010). Studies on paternity skew suggest queen influence on the use of sperm resources: a meta-analysis revealed that in species with low paternity frequencies, paternity skew is higher than expected (Jaffé et al., 2012), sometimes even resulting in monandrous offspring in spite of multiple queen mating (Boomsma and Ratnieks, 1996; Boomsma and Sundström, 1998; Boomsma and Van der Have, 1998).

In this review, we summarize data on male-male competition in ants. Studies on pre-copulatory competition reach back as far as to the beginning of the 20th century, while post-copulatory competition has been addressed only more recently. In the following, we distinguish between male-male competition before and during mating, and post-copulatory male competition. While direct fighting as observed e.g., in *Cardiocondyla* species is probably the most conspicuous form of competition, many more aspects and strategies of male-male competition can be found in ants.

2.3 Pre-copulatory male competition

Interference with other males during mating

Direct competition between ant males has been found for species mating on the ground or in the nest. In several ant species interference of males with their rivals during copulation has been observed. In *Iridomyrmex humilis* (= *Linepithema humile*), a unicolonial ant species with intranidal mating and a heavily male biased sex ratio, mating pairs are frequently disturbed by other males. While no successful displacement of mating males has been observed, the presence of rival male reduces the amount of sperm stored by female sexuals during mating (Keller and Passera, 1992). In *Formica aquilonia*, latency to the first mating is larger in situation with a male-biased sex ratio, indicating some interaction of males during the pre-copulatory phase (Fortelius, 2005). O'Neill (1994) observed males of the species *Formica subpolita* trying to mount a female sexual that was already mating, however, he concludes that males may not aim to remove a rival but simply attempt to mount and copulate themselves. In *Pogonomyrmex*, several males try to gain access to a female sexual and intervene with other males' mating attempts but usually are not able to remove the other male either (see below). Thus it may be difficult to differentiate between targeted attempts to remove a rival male and normal scrambling for mating attempts by males not realizing the queen is already mating.

The influence of male size

Lengthy flight periods restrict male ants in terms of size and mass, forcing them to optimize flight abilities. However, if mating takes place on the ground, these restrictions are relaxed and higher variability in male size can be found (Davidson, 1982).

In the lek-mating *Pogonomyrmex* ants, groups of males compete for female sexuals, but male-male fighting has not been described so far (Hölldobler, 1976; Wiernasz et al., 1995). *Pogonomyrmex* males struggle for access to female sexuals which they grasp and hold firmly with their strong mandibles. Hölldobler (1976) proposes that strong mandibles are selected for as males might be pulled or pushed away by competitors. Female sexuals resist mating for a while after landing on the mating site and thus might select the "strongest" male. In fact, *Pogonomymex* males show a continuous variation in size and several studies have found larger males to be generally more successful in mating (*P. occidentalis*: see Abell et al., 1999, Wiernasz et al., 1995, 2001, 2004; *P. desertorum* and *P. barbatus*: see Davidson, 1982). In addition, it has been shown that the first male copulating with a female sexual invests the most time in the copulation, probably discharging all his sperm. Longer copulations might be adaptive for males to prevent queens from remating; sometimes males even leave parts of their copulatory organs inside the female genital tract (Nagel and Rettenmeyer, 1973; Fukumoto, 1989). Queens mate multiply, but the second or third male spends less time copulating, "probably due to the fact that those males may not have a chance to unload all their sperm and may therefore be more inclined to give up their first female", leaving the chance to remate with another female sexual (Hölldobler, 1976).

An advantage of large body size is also found in *Hypoponera opacior*, where larger males mated more often in comparison to smaller males and hence gained a clear advantage over smaller males (Kureck et al., 2013). Male size variability in some *Myrmica* and *Formica* species is bimodal instead of the continuous variation in most other ant species: they produce a larger class of males in addition to a smaller class. Fortelius et al. (1987) describe rather different tactics of the two male size classes in *Formica exsecta*: large males (macraners) are poor fliers and mate close to the ground near nests, whereas small males (micraners) are dispersers and fly high. Similarly, large and small males are produced in *Myrmica ruginodis* (see Elmes, 1991), but their dispersal strategy seems to be independent of male size (Wolf and Seppä, 2016). However, in both cases the production of these male forms seems to be related to the colony of origin: In *M. ruginodis*, queen and worker size often correlated with male size (colonies producing macrogynes produce larger males and vice versa), and larger males have a distinct advantage in terms of matings (Elmes, 1991). In *F. exsecta*, the production of macraners and micraners may be determined by colony characteristics like size, queen number, polydomy, lifecycle or resource availability, however, results are not fully consistent between studies. Nevertheless, no mating advantage was found for macraners in this species (Fortelius et al., 1987; Brown and Keller, 2000; Vainio et al., 2004). The production of macraner and micraner males may thus rather be seen under the aspect of fitness advantages on the colony level.

Depending on the conditions, larger sized males might not always be at an advantage. In *Cardiocondyla obscurior*, the outcome of fights depends on male age and thus other male traits may be of lesser importance (Cremer et al., 2012). In a recent study on *Cardiocondyla venustula*, no mating advantage was found for larger males in spite of a large variability in male size within colonies (Jacobs and Heinze, 2017). The agility necessary for fighting in small confined spaces may reduce the large-male advantage often described for fighting (e.g., Paxton, 2005) or influence of workers may reduce the importance of male strength itself (Yamauchi and Kawase, 1992). In their studies on body size and sperm content in *Atta colombica*, Fjerdingstad and Boomsma (1997) and Stürup et al. (2011) found high inter-colonial variability in body fresh mass, but other traits, including sperm content, did not vary. From their data, they conclude, that there might be a tradeoff between size and flying abilities. Similarly, O'Neill (1994) did not observe an advantage for larger males during mating swarms of *Formica subpolita* and males did not interfere with other males' matings contrary to the observations made in *Pogonomyrmex* (see Hölldobler, 1976). This seems to be in accordance with Paxton (2005), who suggests that large male mating advantage should be weaker in high male densities with scramble competition, a condition that is met in large mating swarms.

Male fighting (vs. hide and seek)

The strongest form of male competition culminates in fighting between the opponents, sometimes even resulting in death. In several *Cardiocondyla* as well as some *Hypoponera* species, wingless males kill rival males, usually pupae or freshly eclosed callows (*H. punctatissima*: see Hamilton, 1979; *H. bondroiti*: see Yamauchi et al., 1996; *C. mauretanica*, *C. minutior*, *C. emeryi*, *C. kagutsuchi*, *C. tjibodana*: see Mercier et al., 2007, Heinze et al., 1998). Both genera have evolved ergatoid (worker-like) male morphs in addition to or completely without the existence of winged males (Fig. 2.1).

Figure 2.1: Males of the winged and the wingless morph of *Cardiocondyla obscurior* (Picture: J. Giehr)

In addition to the already described worker like morphology (see above), they usually also have a paler coloration in comparison to their nestmates. Thus, mating predominantly takes place in the nest without the need for males to search for female sexuals outside of the colony, and this mating in the confined space of the nest might be one condition favoring direct male competition (Boomsma et al., 2005). Adaptations to this caused a unique feature of wingless males in the genus *Cardiocondyla*: in contrast to all other social hymenopteran males, their testes do not degenerate, but produce sperm throughout their life, allowing them to mate with all virgin queens that they can monopolize inside of the nest. Going hand in hand with this, wingless males can live for up to one year (Yamauchi et al., 2006), in contrast to the comparatively short average lifespan of winged *Cardiocondyla* males (mean lifespan *C. obscurior*: 12.25 ± 5.75 days from Schrempf et al., 2007) and winged ant males in general (Boomsma et al., 2005). Similarly, queens of Cardiocondyla have a relatively short lifespan (*C. obscurior*: max. 56 weeks, Schrempf et al., 2005a; *C. batesii* (corrected for hibernation): max. 112 weeks, Schrempf and Heinze, 2007). As in most of these species older males usually kill their freshly eclosing rivals, the males eclosing first might gain an advantage in comparison to males eclosing later, whereas the size of the males is probably irrelevant (Jacobs and Heinze, 2017, A. Schrempf, unpubl.).

Several different male tactics have evolved in ants from the genus *Cardiocondyla* and male appearance varies with these tactics (e.g., Heinze and Hölldobler, 1993; Heinze et al., 2005). Fighting seems to be ancestral in the genus and species can be divided into a clade producing males with saber-shaped mandibles and a second clade producing males with shear-shaped mandibles. Winged males have been lost in many of the species in both clades (in the *C. argyrotricha* group, *C. mauritanica*, *C. venustula* and the Palearctic group) (Oettler and Heinze, 2010). Males with saber-shaped mandibles kill callow and adult nestmate males. Pupae or callows are easily killed by puncturing the soft cuticle, but males are usually not able to crush the sclerortized cuticle of an adult rival. Hence, they grasp their opponent and transfer secretions of their hindgut on its cuticle inducing worker aggression (described for *C. obscurior*, *C. wroughtonii*, *C.* sp.: see Kinomura and Yamauchi, 1987, Stuart et al. 1987, Yamauchi and Kawase 1992; Fig. 2.2). Males may transfer secretions on each other reciprocally, sometimes even resulting in the death of both males. In these species, colonies thus generally do not contain more than one male at a time, allowing the “winner” male to monopolize all his eclosing sisters. Older males have a clear advantage over young males with a not fully sclerotised cuticle, but this benefit disappears in case the rival has grown older than one day (Cremer et al., 2012). In *Cardiocondyla* species with shear-shaped mandibles, killing of pupae or callows by crushing the soft cuticle is the prevailing male strategy. Especially in larger colonies, some freshly eclosed males manage to hide inside a colony and thus avoid being killed during the first days of their life. As adult male fighting seems to be unusual in this clade, two or three adult males may sometimes be found together in one colony (*C. mauritanica*, *C. emeryi*, *C. kagutsuchi*, and *C. minutior*: see Heinze et al., 1998). However, in some of those species males also occasionally fight against and kill adult rivals, and in at least two species, the transfer of hindgut secretions has been observed (*C. venustula*: see Frohschammer and Heinze, 2009 and *C.* cf. *kagutsuchi*: see M. Suefuji & J. Heinze, unpubl.).

Given the increased chance to survive fighting once the cuticle has hardened, it is in the interest of freshly eclosing males of both clades to hide under the brood pile and stay undetected from an older male, which on the other hand patrols the colony to detect rivals as soon as possible. In accordance with this, it has been shown that under polygynous conditions, the timing of male production is premature, as queens probably try to increase the chances of their own son to eclose early, survive and

Figure 2.2: Wingless *Cardiocondyla obscurior* males in fight (Picture: J. Giehr)

reproduce (Yamauchi et al., 2006; Suefuji et al., 2008). Interestingly, males of the palearctic clade of *Cardiocondyla* characterized by derived monogyny are mutually peaceful and do not engage in fights at all (Schrempf et al., 2005b; Schrempf and Heinze, 2007).

Similar to male fighting in the genus *Cardiocondyla*, wingless males in *Hypoponera punctatissima* and *H. bondroiti* have been observed fighting with other adult males (Hamilton, 1979; Yamauchi et al., 1996). *H. punctatissima* males fight for access to the chamber in which female sexuals are reared and can inflict injuries upon their opponents. Some of them might die from their injuries (Hamilton, 1979). In *H. bondroiti*, dimorphic ergatoid males (major and minor) can be found in one nest. While majors attack each other until one of the males is driven out of the nest, they do not attack minor males, but mount them in some cases. Yamauchi et al. (1996) suggest that the minor males mimic queens and thus are not recognized by majors, similar to winged males in *Cardiocondyla* (see below). Between minors, fighting or mounting has been observed in rare cases. Both male morphs mated with virgin females. In this case, both direct competition (majors) and avoidance of competition (minors) occur within one colony. However, the study by Yamauchi et al. (1996) provides no detailed data on the reproductive success of the respective strategies.

Another form of male killing might have arisen in *Hypoponera opacior*: It has been shown that males embrace other males in their cocoon by inserting their genitalia into the rear end of the pupal cocoon. In this way, more than 70 % of such embraced males are killed (Kureck et al., 2011), and hence it might be an adaptive strategy of males to kill competitors. Similar to the above, early emerging males might gain an advantage, as males that emerge earlier are able to mate more often (Kureck et al., 2013). As an alternative tactic to fighting, males of some of the above mentioned species evolved a strategy to succeed by escaping direct competition: they try to hide from fighter males by mimicking female sexuals. This has been suggested for minor males of *Hypoponera bondroiti* (see Yamauchi et al., 1996) and chemically shown for winged males in *Cardiocondyla obscurior* (see Cremer et al., 2002). These males are not recognized by the wingless males and hence stay concealed in the colony and “sneak” copulations inside the nest before dispersing. Interestingly, the males react quite flexibly to the availability of mating partners and opponents inside of the colony and leave the nest earlier in case that future mating opportunities are low (Cremer et al., 2011; Yoshizawa et al., 2011).

Territoriality

Wingless males of the species *Cardiocondyla venustula* have evolved a strategy hitherto not known for ants: they establish territories (Frohschammer and Heinze, 2009). The colonies of *C. venustula* live in subterranean nests with several small chambers. Male and female sexuals are produced in relatively high numbers during short periods of the year. Freshly eclosing male pupae are usually killed in this species similar to other *Cardiocondyla* species; however, males often do not manage to kill all emerging rivals, probably due to nest structure and number of eclosing males. Unlike in other species like *C. mauritanica* or *C. emeryi*, adult fighting is common and males also occasionally transfer secretions on each other. Some males defend small nest chambers and thereby create a territory, in which they kill eclosing males and mate with virgin females. This seems to be more efficient than patrolling the whole nest. Other males are usually pushed away from these territories by mandible threatening and biting. However, not all males behave in this way, and it remains to be investigated which factors inside of the nest influence the strategies of the different males (Jacobs and Heinze, 2017).

Enforced copulations

In *Hypoponera opacior* and *H. nubatama*, males do not wait until the eclosion of virgin queen pupae; instead, they mate with queens which are still inside of their cocoon to ensure mating. Males of this species do not fight against each other. Yet, by prolonging their copulation for up to 40 h and thereby acting as a "living" mating plug (see below Yamauchi et al., 2001), they can prevent other males from mating. Obviously, female sexuals cannot choose their mating partner when they are in the cocoon, however, several of the queens have been observed to mate a second time after eclosion from the cocoon (Foitzik et al., 2002). It remains to be investigated whether they selectively use the sperm of a preferred male by cryptic female choice.

In both species, two or more males may scramble for access to a cocoon, but only one is copulating with the female as long as she is in the cocoon, and no fighting between adult males has been observed (Foitzik et al., 2002). However, as described above, males embrace nestmate male pupae which afterwards die in a number of cases (35.3 % in Yamauchi et al., 2001, 73 % within two days in Kureck et al., 2011).

The role of workers

Beside direct competition between the males, the interference of workers might contribute to the resulting mating pair. Naturally, this is only possible for species mating inside or close to the nest with workers in direct proximity.

Even though in *Cardiocondyla* workers are utilized by males to kill their rivals (see above, e.g., Kinomura and Yamauchi, 1987), they rather seem to be "tools" of the males, as they normally kill the "marked" male (and do not "choose" to kill e.g., a "less related" male from a different colony; A. Schrempf, unpubl.), and sometimes even kill both males as an outcome of aggressive behavior. So far, there is no evidence of *Cardiocondyla* workers deliberately interfering with a given male's mating success.

In *Cataglyphis cursor*, males aggregate in front of alien nests and try mate with gynes from these nests. While there may be aggression between males (Lenoir et al., 1988), Cronin et al. (2011) observed workers to be highly aggressive towards males, yet, the aggressiveness varied between colonies and no clear difference in behavior towards a specific male was found. However, workers seem to treat males differently depending on their relative weight: while heavier males mate more often than lighter males in the absence of workers, this effect ceases when workers are present. Thus, workers may be able to influence male mating success (Helft et al., 2015).

In *Linepithema humile*, workers have been observed to heavily attack males from other but not from their own supercolonies, and thus they influence mate choice of the queens. This reproductive interference by workers leads to a reduced gene flow between supercolonies (Sunamura et al., 2011). In army ants (e.g., from the genus *Eciton*), queens are flightless and never leave their colony, hence males have to enter the colonies to mate and workers can influence which male inseminates the future queen during colony reproduction (Franks and Hölldobler, 1987), even though mating frequency of queens is extremely high (Kronauer et al., 2004, 2006).

In the ponerine species *Megaponera foetens*, males use trails laid by the workers to locate and enter the nests of alien colonies, thus workers at least influence the recruitment of males. In several other (queenless) ponerine species (e.g., *Ophtalmopone*, *Rhytidoponera*), workers possess spermathecae and are therefore able to mate and produce offspring after mating with foreign males inside the nest (Peeters, 1991).

Competition in male larvae

A recent study by Schultner et al. (2013) suggested that males compete already during the larval stage. They show that in *Formica aquilona*, larvae cannibalize eggs, and that male larvae do this more frequently than female larvae (three times more often). By doing so, males do not only enhance their own survival probability but might at the same time also remove possible competitors later in life. So far, it is unclear whether males prefer male instead of female eggs. Mating in polygynous *Formica aquilona* can be near or even inside of the nest and males might have to compete for access to female sexuals with other males. Males are able to mate with several female sexuals, which also mate multiply. Even though they do not directly attack each other, they compete in securing mating quickly before another male gains access, and increasing male bias decreases mate number in males (Fortelius, 2005).

2.4 Post-copulatory male competition

Mate guarding and mating plugs

Mating plugs produced by the accessory glands are used by males in several insect species to inhibit or at least reduce remating of queens (e.g., Gillott, 2003). In social Hymenoptera, the existence of mating plugs has largely been neglected; however, studies in bumble bees have revealed the function of lipids as mating plugs (e.g., Baer et al., 2000; Sauter et al., 2001) and several investigations in ants also suggest the existence of mating plugs.

In *Hypoponera*, males mate longer in presence of competing males, suggesting that they act as "living" mating plugs (Yamauchi et al., 2001; Kureck et al., 2011). In other species, substances from the accessory glands may serve as mating plugs. In *Diacamma*, *Monomorium* and *Carebara*, accessory gland compounds have been found to form a "sperm plug" or spermatophore (Robertson, 1995; Allard et al., 2002, 2006). In *Carebara*, the mating plugs are of a rather temporary nature. Thus, they may influence remating with subsequent males but do not inhibit remating itself. However, they might serve to prevent sperm leakage out of the vagina, ensuring the maximal number of sperm possible is transferred to the spermatheca (Robertson, 1995). In *Monomorium*, spermatophores also do not inhibit remating, thus they might only be used to transport sperm to the spermatheca or influence, but not prevent further matings (Allard et al., 2006). In *Solenopsis invicta*, fatty acid compounds of the accessory gland fluid are similar to those that have been proven to prevent remating in bumble bees, thus possibly having the same function in ants (Mikheyev, 2003). Generally, it might not be easy to distinguish whether these compounds only function for sperm transfer or whether they serve as mating plugs and inhibit remating. Indeed, different components from the accessory glands may serve different purposes and den Boer et al. (2015) found that mating plug functions are more pronounced in the rather monandrous species whereas sperm transfer, sperm survival and sperm competition are more important functions in highly polyandrous species. In line with this, a study by Baer and Boomsma (2004) suggests that males invest less into accessory glands but more into accessory testes (sperm number) when they are no longer able to influence paternity (in polyandrous species).

Males of other species even go further and perform extreme forms of mate guarding, staying attached to the queen leaving their genitals inside the female genital tract even after their death and thus acting as mating plugs themselves. In *Diacamma* sp., a ponerine ant, males stay attached to the female sexual for as long as 20–24 h. Shortly after the start of the mating, the female sexual returns to the nest. In the nest, the male is finally killed by the workers, leaving his genital inside of the female sexual (Fukumoto, 1989; Allard et al., 2002). It has been shown that in *Diacamma pallidum*, sperm is transferred in the form of a spermatophore already 15 minutes after copulation. The extremely prolonged copulation may be an effective prevention of remating (*Diacamma* females mate only once) but the mating inside the nest might also allow worker influence on mating (Allard et al., 2007). Males of the species *Dinoponera quadriceps* also exhibit suicidal mating: the male mates with a gamergate near her nest, and attached to the female, the male is dragged into the nest where the gamergate cuts off the end of the male's abdomen, leaving parts of his genitalia in her reproductive tract (Monnin and Peeters, 1998). Generally, suicidal mating seems to be restricted to the monogynous species providing only very rare mating opportunities for additional males. In species with multiple gamergates, where the sex-ratio is thus more female-biased, males can mate multiply (Monnin and Peeters, 1998; Allard et al., 2007). In army ants with a highly male-biased sex-ratio, males shed their wings before mating on the ground and stay in copula for a prolonged time (Gotwald Jr, 1995). As described above, *Pogonomyrmex* males in some cases leave their genital organs stuck to the female's mating tract, which however does not seem to inhibit remating completely (Hölldobler, 1976).

Sperm competition

Whenever queens mate multiply, sperm competition between the sperm of males in the female sexual tract might occur. After an initial monandrous phase during the evolution of eusociality, multiple mating in ants has evolved several times (Hölldobler and Wilson, 1990), and even if mate numbers are generally low (Strassmann, 2001), there are also a few highly polyandrous clades (e.g., seed harvester ants, leafcutter ants, army ants, species of *Cardiocondyla*, *Plagiolepis* and *Cataglyphis*; see Page Jr and Metcalf, 1982, Boomsma et al., 1999, Murakami et al., 2000, Kronauer et al., 2004, 2007, Schrempf et al., 2005a, Lenoir et al., 2007, Trontti et al., 2007, Schrempf 2014. A comprehensive review on polyandry in ants has recently been published by Baer (2016). Colonies have to invest into their sterile worker force before they start with the production of sexuals, and the lifelong commitment of the queens and the males makes it necessary that sperm survives inside of the queen throughout her entire lifespan (Boomsma et al., 2005; Baer, 2011, 2014). Even if a male might benefit from outcompeting the sperm of rival males in case he is able to transfer sufficient sperm for the complete lifespan of the queen, intense competition between the ejaculate of several males within the spermatheca is not expected after the mating period and the storage of the sperm (Boomsma et al., 2005; den Boer et al., 2010; Baer, 2014). This does not mean, however, that males do not try to increase their own fitness and indeed, an elegant study by den Boer et al. (2010) confirmed that in highly polyandrous ants (and bees), males manipulate the sperm of opponents with their seminal fluid protein. Sperm survival in *Atta colombica* and *Acromyrmex echinatior* queens was significantly reduced when mixed with alien seminal fluids in comparison to sperm in monandrous species (*Trachymyrmex* cf. *zeteki*). Importantly, they were also able to show that at least in Atta, the spermathecal fluids of the queen inhibit this negative effect. Hence, while males might be able to outcompete some sperm during the sperm storage process, they are probably unable to harm the sperm in the spermatheca after storage (den Boer et al., 2010).

The important role of queens for sperm storage has already been suggested by Sundström and Boomsma (2000) and queens might for example reduce the effect of sperm clumping. Their study suggested that in *Formica truncorum*, it is in the interest of all males to achieve sperm clumping, as this will bias the worker controlled sex ratio towards female offspring because cohorts of highly related individuals would be produced at a time. However, they also suggest that queens oppose this male strategy by probably actively mixing the sperm, resulting in a “compromise between complete sperm clumping (the joint interests of the males mated to a queen) and complete sperm mixing (the queen’s interests), where some queens are more successful than others in mixing sperm and, thus, having more male-biased sexual broods”. Thus, even if sperm clumping is aimed at by males, it may be concealed by mixing by the queen. Several studies have confirmed random sperm use by the queen (Kronauer et al., 2006; Holman et al., 2011), and a recent detailed study on sperm use in *Acromyrmex echinatior* over time also confirmed that sperm is equally mixed and used randomly from the spermatheca (Stürup et al., 2014).

Sperm competition is not only expected to select for male traits against alien sperm, but certainly also for the enhancement of their own sperm fertilization probability. One older and three recent studies independently describe the formation of sperm bundles in adult ant males, in which queens are polyandrous (*Crematogaster victima*: see Boomsma et al., 1999, Oliveira et al., 2014; *Lasius pallitarsis*: see Burnett and Heinze, 2014; *Cataglyphis savignyi*: see Pearcy et al., 2014). While sperm bundles are well known in the testes of premature males due to the development of sperm in cyst cells, sperm cells usually disorganize after maturation in the seminal vesicles (e.g., Moreira et al., 2004). Sperm of haploid insect males is clonal and hence, there is no conflict between individual sperm within an ejaculate, making the evolution of cooperation between sperm cells to enhance a male's fertilization ability in case of intense sperm competition likely. While it is still not clear in *Lasius pallitarsis*, whether sperm bundles are just the result of incomplete decomposition of cyst cells after maturation or whether sperm bundles are the result of sperm competition to increase mobility (Burnett and Heinze, 2014), Pearcy et al. (2014) were able to show for *Cataglyphis savignyi* that sperm swimming speed in bundles is indeed faster in comparison to single sperm. As it is a well-known fact that sperm velocity transfers into fertilization success (Birkhead et al., 1999; Gasparini et al., 2010), it is very likely that sperm bundles in all of these species are formed to increase the fertilization success of individual ant males. On the species level, clear adaptations to the level of sperm competition have been found in males from *Cataglyphis* species. Males from species experiencing higher sperm competition have a higher sperm production and thus ejaculate size compared to those from species with less competition. Sperm length and male size also varied with competitive situations; however, this effect was much weaker (Baer and Boomsma, 2004; Aron et al., 2016).

During postcopulatory selection, queens are involved as a third party via cryptic female choice (CFC). While it has been shown that queens disable negative effects of seminal fluids on rival sperm (den Boer et al., 2010), queens might have no influence on sperm displacement. However, with or without active sperm choice of females it might be that sperm of superior males is differently used for insemination. Moreover, one might argue that sperm of high quality males survives longer and hence is the only one to be able to fertilize eggs at the end of the queens' life (at the time where often sexuals are produced). Yet, more studies are needed to understand the dynamics of male-male competition and cryptic female choice in social insects and to investigate whether CFC might select for higher quality sperm in males.

2.5 Conclusion and future perspectives

With an increasing number of studies that have been published in recent years, it has become evident that competition between ant males is more common than previously assumed. Indeed, competition might already take place at the larval stage (Schultner et al., 2013). More studies are needed to investigate whether egg eating actually represents a form of selective killing of rivals and whether this phenomenon is unique to *Formica* ants or whether it is a general trait in species where larvae are able to move and feed by themselves.

After eclosion, ant males might differ in quality with regard to mature sperm number, size and viability. Even though the testes of ant males degenerate before or shortly after maturation (Hölldobler and Bartz, 1985), males might nevertheless produce more sperm in a given period of time and / or higher quality sperm when reared in an environment that predicts future sperm competition conditions. For example, it has been shown in flatworms that sperm is produced faster under sperm competition conditions (Giannakara et al., 2016). It remains to be investigated whether also ant males “react” to the level of sperm competition on an intraspecific level, e.g., in *Hypoponera* or *Cardiocondyla*, depending on the number of competitors inside the colony. Under the assumption that only healthy, well-fed larvae can produce optimal sperm cells, competition between larvae, e.g., in begging for food, might occur and needs further investigation. Under such conditions, workers might be able to support larvae differently depending on relatedness (in case they are able to discriminate kin, see e.g., Nehring et al., 2011, Helanterä et al., 2013; but see Urbani, 1991 and Schultner et al., 2013). Theory predicts that sperm competition selects for an optimal sperm phenotype (Parker, 1993), and a species comparison by Fitzpatrick and Baer (2011) indeed confirmed that variance in sperm length is lower in social insect males that experience sperm competition.

In solitary insects, it has also been shown that males are able to adjust ejaculate size and viability directly to the level of sperm competition (Thomas and Simmons, 2007; Martin and Hosken, 2002). The above-mentioned studies by Baer and Boomsma (2004) and Aron et al. (2016) show that at least concerning between species comparison, adaptation to competition can be found in attine fungus growing and *Cataglyphis* ants. Again, further studies are needed to investigate, whether males adjust their sperm number and viability to competition on an intraspecific level – on condition that sperm number is sufficient for the offspring production of the female mating partner.

Studies on post-copulatory traits in polyandrous species as conducted by Baer and colleagues can help to assess the magnitude of male manipulations after and during the storage process, respectively, and might reveal, in addition, previously undetected male traits to manipulate their reproductive success. For example, similar to solitary insects, sperm displacement might be a strategy of males mating subsequently (again only provided that a single male can transfer sufficient sperm for the offspring that a queen produces during her complete lifespan).

It is similarly conceivable that queens might sometimes reject sperm of males, which transfer “low” quality sperm, but studies on cryptic female choice are rare. This could be investigated in the future e.g., by offspring analysis of queens that are allowed to mate with known males whose ejaculate quality has been manipulated. Species with intranidal mating and the possibility to perform matings in the laboratory will be valuable model systems for future research. A very recent study in *Drosophila* revealed that females are even able to counter-act to mechanisms of chemical mate-guarding (Laturney and Billeter, 2016). In several ant species, females change their “bouquet” after mating and become unattractive for males. It remains to be investigated whether similar antagonistic co-evolution of males and females on the “chemical level” is existent in ants. Advances in proteomics will allow deeper insights into molecular pathways of sexual selection. As an example, the comparison of seminal fluids and spermathecal fluids of additional species may be used to detect general patterns in sperm competition and / or cryptic female choice in social insects and to compare them with solitary insects where life history is different.

Ants exhibit an enormous variety of mating systems, thus it will be crucial to include as many different study species as possible in further studies to compare levels of sexual conflict and sexual selection. Insights into intra- and interspecific differences of male-male competition and their evolutionary background may contribute to a better understanding of the evolution of sexual selection in social insects and beyond. The availability of more and more ant genomes will provide the opportunity to analyse these evolutionary processes on the genomic and / or proteomic level.

2.6 Acknowledgments

We thank two anonymous referees for helpful comments, which significantly improved the manuscript. Furthermore, we would like to thank Dorothee Jacobs for language correction.

3 Between fighting and tolerance: reproductive biology of wingless males in the ant *Cardiocondyla venustula*

Susanne Jacobs & Jürgen Heinze

Institut für Zoologie, Universität Regensburg, Germany

3.1 Abstract

Male reproductive tactics vary widely across the species of the ant genus *Cardiocondyla*, from obligatory lethal combat among co-occurring males to complete mutual tolerance. The African species *C. venustula* Wheeler, 1908 has an intermediate phylogenetic position between taxa with fighting males and taxa with tolerant males and also shows an intermediate male behavior. Males from two native populations in South Africa and a population introduced to Puerto Rico attacked and killed freshly eclosing rivals but rarely engaged in deadly fights with adult competitors. Instead, several males per colony established small "territories" in their natal nests and defended them against other males. Males with a stable territory had more contact with female sexuals than non-territorial males and more frequently engaged in mating attempts. In controlled choice experiments, female sexuals did not show any preference for particular males. We suggest that male territoriality in *C. venustula* is an adaptation to the seasonal production of large numbers of female sexuals by multiple mothers.

Key words Cardiocondyla; ergatoid males; Formicidae; male competition; sexual selection; territoriality

3.2 Introduction

Sexual displays, courtship, and competition for access to mating partners are among the most elaborate and diverse behavior in animals (Andersson, 1994). Though ants excel in other behavioral categories (Hölldobler and Wilson, 1990), their sexual behavior is often disappointingly monotonous. Across species, mating typically occurs away from the natal nest, either during large-scale nuptial flights, where thousands of sexuals from many colonies gather for a few hours of frantic activity, or close to the ground, where scattered female sexuals attract males with sexual pheromones. In both scenarios, males can do little to increase their reproductive success, because defending territories and monopolizing females is impossible (Boomsma et al., 2005). Typical ant males are not equipped with weapons or extravagant sexual ornaments. Because their sperm supply cannot be replenished and suffices only for a few copulations, males have been regarded as "flying sperm-bearing missiles constructed only for the instant of contact and ejaculation" (Hölldobler and Wilson, 1994; but see Shik et al., 2013). Consequently, the importance of sexual selection in ants remains poorly understood (e.g., Baer (2014)).

The situation is considerably different when mating occurs in the nest. Here, males can try to increase their reproductive success by excluding rivals from mating with young queens. In the genus *Cardiocondyla* this has led to the evolution of wingless (ergatoid) males with livelong spermatogenesis (Kugler, 1983; Heinze and Hölldobler, 1993). Ergatoid male strategies range from lethal fighting in several paleotropical species with multi-queen colonies and year-round production of sexuals (e.g., *C. obscurior*, *C. emeryi*, and *C. minutior*; Kinomura and Yamauchi, 1987; Stuart et al., 1987; Heinze et al., 1998) to the peaceful coexistence among males in several derived species with single-queen colonies and seasonal production of sexuals, such as *C. batesii*, *C. elegans*, and *C. nigra* (Schrempf et al., 2005b; Lenoir et al., 2007; Oettler and Heinze, 2010; Schrempf, 2014).

The African species *Cardiocondyla venustula* occupies an intermediate phylogenetic position between species with fighting males and species with mutually tolerant males (Oettler and Heinze, 2010) and could therefore be a key to understanding the evolution of male reproductive strategies in this genus. First behavioral studies on *C. venustula* revealed that adult males only occasionally engage in lethal fighting but defend “territories” in their natal nests (Frohschammer and Heinze, 2009). Like the fighting males of *C. mauritanica*, *C. kagutsuchi*, and several other species (Heinze et al., 1993; Yamauchi and Kinomura, 1993), males of *C. venustula* crushed the still unhardened cuticle of freshly eclosed rivals with their strong, shear-shaped mandibles. Most adult males stayed consistently in particular areas of the multi-chambered laboratory nests and defended their “territory” against intruders by short, non-lethal biting attacks (Frohschammer and Heinze, 2009). Because these observations were based on experimental colonies originating from a single field colony collected in a population introduced to Kaua’i, Hawai’i, it remained unknown whether territoriality is a consistent feature of this species or an idiosyncratic adaptation to a novel and untypical environment. Furthermore, parameters that might affect male behavior, such as colony structure and the timing of the production of sexuals, remained unknown for this species.

We therefore studied colony structure and male behavior in two native South African and one introduced Caribbean population of *C. venustula.* We expected to find male territoriality in all populations of this species and a positive association between male territoriality and reproductive success. Furthermore, the coexistence of several males in a confined space allows virgin female sexuals to assess male quality and to choose the best mate. We therefore conducted controlled female choice experiments to determine whether female sexuals show a consistent preference for particular males.

3.3 Material and Methods

Study species and colony maintenance

Cardiocondyla venustula, originally described from Puerto Rico, is a tramp species that originates from Africa and was later introduced to the Caribbean islands, Hawai'i, and other parts of the tropics and subtropics (Seifert, 2003). In May 2012, we collected 101 colonies of *C. venustula* in marsh flats and adjacent grassland near Coco Beach, Rio Grande, Puerto Rico (N 18° 24′ 52″, W 65° 47′ 55″, elevation 1 to 2 m). In April 2011 and February / March 2013 we collected 249 colonies of *C. venustula* from sparsely vegetated patches, such as unpaved parking lots or driveways, at Hlalanathi Drakensberg resort, uThukela river, KwaZulu-Natal (S 28° 39′ 2″, E 29° 2′ 3″, elevation 1260 m), Rietvlei Dam Municipal Nature Reserve near Pretoria, Gauteng (S 25° 53′ 35″, E 28° 17′ 44″, elevation 1520 m) and a few other places in the Drakensberg. Nests were found in the soil down to a depth of 30 cm and consisted of several pea-sized chambers connected by narrow corridors. Upon collection, colonies contained of up to 100 workers, 1 to 10 dealate queens, up to 10 winged female sexuals, and up to four males. All males had the typical habitus of ergatoid males, even though some males from South Africa had more pronounced thoracic sutures and vestigial wings (e.g., Heinze et al., 2013).

In the lab, colonies were housed in plastic boxes with a plaster floor. A small cavity in the plaster covered by a microscope slide served as nest site (Fig. 3.1). The colonies were kept in incubators at a 12 h day (26 °C) / 12 h night (24 °C) cycle and fed three times per week with honey and chopped cockroaches, water was provided ad libitum. Cardiocondyla colonies can be kept in such artificial nests over several years and though 10 generations of sib-mating may lead to subtle signs of inbreeding depression (Schrempf et al., 2006), there is no evidence that prolonged laboratory rearing affects male fighting or courtship behavior. Colonies used in this experiment

Figure 3.1: Part of a three-chambered laboratory nest with an ergatoid male (lower left), three winged female sexuals, aworker and brood of the ant *Cardiocondyla venustula*

came from Hlalanathi (2011, $n = 3$; 2013, $n = 9$), Rietvlei (2013, $n = 5$), and Puerto Rico ($n = 9$). Experimental plaster nests for the observation of males consisted of six interconnected nest chambers that resembled natural nest chambers in size and were covered by a glass plate and a piece of red plastic. Corridors between the chambers were so narrow that workers could deposit brood only in the chambers but not in the connecting tunnels. Field-collected colonies of suitable size (i.e., with 25 or more workers and enough brood to fill the six nest chambers) were transferred into experimental nests. Behavioral experiments were started as soon as a colony contained at least two males or male pupae. At this stage, all colonies also contained one to five mature queens, brood, workers, and young female sexuals or female sexual pupae. Voucher specimens were deposited at Senckenberg Museum für Naturkunde, Görlitz, and Iziko Museums of Cape Town.

Study design for male territoriality

The nest design with six small chambers allowed males to space out in their nest as described in Frohschammer and Heinze (2009). To be able to track the location of males, males had to be sufficiently distinct in size, color or visible injuries or they were individually marked with thin copper wires tied between petiole and postpetiole. Previous studies had not indicated any influence of marking on male behavior (unpubl. observations). Male positions in the nest were checked by eye with the help of a stereo microscope during 5 to 10 scans per day over 10 to 21 days (in total 5,606 data points, 4,009 from Hlalanathi, 602 from Rietvlei, and 995 from Puerto Rico). Additionally, the presence or absence of brood and the number of queens in the occupied chambers were noted. Interactions between males were monitored during the scans.

As observations of physical defense of territory borders during the scans were too scarce to assess territoriality for all males, males that stayed in one chamber (or a home range spanning more than one chamber) for more than consecutive 24 h were considered as “territorial”. Males that never stayed at one place for more than 24 h,were defined as “non-territorial”. The median life span of *C. venustula* males was 3 days (quartiles 1, 8; maximum 34 days, $n = 230$), so 24 h are a reasonable fraction of the male’s life span. Males, which were found dead but intact during the experiment, were collected and stored in 100 % ethanol. Body size was assessed by measuring the head width between the eyes using a Keyence VHX-500FD digital microscope at a magnification of 200x. In seven colonies, we also checked the position and of individually marked female sexuals during the scans for 10 to 20 days (1 to 6 female sexuals per colony, in total 20). The mobility of female sexuals and males (i.e. changing chambers between consecutive scans) was compared. Colonies were manipulated as little as possible because the ants reacted sensitively to any disturbance and started to relocate their brood.

Female choice experiments

We investigated whether female sexuals differentiate between males with opposite features. We chose the following traits, which at the same time might affect offspring quality and were easily to determine: origin (nestmate or non-nestmate – female sexuals might prefer alien males to avoid inbreeding), age (2 days vs. 10 days – choice might be based on male age Beck and Powell, 2001; Pizzari et al., 2008), body size / presence of vestigial wings (small without wings, large with wings; Heinze et al., 2013; larger insect males are often preferred by females, e.g., Thornhill and Alcock, 1983), and location in the nest (in a chamber with or without brood; female sexuals might prefer males that had established territories in the safe center rather than the periphery of the nest).

Choice experiments were executed in small plastic arenas that contained three chambers (each approximately $1\,\mathrm{cm}^2$) separated by wire mesh covered with a glass plate. Through the wire mesh antennation was possible, but the ants were confined to their chamber. Pupae of female sexuals and males were placed into separate nests with a few workers and brood until they eclosed. Experiments started 3 days after eclosion at the earliest. The female sexual was placed into the middle chamber; two males with opposing traits were transferred into the marginal chambers. Each female sexual was only used once during the whole experiment. Behavior and location of the female sexual were recorded directly and by video for ten minutes. The time spent in either half of the middle chamber was either manually determined or tracked with the software Biotrack Pack 1.5 (Feldman et al., 2012).

Statistics

The distribution of males across chambers was compared to a standard binomial distribution to check whether more chambers where consistently occupied by only a single male than expected from a random distribution of males. Furthermore, the probability of finding a male in the same chamber for several consecutive observations by chance was compared to the actual distribution ($P = (\frac{1}{6}) * (y - 1) * Nm * ns$ with y = number of scans necessary to define male as territorial, Nm = number of males in the colony, ns = number of scans per colony). Unfortunately, the low variability of genetic markers and the high relatedness among nestmates did not allow a direct estimation of the reproductive success of individual males of *C. venustula*. We therefore used the mean number of winged female sexuals in the chamber of a particular male as an estimate of male mating chances. Due to different total numbers of males and young queens in the different colonies,“estimated mating chances” could not be compared directly. Therefore, we calculated the highest mean number of young queens per male in the colony and defined three categories relative to this maximum value ($C1 > \frac{2}{3}max$; $\frac{2}{3}max < C2 < \frac{1}{3}max$; $C3 < \frac{1}{3}max$). In addition, we compared the number of mating attempts between territorial and non-territorial males by a Wilcoxon test. All statistic analyses were performed in R 3.0.2.

3.4 Results

A large percentage of the collected colonies contained multiple dealate queens, and dissections of 25 queens from five colonies clearly substantiated that *C. venustula* is facultatively polygynous (3 of 5, 4 of 4, 7 of 7, 4 of 4, and 3 of 5 queens per colony were inseminated and their ovaries contained mature eggs). Behavioral observations suggest that female sexuals mate in their natal nest, shed their wings, and leave the nest on foot. During our field studies we repeatedly observed dispersing, dealate queens. All dissected dealate queens found outside the nest chamber in the laboratory were inseminated ($n = 6$), suggesting that dispersing queens in the field also had mated. We could not determine whether young queens seek adoption into alien established colonies, as suggested by the co-occurrence of different mtDNA haplotypes in some colonies (Heinze et al., 2013), or establish new colonies solitarily.

Field colonies contained sexuals in spring and early summer (casual observations in Hlalanathi in 2008 and collections in 2011, 2012, and 2013). Under laboratory conditions, a few males and female sexuals were produced year-round, but their numbers peaked in early (2014) and late summer (2015), suggesting seasonality of sexual production. During the main time of sexual production we counted between 2 and 18 males (median, quartiles 4, [3, 6.5]) and between 1 and 42 female sexuals (median, quartiles 6, [3, 10]) in each of the 26 more intensely studied laboratory colonies (12 from Hlalanathi, 5 from Rietvlei, 9 from Puerto Rico). The sex ratio in these colonies (female sexuals / all sexuals) ranged between 0.2 and 0.89 (median, quartiles 0.56, [0.38, 0.66]). Sex ratios did not differ between the three populations (Anova: $F = 0.753$, $P = 0.484$, $df = 2$, $error\, df = 20$).

We observed the behavior of a total of 151 males (71 from Hlalanathi, 18 from Rietvlei, 62 from Puerto Rico). Across the studied colonies, the tendency of males to stay in a particular chamber differed considerably. For example, 15 males (9.9 %) changed chambers between less than 10 % of the consecutive scans, while the eight most mobile males (5.6 %) were found in different chambers in 80 % of consecutive scans. Territorial males were found in all but four of the 26 experimental colonies. Several males consistently were found in two adjacent chambers, indicating that their territory might have spanned both chambers (see also Frohschammer and Heinze, 2009). The probability of finding randomly moving males in the same chamber for at least 24 hours by chance was $P < 0.0001$ (Wilcoxon paired test comparing the observed and expected values for each colony, $V = 336$), considering the number of males and the total number of scans per colony. We therefore considered such males as "territorial".

Of these territorial males, 36 % (20) stayed in their "territory" throughout their whole life spans, and died when or shortly after their territory was taken over by another male. Other males occupied a territory only during parts of their life, and moved around in the nest before (21 %, $n = 12$), after (14 %, $n = 8$) or both before and after being territorial (29 %, $n = 16$). Colonies from Hlalanathi (H) had higher percentages of territorial males (38 of 71) than colonies from Rietvlei (R; 4 of 18) and Puerto Rico (PR; 14 of 62). However, only the difference to the latter was significant (Fig. 3.2; Anova: $F = 4.537$, $P = 0.022$, $df = 2$, $error\,df = 23$, post-hoc Tukey test: PR-H, $P = 0.025$; R-H, $P = 0.136$; R-PR, $P = 0.944$).

Males were seen attacking intruders and freshly eclosing male rivals. Aggression among males occurred in at least 23 of the 26 colonies (11 of 12 colonies from Hlalanathi, 3 of 5 colonies from Rietvlei, and 7 of 9 colonies from Puerto Rico, pairwise comparisons by Fisher's exact test, all $P > 0.15$). Male antagonism was directly observed in 25 instances (0.46 % of all observations) and ranged from aggressive displays (mandible threatening) to short biting and holding each other by the head, antennae, legs or petiole for several minutes. Twice we observed a male besmearing another male with hindgut secretions. This led to intensive grooming of the male by workers but not to violent worker attacks like in *C. obscurior* (Yamauchi and Kawase, 1992). The occurrence of injured males (i.e., missing one or more legs, $n = 45$, missing an antennae, $n = 16$, or with caved-in abdomen, $n = 4$) and numerous killed males (in total more than 180 corpses in all 26 experimental colonies during the observation period) indirectly proved the existence of regular attacks, in particular against young, freshly eclosed males. Even severely injured males could survive for several days. Occasionally males intruded into territories that had been occupied by a territorial male for several days without being attacked (0.55 % of all observations, $n = 31$).

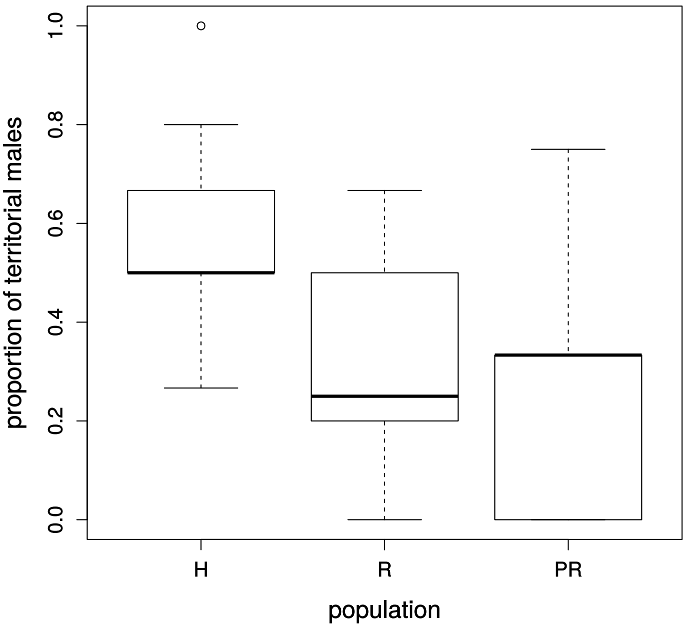


Figure 3.2: Proportion (median, quartiles and range) of territorial males in colonies of the ant *Cardiocondyla venustula* from two native populations in South Africa (H Hlalanathi, R Rietvlei) and one population introduced to Puerto Rico (PR)

Usually these males were observed in the chamber only during a single scan and they never stayed in the chamber as long as the territory owner. In addition to aggressive interactions between males we also observed occasional aggression between workers and males ($n = 35$, 0.62 % of all observations), probably caused by besmearing or by males unintentionally biting workers in the course of a male-male fight. The occurrence of aggression or territoriality in a colony was not associated with the absolute number of males or female sexuals per colony (Spearman's rank test, males: $rS = 3619.7$, $P = 0.243$, $\rho = -0.237$; female sexuals: $rS = 3538.9$, $P = 0.304$, $\rho = -0.209$), numerical sex ratio ($rS = 2707.5$, $P = 0.718$, $\rho = 0.074$), or colony size (three size categories, Kruskal-Wallis rank sum test, $\chi^2 = 1.933$, $df = 2$, $P = 0.380$). Male body size ($n = 45$) was not different between territorial and non-territorial males (overall analysis, t-test: $t = -1.199$, $df = 42.0$, $P = 0.237$; pairwise analysis per colony, paired t-test: $t = -1.0796$, $df = 6$, $P = 0.322$). Similarly, the absence or presence of vestigial wings in the males from Hlalanathi did not affect their propensity to be territorial (35 short-winged males, 25 ergatoid males, proportion of winged/ergatoid territorial males per colony tested by Kruskal-Wallis rank sum test,

$\chi^2 = 1.030$, $P = 0.310$).

Similar to males, female sexuals occasionally stayed in one place for a longer period of time, while others moved freely from chamber to chamber. While the average mobility did not differ between males and young queens, no young queen changed their location between less than 20 % or more than 70 % of the scans (Fig. 3.3). While mobility was normally distributed in female sexuals (Shapiro-Wilk normality test $P = 0.706$), male mobility was not ($P = 0.037$). This means that young queens express a "moderate" movement behavior, whereas in males extreme values (i.e., territorial males and highly mobile males) can be observed. Several female sexuals or queens were often seen together in one chamber without any signs of aggression (e.g. up to 9 female sexual and 3 adult queens in one chamber throughout several scans), i.e., in contrast to males, neither female sexuals nor queens defended territories.

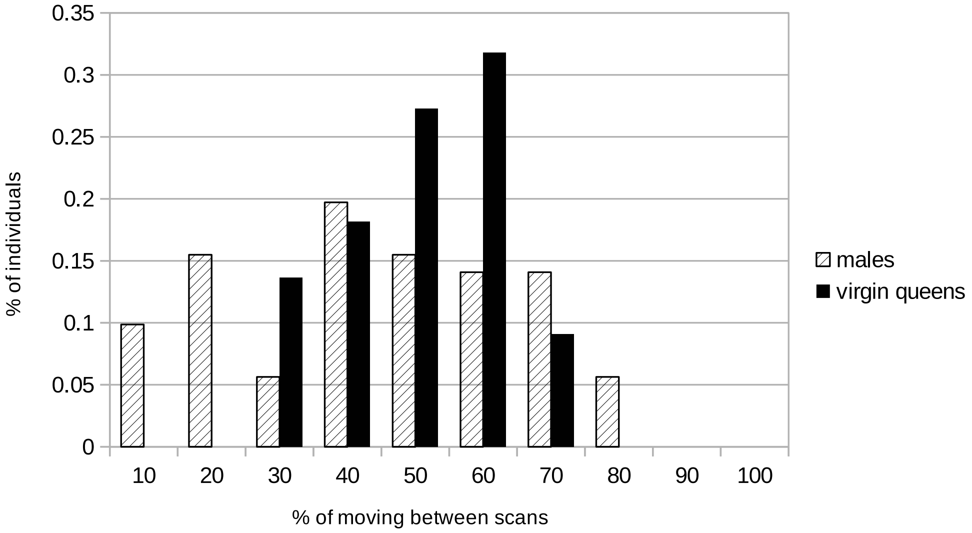


Figure 3.3: Movement pattern of males and female sexuals / young queens of *Cardiocondyla venustula*. Shown is the percentage of individuals that changed location in the multi-chamber experimental nests between different scans

From the number of female sexuals located in the same chamber as a particular male it became evident that some males had more access to mating partners than others. Territorial males constituted a higher percentage of the group of males with the most frequent contact to female sexuals (Fig. 3.4; Kruskal-Wallis test, $\chi^2 = 14.42$, $df = 2$, $P = 0.0007$; post-hoc Wilcoxon with Bonferroni-correction: C1 – C2: $W = 70.0$, $P = 0.085$, C1 – C3: $W = 97$, $P = 0.007$, C2 – C3: $W = 48$,

$p = 0.640$). Mate guarding was not observed, i.e., males and female sexuals moved independently of each other. Mating attempts (hammering, kissing, stridulation, as described by Mercier et al., 2007) were seen regularly (1.80 % of all observations, $n = 104$). Most of these mating attempts were conducted by territorial males (72 of 98 mating attempts in which the male's status was known). In eight of nine colonies, in which mating attempts were observed, territorial males engaged in more mating attempts than non-territorial males (Wilcoxon signed ranks test, $W = 4$, $P = 0.019$). Successful mating was rare during the observation periods (0.16 % of all observations, $n = 9$), even though dissections revealed sperm in the spermatheca of female sexuals already shortly after emergence. During our observations, female sexuals tended to move away or to retract their abdomen before the male succeeded in inserting its genitalia. Nearly as frequently males tried to "mate" with workers, adult queens, other males, or even pupae and dead ants (0.89 % of all observations, $n = 50$). Experiments in which female sexuals were given the chance to choose between two different males did not reveal any female choice concerning the tested criteria. In the arena, female sexuals seemed to move around randomly, crossing the middle line between the two males up to 93 times within the ten minutes (mean

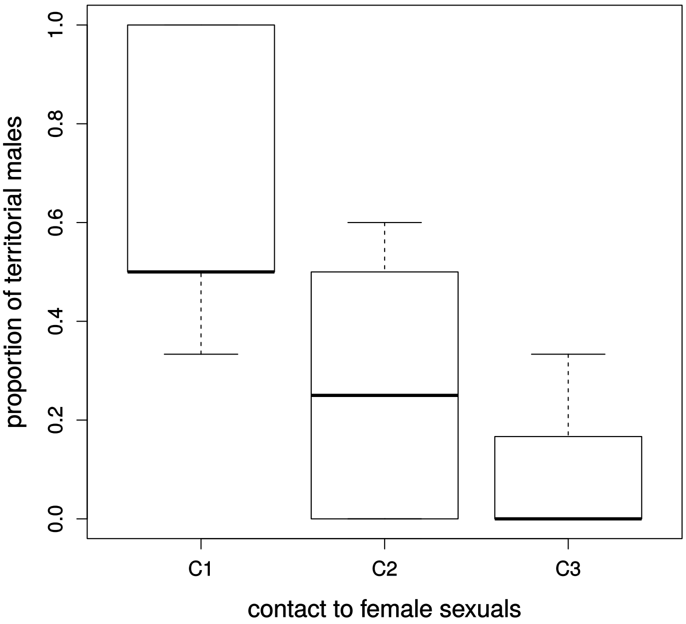


Figure 3.4: Proportion of territorial males (median, quartiles and range) in colonies of the ant *Cardiocondyla venustula* among the males with the highest, medium, and the lowest contact rate to female sexuals. For details see text.

34.4). They did not spend longer time close to any of the males. We found no significant preference for either of the male categories (young vs. old: one-sample t-test, $t = 0.544$, $df = 9$, $P = 0.599$; small, wingless vs. large, winged: $t = 0.353$, $df = 9$, $P = 0.732$; non-nestmate vs. nestmate: $t = -0.353$, $df = 9$, $P = 0.732$). There was a non-significant trend for female sexuals to stay closer to males from a chamber without brood than to males from a chamber with brood ($t = -2.1506$, $df = 9$, $P = 0.060$; Fig. 3.5).

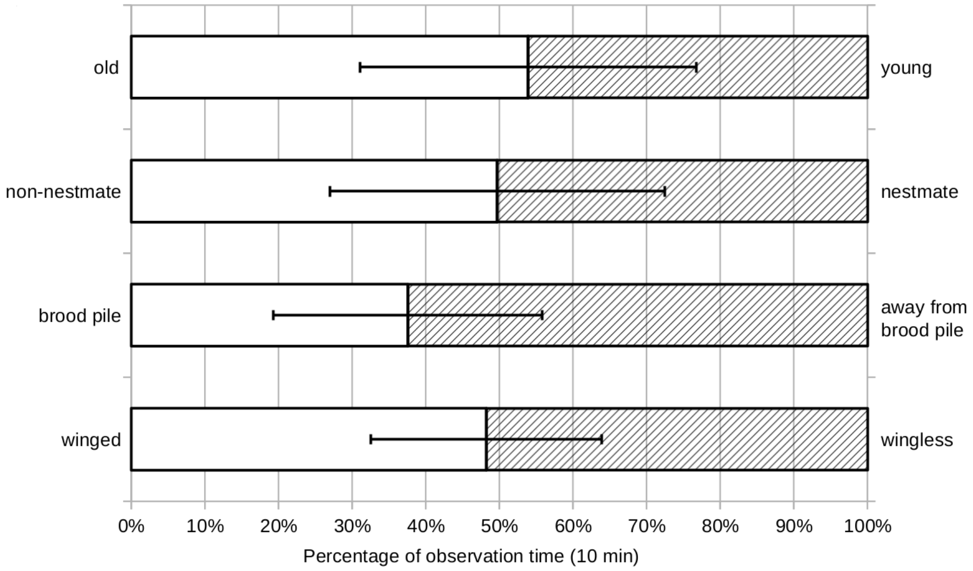


Figure 3.5: Percentage of observation time (*mean* ± *S.D.*) spent by female sexuals in a choice experiment in the vicinity of one of two males of different age (2 vs. 10 days), origin (nestmate or non-nestmate), location in the nest (near brood pile or away from it), or size and presence or absence of wings

3.5 Discussion

Our study shows that male behavior in the ant *Cardiocondyla venustula* varies considerably among individuals. In both native and introduced populations we found males that stayed preferentially in one part of their natal nest and defended it against intruders, as previously observed in one colony from a population introduced to the Hawai'ian island of Kaua'i (Frohschammer and Heinze, 2009). Other males, however, did not show any local preference but floated freely among different nest chambers. Territorial males had more contact to female sexuals and also engaged in mating attempts more often. Though the actual reproductive success could not be determined, this suggests that territoriality is associated with higher mating chances. In contrast to our expectations, female sexuals did not actively choose among individual males.

In general, male competition may be affected by the availability of mating partners and the number and, less strongly, the relatedness among rival males (e.g., Anderson et al., 2003; Reinhold, 2003; Pizzari et al., 2015). In the following, we first explore what might have led to the evolution of male territoriality in *C. venustula* and then discuss a few aspects of our findings in more detail. Depending on the spatiotemporal distribution of receptive females, male Hymenoptera guard and defend female emergence sites or areas where females are likely to forage or lay eggs (reviewed by Paxton, 2005; Alcock, 2013). For example, males of several paper wasps, bumblebees, and beewolves establish territories on perching sites and ward off intruders (Alcock, 1975; Beani and Turillazzi, 1988; O'Neill et al., 1991; Polak, 1993), and wingless males of the communal bee *Macrotera portalis* guard tunnels to subterraneous oviposition sites (Danforth, 1991). In contrast, when female emergence sites are clumped, as in Dawson's burrowing bee (Paxton, 2005; Alcock, 2013) or parasitoid wasps (e.g., Leonard and Boake, 2006), males might be better off guarding and defending the latter instead of waiting for the unpredictable arrival of foraging females. Across species, the behavior of *Cardiocondyla* males appears to vary similarly with the availability of receptive female sexuals and numerical sex ratios. The phylogeny of *Cardiocondyla* (Oettler and Heinze, 2010) suggests an origin of this genus in tropical Southeast Asia, and studies on *C. obscurior* and other originally paleotropical species show that colonies reliably produce small numbers of female sexuals year-round. For example, natural colonies of *C. obscurior* contain only a few female sexuals and a single male throughout most of the year (mean number of female sexuals from in 71 colonies with at least one female sexual or male: 4.6; mean sex ratio: 0.50; data from the introduced population in Brazil; Heinze and Delabie, 2005). This allows males to monopolize mating with a small, but continuously replenished supply of female sexuals and selects for long life and lethal fighting among adult males. In contrast, when female sexuals are produced seasonally and in very large numbers, as in palearctic *Cardiocondyla* (mean number of female sexuals in 42 natural colonies: 76.6; sex ratio: 0.91; Lenoir, 2006; Lenoir et al., 2007), a single male may not be capable of defending and inseminating all of them. Monogyny in the palearctic species and the resulting close relatedness among colony males may have facilitated the evolution of male tolerance.

The peculiar male territoriality of *C. venustula* may be an adaptation to the more seasonal production of female sexuals in multi-queen colonies. On the one hand, sex ratios are more balanced than in palearctic *Cardiocondyla* as competing queens are selected to produce more males than are needed to guarantee the insemination of all female sexuals (e.g., Cremer and Heinze, 2002). On the other hand, because of the relatively large number of female sexuals and the complexity of the nest with multiple chambers, males cannot eliminate all adult rivals and monopolize a "nest-wide harem". In such a situation, all surviving males profit from the killing of rivals, but only the male involved in the fight suffers the risks of injury or death (Kureck et al., 2011). Outcome of this "tragedy of the commons" might be the absence of all-round competition among adult males (Rankin et al., 2007) and the guarding of smaller areas in the nest. Killing defenseless callows imposes less risk but still requires considerable investment into the search for freshly eclosed opponents. In *C. venustula*, callow killing occurred in small colonies and colonies with many simultaneously eclosing males, but usually several males eclosed safely and hardened their cuticles. Males then spaced out in different chambers or remained without territory.

The occurrence of male territorial behavior in both native and introduced populations suggests that it is a robust and successful strategy. We can only speculate about the cause of variability among and within populations. Climatic differences, e.g., between tropical Puerto Rico and temperate Hlalanathi, Drakensberg, might affect the production pattern of sexuals and thus male territoriality. However, there are also differences between colonies within a population, which might originate from different individual or colony personalities, different colony history, or a differing genetic background.

Territorial males appear to have more access to female sexuals by excluding other males from approaching them. Unfortunately, the hesitancy of female sexuals to mate during the observation sessions and the notoriously low variability of genetic markers throughout *Cardiocondyla*, together with frequent inbreeding and high nestmate relatedness (e.g., Heinze et al., 2014; Schmidt et al., 2016), did not allow quantifying the reproductive success of individual males or the mating frequency of queens.

Mating frequency varies across species of *Cardiocondyla* (e.g., Schrempf et al., 2005b; Lenoir et al., 2007; Schmidt et al., 2016), and a few behavioral observations suggest multiple mating. However, this does not necessarily mean that queens use sperm from different males to inseminate their eggs (e.g., Baer, 2014; Schmidt et al., 2016).

At present it remains unclear, which physical properties determine whether a male can obtain or territory or not. We did not find any association between body size and “territory” ownership despite the high variability in male size within and between colonies. Larger males are generally expected to be more successful in competition for access to females (Paxton, 2005; Kemp and Alcock, 2003), but in *Cardiocondyla* size advantages might be overridden by other factors like age (Cremer et al., 2012; Frohschammer and Heinze, 2009). Additionally, large size might not always be beneficial and smaller and more agile males might have an advantage in the confined space of the nest chambers and intersects. In choice experiments, female sexuals neither favored large, winged males over small, wingless males nor did they show any preference concerning the origin or age of males. This matches the scarcity of clear pre-copulatory female choice in social Hymenoptera (Keller and Passera, 1993; Oppelt et al., 2008; Baer, 2014). The frequent observation of males attempting to copulate with dealate queens, workers, pupae, and even dead individuals (see also Heinze et al., 1993) indicates that female sexuals do not signal their receptivity and that males try to increase their mating chances by more or less randomly courting potential partners.

To conclude, our study again highlights the suitability of the male-polyphenic genus *Cardiocondyla* to explore the evolution of alternative reproductive strategies and sexual selection in social insects. The large number of unstudied *Cardiocondyla* in sub-Saharan Africa (e.g., Bolton, 1986; Rigato, 2002; Seifert, 2003) makes the Afrotropic ecozone a particularly promising area for future research into the evolutionary pathways of male morphology and behavior in this genus.

3.6 Acknowledgments

We thank Bartosz Walter and Bert Rivera Marchand for help with obtaining collecting permits in Puerto Rico and Robin Crewe for help with obtaining the relevant permits in South Africa, and Sonja Dorfner, Marion Fuessl, Irene Fuhrmann, Katrin Kellner, Jan Oettler, Lukas Schrader, Jon Seal, Bartosz Walter, Ursula Wandinger, and Tina Wanke for valuable help in the field and/or the lab. Collecting in Puerto Rico was made possible through per- mit 2012-IC-036 by Departamento de Recursos Naturales y Ambientales, collecting in South Africa by Ezemvelo KZN Wildlife permit OP3198/2010 and the Rietvlei Nature Reserve permit SP 16-10-2012. Riaan Marais and the managers of Hlalanathi Drakensberg Resort allowed collecting ants from their land. Our study was supported by Deutsche Forschungsgemeinschaft (He1623/34).

4 Population and colony structure of an ant with territorial males, *Cardiocondyla venustula*

Susanne Jacobs & Jürgen Heinze

Institut für Zoologie, Universität Regensburg, Germany

4.1 Abstract

Background

Many species of social insects have large-scale mating and dispersal flights and their populations are therefore often relatively homogenous. In contrast, dispersal on the wing appears to be uncommon in most species of the ant genus *Cardiocondyla*, because its males are wingless and the winged queens mate in their natal nests before dispersing on foot. Here we examine the population structure of *C. venustula* from South Africa. This species is of particular interest for the analysis of life history evolution in *Cardiocondyla*, as it occupies a phylogenetic position between tropical species with multi-queen (polygynous) colonies and fighting males and a Palearctic clade with single-queen colonies and mutually peaceful males. Males of *C. venustula* exhibit an intermediate strategy between lethal fighting and complete tolerance – they mostly engage in non-lethal fights and defend small territories inside their natal nests. We investigated how this reproductive behavior influences colony and population structure by analyzing samples on two geographic scales in South Africa: a small $40 \times 40\,\mathrm{m}^2$ plot and a larger area with distances up to 5 km between sampling sites in Rietvlei Nature Reserve near Pretoria.

Results

Colonies were found to be facultatively polygynous and queens appear to mate only with a single male. The extraordinarily high inbreeding coefficient suggests regular sib-mating. Budding by workers and young queens is the predominant mode of colony-founding and leads to high population viscosity. In addition, some queens appear to found colonies independently or through adoption into foreign nests.

Conclusion

While *C. venustula* resembles tropical *Cardiocondyla* in queen number and mating frequency, it differs by the absence of winged disperser males. Dispersal by solitary, mated queens on foot or by short flights and their adoption by alien colonies might promote gene flow between colonies and counteract prolonged inbreeding. The abundance of suitable habitat and the high density of nests facilitate the spread of

this species by budding and together with the apparent resistance against inbreeding make it a highly successful pioneer species and invader of degraded and man-made habitats.

4.2 Background

The population structure of social insects is strongly shaped by their specific life-histories, in particular their mating behavior, dispersal abilities, and mode of colony founding. Many ant species are characterized by large-scale mating and dispersal flights and lack strong spatial population structure (e.g., Sundström, 1993; Seppä and Pamilo, 1995; Ross et al., 1997). In others, young queens mate in or near their natal nests and establish new colonies close by. This leads to high differentiation among populations at least at the level of maternally inherited mtDNA (e.g., Chapuisat et al., 1997; Doums et al., 2002; Sundström et al., 2003; Jaffé et al., 2009). Gene flow and outbreeding in the latter case are typically promoted by the dispersal of winged males (Boomsma et al., 2005; Helms IV, 2018).

Winged males have been replaced by wingless "ergatoid" males in most species of the ant genus *Cardiocondyla* (Kugler, 1983; Seifert, 2003; Oettler and Heinze, 2010; Heinze, 2017). Mating occurs inside the nest and mostly involves sibs, and the frequent occurrence of solitarily walking winged and dealate queens (which have lost their wings after mating) (Heinze, 2017) suggests limited dispersal capability and a highly viscous population structure. This stands in striking contrast to reports that *Cardiocondyla* are often among the first ants colonizing disturbed or rehabilitated habitats and that several species are highly successful "tramp species" with an almost worldwide distribution (Hoffmann and Andersen, 2003; Van Hamburg et al., 2004; Heinze et al., 2006; Wetterer, 2015). While the reproductive behavior of *Cardiocondyla* has been studied in detail in a number of species (e.g., Kinomura and Yamauchi, 1987; Heinze et al., 1993, 1998; Yamauchi et al., 2006), little is known about the genetic structure of populations and how far queens disperse.

The African species *Cardiocondyla venustula* is of particular interest in this context because of its placement in the phylogeny of the genus: it is positioned between tropical taxa, where colonies typically contain multiple, singly-mated queens and wingless males engage in fatal fighting over access to freshly emerging virgin queens, and a Palearctic clade where colonies have a single, multiply-mated queen per colony and mutually tolerant males (Oettler and Heinze, 2010; Heinze, 2017). Analyzing the social and genetic structure of *C. venustula* colonies and populations therefore is of considerable importance for unravelling the evolution of different life histories in this genus.

Behavioral studies revealed that males of *C. venustula* are always ergatoid and show an "intermediate" behavior between fatal fighting and complete tolerance: they defend small areas in the nest against nestmate males, engaging in regular, but typically non-lethal fighting with nestmate males (Frohschammer and Heinze, 2009; Jacobs and Heinze, 2017). Female sexuals (young, winged queens) were observed interacting with several males, but it remained unclear if they mated multiply. However, from male territoriality we hypothesized that queen mating frequencies would be low as in species with lethally fighting males.

Field studies gave conflicting results concerning queen number: while Wheeler (1908) reported on regular single-queening in an introduced population in Puerto Rico, our own observations revealed the regular presence of multiple queens per colony both in introduced and native populations (Jacobs and Heinze, 2017; Heinze et al., 2013). The finding of dense populations of *C. venustula* near Pretoria and Bergville, South Africa, allowed us to conduct detailed analyses of the genetic structure of colonies and populations and how they are influenced by male territoriality and female dispersal on foot.

4.3 Methods

Sampling and field observations

Samples for DNA analysis were collected in Rietvlei Nature Reserve, Pretoria, Gauteng, South Africa, and Hlalanathi Drakensberg Resort, Bergville, KwaZulu-Natal, South Africa, in February and March 2013. Workers were easily spotted when foraging on bare or sparsely vegetated ground, e.g., on unpaved parking lots. They were followed back to their nests, which consisted of two or three pea-sized holes in the ground down to a depth of 20 cm (Heinze et al., 2013). Subsequently, these nests were carefully excavated and the ants were either directly transferred into a tube with 100 % ethanol or transferred to the laboratory alive for behavioral experiments. Of the latter colonies, a few individuals were also stored in 100 % ethanol immediately after collection.

To analyze genetic population structure, relatedness and dispersal on a small scale, we measured the distances between all 57 colonies found in February 2013 in an area of $40 \times 40\,\mathrm{m}^2$ at the main gate of Rietvlei Nature Reserve (Riet: S 25.88250, E 28.26417, 1515 m). A few colonies from sampling areas in and near Rietvlei Nature Reserve, 0.5 km to 5.5 km away from the plot at the entrance building, were used to estimate gene flow and population structure on a larger scale (C: Rietvlei Coffee Shop, $n = 13$, S 25.87722, E 28.30083, 1511 m; CC: Coots Corner Bird Hide parking lot, $n = 4$, S 25.88139, E 28.26861, 1477 m; IV: Island View Bird Hide parking lot, $n = 8$, S 25.87639, E 28.28056, 1481 m; MD: Marais Dam parking lot, $n = 8$, S 25.90528, E 28.30833, 1511 m; PH: Pheasant Hill B & B, $n = 2$, S 25.88722, E 28.25778, 1508 m). Additionally, colonies from Hlalanathi Drakensberg Resort were used for estimation of relatedness and inbreeding ($n = 82$, S 28.65903, E 29.03136, 1288 m).

DNA extraction, microsatellite genotyping, and mtDNA sequencing

Before DNA extraction, samples were frozen in liquid nitrogen and crushed with a pestle in order to destroy the hard cuticle. Thereafter, DNA was extracted from older or damaged whole ants or pupae using a CTAB protocol (Sambrook et al., 1989) and from fresh and intact specimens using the Macherey Nagel XS Tissue Kit following the protocol for tissue samples. Seven microsatellite markers developed for *Cardiocondyla* were found to be sufficiently polymorphic for population genetic analysis in *C. venustula* (CE2-3A, CE2-4E, CE2-5D, CE2-12D (Lenoir et al., 2005); Card 8 (Schrempf et al., 2005b); Cobs 3 and Cobs 13 (Schmidt et al., 2016), see Table 4.1 for details). PCRs were conducted with a total volume of 15 µl using 7.5 µl GoTaq PCR mix (Promega, Madison, WI), 5 µl water, $0.5\,\mathrm{pmol}\,\mu\mathrm{l}^{-1}$ of each primer, and 1 µl of the DNA solution. Forward primers were end-labelled with fluorescent dye (FAM, Eurofin Genomics, Ebersberg). PCRs were run using an initial denaturation step at 94 °C for 4 min followed by 36 cycles with 70 s at 94 °C for denaturation, 45 s at the specific annealing temperature, and 25 s at 72 °C for elongation, and a final elongation step at 72 °C for 3 min. Fragment lengths were determined on an ABI

PRISM using a TAMRA labelled size standard. Alleles were scored using Genescan 3.1 software (PR Biosystems).

Table 4.1: Number of alleles N_A , allele sizes, expected (H_{exp}) and observed heterozygosity (H_{obs}), and inbreeding coefficient for microsatellite markers in the ant *Cardiocondyla venustula* from two populations in South Africa

Marker	$\mathbf{N_A}$	**allele size**	$\mathbf{H_{exp}}$	$\mathbf{H_{obs}}$	**F**
Hlalanathi					
Ce2-3a	10	80–102	0.723	0.198	0.728
Ce2-4e	2	101–107	0.426	0.051	0.882
Ce2-5d	4	192–198	0.283	0.171	0.397
Ce2-12d	15	132–206	0.847	0.207	0.756
Card8	6	121–133	0.702	0.171	0.758
Cobs3	11	103–139	0.664	0.207	0.689
Cobs13	7	72–88	0.562	0.049	0.914
Rietvlei					
Ce2-3a	4	84–92	0.043	0.043	-0.010
Ce2-4e	1	107	–	–	
Ce2-5d	1	194	–	–	
Ce2-12d	2	132–134	0.366	0.100	0.728
Card8	2	121–127	0.291	0.132	0.549
Cobs3	9	121–139	0.691	0.253	0.636
Cobs13	4	76–84	0.488	0.228	0.533

We analyzed a 795 bp-fragment of the COI/COII gene. Amplification of mtDNA was conducted in a total volume of 25 µl using 12.5 µl GoTaq PCR mix, 8.5 µl water, 0.5 pmol μl^{-1} of each primer, and 1.5 µl of the DNA solution.

Due to amplification problems with standard primers, we designed new primers using conserved regions in the previously obtained COI/COII-sequences: COICv-f (5'-AT TATCGCCGTCCCTACAGG-3') and COICv-r (5'-TCGGATGGGGAAGTTAT AAGGT-3'). PCRs were run using an initial denaturation step at 94 °C for 4 min followed by 39 cycles with 75 s at 94 °C for denaturation, 45 s at the specific annealing temperature, and 90 s at 94 °C for elongation, and a final elongation step at 72 °C for 7 min. PCR products were purified using the Macherey-Nagel Gel and PCR clean-up kit following the manufacturer's instructions and sent to LGC Genomics, Berlin, for sequencing. The trace files of obtained sequences were checked by eye for sequence quality.

Analysis of mating frequency

Field observations and dissections suggested that colonies of *C. venustula* might contain multiple, fertile queens. Polygyny was additionally corroborated by the analysis of workers by microsatellite genotyping. In addition, we investigated by COI sequencing whether colonies may contain individuals belonging to several unrelated lineages.

Queens and their worker offspring from natural colonies were genotyped to determine queen mating frequencies. However, these genotypes did not always allow determining whether workers were offspring of one queen, which had mated with several related males, or offspring of multiple related queens sharing the same mitochondrial haplotype, of which several might have been missed during or had died before collection. We therefore investigated whether female sexuals mate with multiple males in the laboratory. We set up experimental colonies, each consisting of ten workers, brood, and a queen pupa, in small nests (diameter appr. 1.5 cm) and later added one male each from two unrelated colonies with different genotypes, using colonies from both the populations at Rietvlei and at Hlalanathi, KwaZulu-Natal. To avoid males killing each other we first kept male pupae separately in nests with some brood and workers from their natal colony. After they had eclosed and hardened their cuticula, they were transferred into the experimental colonies with the young queen pupae. Experimental colonies, in which one of the males had died prematurely, were excluded from the analysis. Thus, each female sexual was provided with simultaneous access to two males until it was mated. Mating events are inconspicuous and difficult to observe (Jacobs and Heinze, 2017). We therefore waited for several days until all queens had shed their wings – which they usually do only after mating – and then stored them in 100 % ethanol before genotype analysis of stored sperm following Lenoir et al. (2007).

As the small amount of sperm DNA might not always give a reliable estimate of mating frequencies (because of large allele dropout or non-detection of alleles due to varying amounts of sperm from individual males), we also determined mating frequency from offspring genotypes. To do so we transferred ten additional, dealate queens into small colonies containing 20 workers and large larvae or worker pupae. From six of these queens we managed to collect and genotype five to eight 2nd instar or larger larvae. Thereafter, the queens were killed and genotyped.

Population genetic analyses

We used the software STRUCTURE (Pritchard et al., 2000) to find potential population structure on two scales, the $40 \times 40\,\mathrm{m}^2$ plot and the whole Rietvlei Reservation Area. The results were analyzed with STRUCTURE harvester (Earl and von Holdt, 2012) in order to find the optimal number of clusters (K). In addition, we assessed K with the function find.clusters implemented in the package “adegenet” (Jombart, 2008) in R (R Core Team, 2008). Population genetic parameters, intra- and intercolonial relatedness were calculated in SpaGeDi 1.5a (Hardy and Vekemans, 2002). Isolation by distance was tested via Mantel test comparing genetic and geographic distance matrices with the software Arlequin 3.1 (Excoffier and Lischer, 2010) with 10,000 permutations. As a measure for genetic distance in the $40 \times 40\,\mathrm{m}^2$ plot codominant pairwise distances were calculated in GenAlEx 6.5 (Peakall and Smouse, 2006), for the inter-population test, Slatkin’s linearized F_{ST} (Slatkin, 1995) was calculated as implemented in Arlequin. F-statistics were calculated using GDA 1.1 with bootstrapping over loci to obtain confidence intervals (Lewis and Zaykin, 2001). Significance of pairwise differentiation between sampling sites was calculated by permutation in Arlequin (Excoffier and Lischer, 2010). Microsatellites were tested for the existence and frequencies of null-alleles using INEST 2.2 (Chybicki and Burczyk, 2008).

4.4 Results

Field observations

Solitary queens, both winged and dealate, were regularly seen walking on the ground; one winged queen was observed flying for approximately 20 cm from the top of a blade of grass. These observations and dissections of winged, dispersing queens in the lab suggest short-range dispersal by queens both before and after mating (see also Heinze et al., 2013).

Of the 92 colonies collected in Rietvlei Nature Reserve, 30 contained a single dealate queen, 17 contained multiple, up to seven dealate queens, and the rest were queenless. The latter were probably incompletely sampled and were directly transferred into EtOH for genetic analyses.

Population structure

We obtained microsatellite genotypes of one worker each for 92 colonies from Rietvlei Nature Reserve (57 colonies from main gate, 13 colonies from C, four colonies from CC, eight colonies from IV, eight colonies from MD, two colonies from PH). Estimation of null allele frequencies in INEST did not support the existence of null alleles in any of the markers. Mean F_{ST}-values indicated considerable differentiation between collecting sites (mean $F_{ST} = 0.308$, 95 % confidence interval 0.136–0.456; Table 4.2). F_{ST}-values were positive for all pairs of sites, and permutation tests revealed significant differences in 11 of 15 pairwise comparisons. STRUCTURE analysis proposed two clusters in the intensively sampled $40 \times 40\,\mathrm{m}^2$ plot. While the "find.clusters" algorithm in DAPC did not provide clear clustering, manually setting the number of clusters to two revealed the same grouping as the one calculated by STRUCTURE. Plotting genetic clusters against sampling location in the $40 \times 40\,\mathrm{m}^2$ plot showed that cluster 1 (blue squares in Fig. 4.1) appeared to be mostly concentrated in two distinct spots in the sampling area while cluster 2 (black circles in Fig. 4.1) was distributed relatively evenly over the whole sampling area.

Table 4.2: Pairwise F_{ST}-values and geographic distance between sampling sites of the ant *Cardiocondyla venustula* at Rietvlei Nature Reserve, South Africa. Significant F_{ST}-values marked with *, significance level 0.05

	Main gate	**PH**	**MD**	**C**	**IV**	**CC**
	$n = 57$	$n = 2$	$n = 8$	$n = 12$	$n = 8$	$n = 4$
Main gate	-	0.8 km	5.1 km	3.7 km	1.8 km	0.5 km
PH	0.15	-	5.5 km	4.5 km	2.6 km	1.2 km
MD	0.21*	0.56*	-	3.2 km	2.0 km	4.8 km
C	0.01	0.15	0.24*	-	2.1 km	3.3 km
IV	0.49*	0.78*	0.86*	0.53*	-	1.3 km
CC	0.42*	0.54	0.62*	0.39*	0.69*	-

Across all collecting sites in Rietvlei, STRUCTURE suggested six clusters. Similar to the analysis in the smaller plot, "find.clusters" in DAPC did not reveal clear clustering. However, when K was set to six, results were concordant with those from STRUCTURE. While F_{ST}-values suggested significant differentiation among sampling sites, individuals from different sampling sites clustered together in STRUCTURE

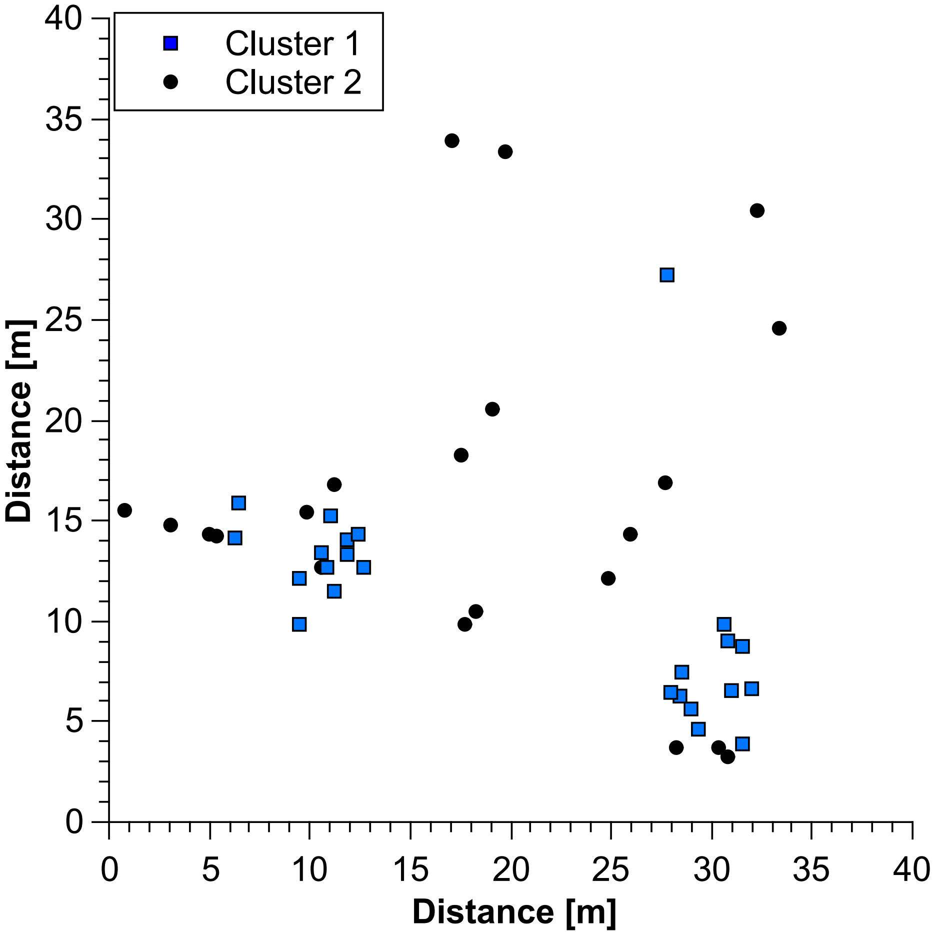


Figure 4.1: Distribution of clusters ($K = 2$) found by STRUCTURE clusters in the $40 \times 40\,\mathrm{m}^2$ grid at main gate. Coordinate points (circles and squares) represent one sampled colony each

analysis (Fig. 4.2). A comparison of pairwise genetic and geographic distances suggested a weak trend of isolation by distance in the $40 \times 40\,\mathrm{m}^2$ plot ($p = 0.06$, $r^2 = 0.01$). When comparing pairwise F_{ST}-values and geographic distances within the whole sampling area, no such effect was visible ($p = 0.40$, $r^2 = 0.01$).

Due to amplification problems, mtDNA sequences were available only for a subset of 44 individuals from 35 colonies (21 from main gate, two from C, four from CC, four from IV, three from MD, one from PH). In the $40 \times 40\,\mathrm{m}^2$ plot we found six haplotypes, one of which, h2, exhibited a similar distribution to that observed in

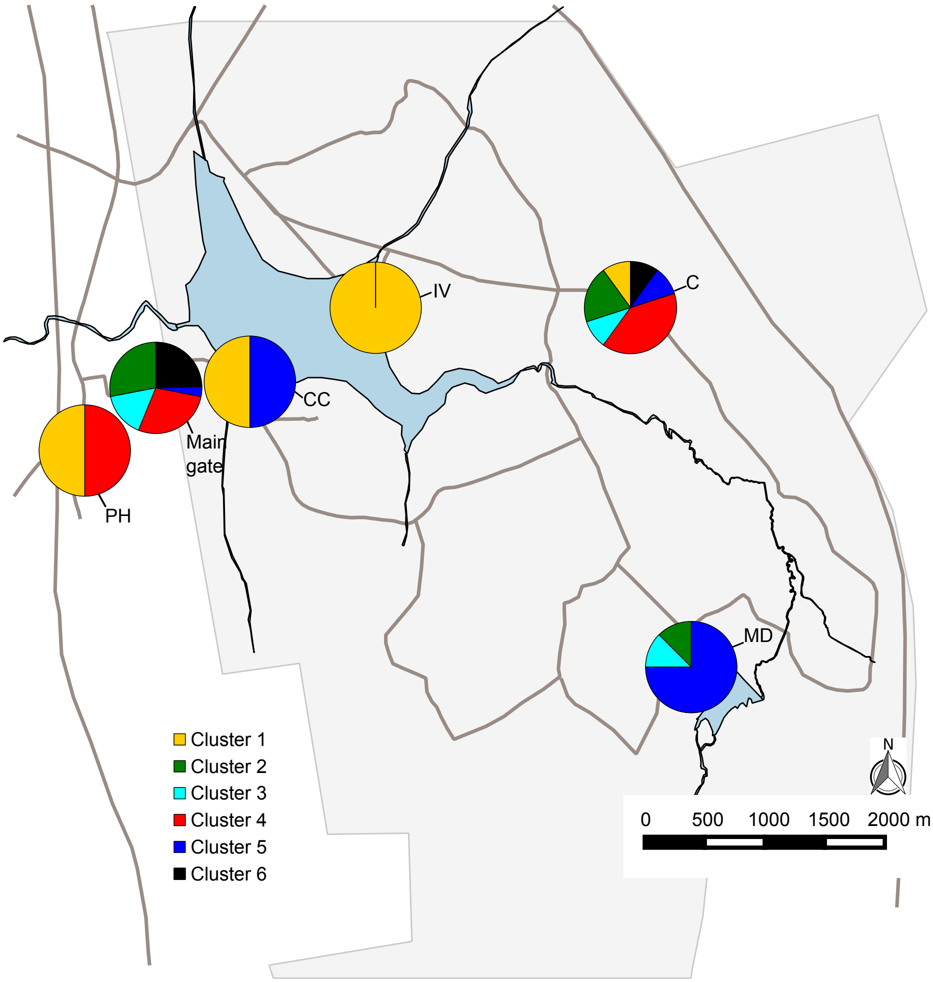


Figure 4.2: Location of sample sites with distribution of clusters ($K = 6$) found by STRUCTURE. Pie charts represent sample sites (not to scale) with the relative abundance of samples assigned to the respective cluster. Rietvlei nature reserve is depicted in light gray, water surfaces are depicted in blue. Sample site abbreviations: PH = Pheasant Hill B & B, CC = Coots Corner Bird Hide parking lot, IV = Island View Bird Hide parking lot, C = Rietvlei Coffee Shop, MD = Marais Dam parking lot

microsatellite cluster 1, occurring mainly in two spots in the sampling area. The other haplotypes were scattered (Fig. 4.3).

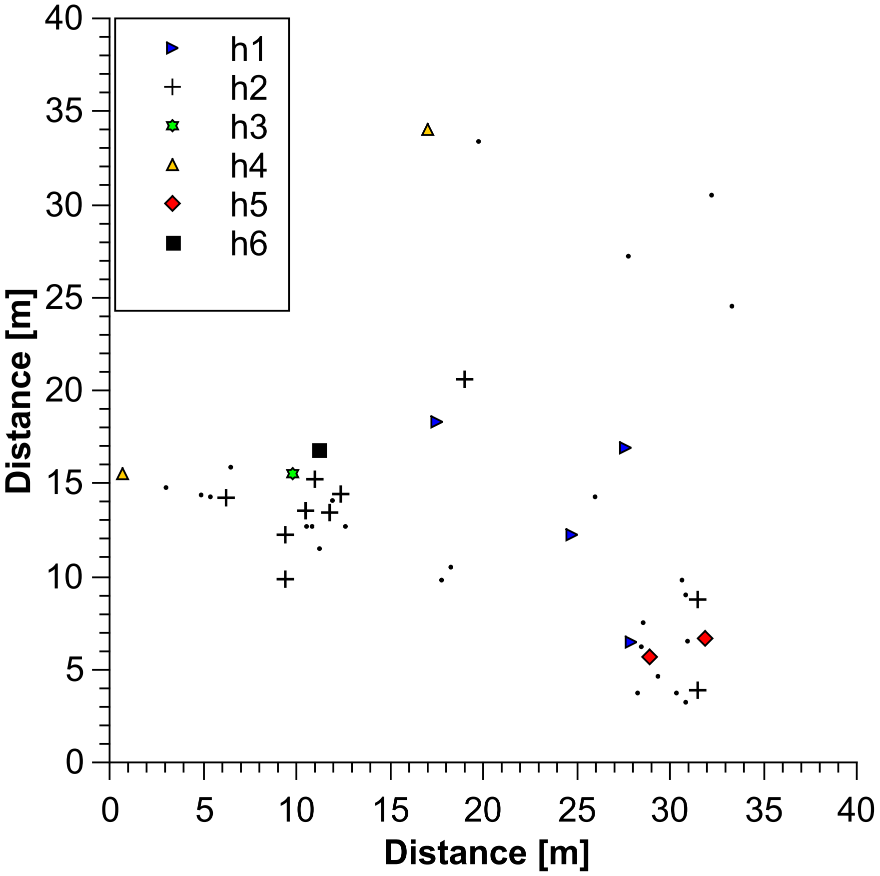


Figure 4.3: Distribution of mtDNA-haplotypes in the $40 \times 40\,\mathrm{m}^2$ grid at main gate. Coordinate points represent one sampled colony each, dots represent samples used in microsatellite, but not in mtDNA analysis

Across all sampling sites, seven haplotypes were found (GenBank Accession numbers MK138574 – MK138580). Interestingly, we found only a single haplotype at several of the less intensively sampled sites (Fig. 4.4). Comparison of genetic clusters found by STRUCTURE analysis of the microsatellite genotypes with those found in mitochondrial haplotypes revealed an independent distribution of both marker types (Mantel test for correlation between F_{ST}-values: $p = 0.42$, $r^2 = 0.005$). Isolation by distance was similarly low in mtDNA markers as in microsatellite markers. For the $40 \times 40\,\mathrm{m}^2$ plot, isolation by distance was significant but weak ($p = 0.05$, $r^2 = 0.02$). Between the sampling sites, no significant effect was found ($p = 0.40$, $r^2 = 0.005$). Maternally inherited mtDNA and the bi-parentally inherited genomic microsatellites and their spatial distribution thus revealed only subtle differences, suggesting a female-biased dispersal and supporting the observations of dispersing queens in the field.

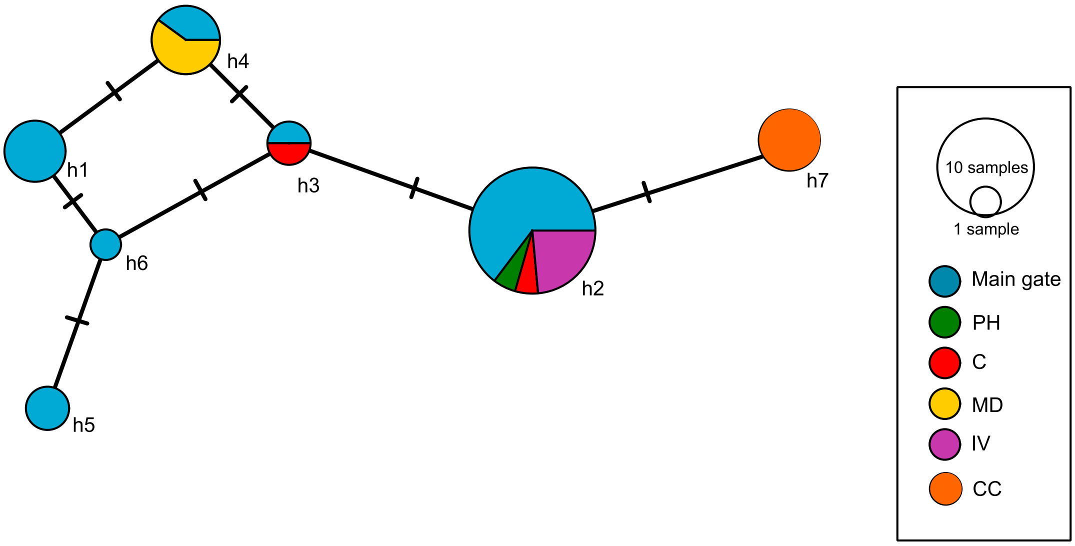


Figure 4.4: Haplotype network for the 795-bp fragment of the COI-gene. Pie charts represent one haplotype each, sample sites are represented by the respective color. Sample site abbreviations: PH = Pheasant Hill B & B, CC = Coots Corner Bird Hide parking lot, IV = Island View Bird Hide parking lot, C = Rietvlei Coffee Shop, MD = Marais Dam parking lot

Relatedness and colony structure

For the analysis of relatedness and colony structure, we obtained microsatellite genotypes from 8 to 23 workers per colony, analyzing ten colonies from Rietvlei (117 genotypes in total) and twelve colonies from Hlalanathi (112 genotypes in total). F_{IS} and background allele frequencies were calculated using one individual per colony for all sampled colonies in the respective populations (91 colonies from Rietvlei, 82 colonies from Hlalanathi). Microsatellite genotypes showed high levels of inbreeding in all sampled areas in Rietvlei (overall $F_{IS} = 0.495$, 95 % confidence interval 0.366–0.562) as well as in Hlalanathi (overall $F_{IS} = 0.551$, 95 % confidence interval 0.422–0.614), suggesting 86.1 and 83.1 % sib-mating, respectively (following Suzuki and Iwasa, 1980).

Though field observations revealed the frequent presence of multiple queens within a single colony (see above), relatedness was usually high (Rietvlei: mean $0.867 \pm S.E.\,0.079$, ranging from 0.030 ± 0.291 to 1 ± 0; 5 colonies only from Rietvlei main gate: mean $0.767 \pm S.E.\,0.133$, ranging from 0.567 ± 0.175 to 0.983 ± 0.018; Hlalanathi: mean $0.786 \pm S.E.\,0.096$, ranging from 0.456 ± 0.185 to 1 ± 0). This matches the high inbreeding coefficient and the assumption that workers may occasionally be offspring of multiple, usually related queens, most of which have mated with the same or several closely related males. Correction for inbreeding (Pamilo, 1985) yielded a mean relatedness of 0.454 (Rietvlei) and 0.261 (Hlalanathi). The co-occurrence of two different mtDNA haplotypes in workers of a previously studied colony (Heinze et al., 2013) indicates that colonies may contain multiple unrelated queens or occasionally adopt alien workers.

The non-detection error arising from the low variability of microsatellites typical for most *Cardiocondyla*, the high inbreeding coefficient, and the fluidity of colony structure all make it difficult to exactly determine the number of matrilines and patrilines. We therefore staged mating experiments in the lab to determine whether female sexuals (young, winged queens) mate with multiple or single males. In the spermathecae of queens that had been given the possibility to mate with two genetically different males, we did never find more than one paternal allele, i.e., there was no evidence of multiple inseminations. Similarly, the five to eight offspring larvae produced each by the six queens that had been exposed to two males were all offspring of only one father (total number 36 larvae).

4.5 Discussion

The reproductive biology of ants of the genus *Cardiocondyla* with stationary wingless males, mating in the nest, and dispersal on foot by winged, mated and / or unmated queens is quite unusual for ants and social insects in general (Heinze, 2017). As we here show specifically for *C. venustula* from Rietvlei Nature Reserve in South Africa, this leads to high levels of inbreeding and nestmate relatedness.

Unfortunately, the low genetic diversity, already known from previous studies in other *Cardiocondyla* species (e.g., Schmidt et al., 2016; Heinze et al., 2014; Schrempf, 2014) together with sib-mating and the short lifespan and rapid turnover of queens (Oettler and Schrempf, 2016) makes it difficult to resolve colony and population structure to a high degree of accuracy. Nevertheless, our findings are in agreement with previous assumptions about the dispersal biology of this genus, with local mating, short-range dispersal by queens, colony budding, and occasional long-range dispersal by queens on the wing or mediated by human activities. Population viscosity was surprisingly small both at the level of nuclear microsatellites and mtDNA haplotypes, and no significant structure was seen when colonies from more distant collecting sites were included in the analysis.

A detailed survey in a $40 \times 40\,\mathrm{m}^2$ plot with almost 60 colonies showed that colonies with similar microsatellite genotypes and mtDNA haplotypes may occasionally cluster. This matches our field observations: while wingless males are only very rarely observed outside the nest (e.g., Lenoir et al., 2007; Bolton, 1982), winged and wingless queens are regularly seen walking over distances of a few meters. Winged queens may even be drifted over longer distances by wind (Heinze, 2017), explaining the relatively weak isolation by distance. The clumped distribution of cluster 1 and haplotype 2 in the $40 \times 40\,\mathrm{m}^2$ suggest that new colonies may be founded by budding, i.e., young queens disperse together with several workers from their natal nest after mating. In the laboratory, new colonies are easily produced by splitting multi-queen colonies (e.g., Heinze et al., 1993), supporting the hypothesis that budding is a regular mode of founding in this genus. Whether colonies of *C. venustula* may inhabit several neighboring nests (polydomy), as suggested for populations of this species introduced to Puerto Rico (Wheeler, 1908; Wilson, 1959), remains unclear. Workers in Rietvlei exhibited aggression or backed off when confronted with a worker from an adjacent nest in a small confined space in the field (S. Jacobs, unpublished observations), making it unlikely that buds remain connected to the natal nest.

The rare co-occurrence of different mtDNA haplotypes within the same colony (Heinze et al., 2013) might suggest that in addition to colony founding by budding solitarily dispersing queens occasionally may be adopted into alien nests. This would explain the lack of a strong correlation between mtDNA and microsatellite clusters and also promote gene flow between colonies. In the laboratory, groups of multiple queens of *C. venustula* and other *Cardiocondyla* were able to rear larvae (Schrempf and Heinze, 2007, S.J., unpubl. observations). Hence, cooperative founding by unrelated dispersing queens provides an alternative way of colony founding and might also contribute to the existence of multiple mtDNA haplotypes in one colony.

Though F_{ST}-values were significant, we could not reveal a clear genetic structure across all sampling sites in Rietvlei Nature Reserves. The population from Island View (IV) did not show any variability in the analyzed markers despite a sufficiently large sample size at least for microsatellite genotyping. This might indicate that populations are occasionally founded by only one or a few related queens (with or without workers). Isolated patches might be colonized via several stepping stones of small, sparsely vegetated patches. In addition, the worldwide spread of several *Cardiocondyla* tramp species (Heinze, 2017; Wetterer, 2012, 2014a,b) including *C. venustula*, suggests that the accidental transfer of solitary queens or colony fragments with soil, seedlings, or garbage may ease the colonization of new habitat and may have contributed to these apparent founder effects. The network of unpaved roads in Rietvlei Nature Reserve may also have facilitated the dispersal of *C. venustula* as such roads provide the ideal habitat for these ants.

The inbreeding and relatedness coefficients obtained by microsatellite genotyping for nestmates of *C. venustula* were higher than in other *Cardiocondyla* species (Schrempf et al., 2005b; Schmidt et al., 2016; Lenoir et al., 2007; Schrempf, 2014), including the related *C. shuckardi* from Madagascar (Heinze et al., 2014). The inbreeding coefficient suggests more than 80 % sib-mating. Generally, *Cardiocondyla* show a high tolerance to inbreeding. In *C. obscurior*, no diploid males were found even after several generations of sib-mating in the lab (Schrempf et al., 2006). In contrast to honeybees and presumably also other social Hymenoptera (Van Wilgenburg et al., 2006), *Cardiocondyla* does not exhibit single-locus complementary sex determination and inbreeding therefore does not lead to the production of non-viable or sterile diploid males. Resistance towards inbreeding and genetic impoverishment has previously been suggested to facilitate the successful establishment of small propagules in novel environments (Heinze et al., 2006; Baker, 1955; Yamauchi and Ogata, 1995; Passera, 1994; Evans et al., 2011), and our study again highlights that sib-mating in *Cardiocondyla* is not a consequence but a precondition of invasiveness (see also Eyer et al., 2018).

Nevertheless, exclusive inbreeding may occasionally have adverse effects also in *Cardiocondyla*, including reduced queen life span and a lower fecundity of males (Schrempf et al., 2006). These are presumably lessened by occasional outbreeding events, e.g., through the adoption of alien queens as described above. Winged queens have regularly been observed to disperse on foot, and some of them may enter and mate in alien nests, similar to what has been observed in Palearctic *Cardiocondyla* (Lenoir et al., 2007; Schrempf, 2014). Wingless males of *C. venustula* may therefore not only compete for mating with their sisters (Frohschammer and Heinze, 2009; Jacobs and Heinze, 2017) but also for mating with alien queens. The presence of multiple fertile queens in field and also lab colonies (unpublished data) suggests that *C. venustula* is facultatively polygynous, in contrast to the obligatorily monogynous Palearctic clade. While workers typically are expected to oppose the adoption of alien queens on relatedness grounds (e.g., Stuart et al., 1993; Gadau et al., 1998), *C. venustula* workers would be closely related to an adopted queen's offspring in case it has mated with one of the colony's males.

The low variability of genetic markers and the presumed high relatedness among nestmate males did not allow determining the mating frequency of field-collected queens. Sperm and offspring analyses suggest that queens, which in the lab had been given the chance to mate with two genotypically distinct males, mated only once. Monogamy in *C. venustula* is in accordance with males successfully increasing their fitness by defending small "territories" inside the nests and monopolizing mating with female sexuals inside the nest. While obligatorily monogynous *Cardiocondyla* are polyandrous (Schrempf et al., 2005b; Lenoir et al., 2007; Schrempf, 2014), facultatively polygynous *C. obscurior* (Schmidt et al., 2016) and, as we here show, *C. venustula* appear to be monandrous. This matches previous observations of the correlation between queen number and mating frequency in other ants (e.g., Hughes et al., 2008b).

4.6 Conclusion

Our study shows that *C. venustula* resembles other tropical species of the genus in the presence of multiple queens per nest and single mating by queens. Though lethal fighting among *C. venustula* males appears to be uncommon, the aggressive defense of small areas in the nest appears to allow them to monopolize mating with many female sexuals. The very high levels of inbreeding and nestmate relatedness indicate that most matings involve siblings, but the co-occurrence of multiple mtDNA haplotypes in a single colony suggests that dispersing queens may occasionally be adopted by alien colonies. This can promote gene flow and alleviate the negative effects of prolonged inbreeding. Dispersal by young queens over short distances and budding appear to be the prevalent modes of colony founding. Though it still remains to be clarified how long-range dispersal is achieved, the preference of *C. venustula* for ruderal, degraded patches with sparse vegetation certainly facilitates the colonization of novel habitats, such as rehabilitated mines, parks, or roadsides. Human-assisted transfer in potted plants may have allowed it to spread from its Afrotropical origin to Florida and several Caribbean and Pacific Islands (Seifert, 2003; Wetterer, 2015) and may also have contributed to the limited isolation by distance across all collecting sites in Rietvlei.

Acknowledgments

We thank Sonja Dorfner, Lukas Schrader, and Tina Wanke for help in the field, Riaan Marais for permission to work at Rietvlei Nature Reserve (SP 16-10-2012) and the administration for Hlalanathi Drakensberg Resort for permission to collect ants on their property. Robin Crewe helped obtaining permits for collecting and exporting ants from the Premier of the province of Gauteng (CPF6 0173 and CPB5 003499) and Ezemvelo KZN Wildlife (OP 4720/2012). Two reviewers and Qiao Wang made helpful suggestions on the manuscript.

5 General Discussion

5.1 Sexual selection in *Cardiocondyla venustula*

The study of male behavior in *Cardiocondyla venustula* revealed a previously unknown behavior for ant males: defending small territories within their natal nests (Frohschammer and Heinze, 2009, Chapter 3). Male territoriality was observed in both native and introduced populations. This peculiar behavior adds to a number of idiosyncratic features of the genus *Cardiocondyla*, probably best-known for the peculiar male diphenism and ergatoid males with lifelong spermatogenesis, a trait unique for social Hymenoptera (Heinze and Hölldobler, 1993). The evolution of male mating strategies is presumed to be mainly influenced by the availability of virgin queens and the numerical sex ratio, resulting in the ability of males to monopolize matings. These factors vary greatly between species, for example, due to the influence of local mate competition (Cremer and Heinze, 2002; Schrempf et al., 2005b) or seasonality of reproduction (Heinze, 2017). Species of the genus *Cardiocondyla* thus open up the opportunity to study a broad range of aspects of male competition, a topic that has largely been neglected in social insect research (Chapter 2).

Compared to the pilot study, the experimental nests described in Chapter 3 were designed to more closely resemble the natural nests found during the collection of colonies from the species in terms of corridor width and chamber diameter. As described in Chapter 3, male behavior was highly variable within, but particularly between colonies. Differences in male behavior within a colony – territorial males vs. "floaters" – may represent alternative reproductive tactics, with "floaters" sneaking matings as known from several species (Gross, 1996). Causes of the high between-colony variability, which had already been observed in the pilot study by Frohschammer and Heinze (2009), did not become obvious during the experiments. Male behavior did not depend on factors like colony size, presence or absence of virgin queens in the experimental nests. Generally, males in small colonies appear to be more aggressive than males from larger colonies (unpublished results). However, the high intra- and intercolonial variations, probably influenced by several co-varying factors, did not allow for statistically significant conclusions. Inter-individual variation and behavior syndromes or “colony personality” that may account for variability in behavior have only recently commenced to be studied (e.g., Jeanson and Weidenmüller, 2014; Scharf et al., 2012; Pinter-Wollman, 2012). Yet, genetic differences as one of the major causes are presumably small in the highly inbred *C. venustula*. Apart from that, a number of different parasites are able to significantly alter ant behavior including aggression and may thus add to variation (de Bekker et al., 2018).

Unlike in most *Cardiocondyla* species, the size of males in *C. venustula* and the closely related *C. shuckardi* exceeds the size of workers notably (Schmidt and Heinze, 2018). Assuming that the production of larger males imposes higher costs on colonies, this suggests that large male size represents a sexually selected trait, comparable to size differences in harvester ants as described by Davidson (1982); Wiernasz et al. (1995). As described in Chapter 3, we could not find large size to be advantageous in fighting in our experimental setting. Whether this is an artifact of the experimental conditions or whether large size may also be disadvantageous under natural conditions in some cases, for example, by providing higher agility of smaller males in small nests with narrow corridors, remains to be studied. While the testes size did not vary between males of different body size in a small sample, large, brachypterous males were found to produce significantly longer sperm than smaller, wingless males (unpublished data). In this sample, the variance of sperm length within the single males was large. High variance of sperm length has also been shown in other *Cardiocondyla* species (e.g., Metzler et al., 2018) and it may vary with different factors, such as age (Heinze et al., 2018). According to Fitzpatrick and Baer (2011), a high degree of intra- and inter-individual variance in sperm length is also in accordance with monandry in *C. venustula* which is suggested by population genetic analysis (Chapter 4).

Albeit in our experiments, preference for specific nest chambers (for example, close to the entrance of the nest) was found neither in males nor in female sexuals, nest chambers may vary in quality under natural conditions. Upon excavation, alate females often aggregated in the uppermost nest chamber, thus, it could be concluded that chambers closer to the surface are more attractive to males. If unmated foreign female sexuals are able to enter the colony, as seen in other *Cardiocondyla* species (Lenoir et al., 2007; Schrempf, 2014) and as suggested by the finding of different mtDNA lineages within one colony (see Chapter 4), this effect might be even stronger. However, as we did not dissect these alate females in the field but rather collected them together with the rest of the colony to obtain colonies for subsequent lab experiments, we cannot conclude whether these were already mated and preparing for dispersal.

In the course of the behavioral observations, there was no clear evidence for pre-copulatory female choice, but queens frequently impeded mating by retracting their gaster when the male tried to insert its genitalia, preceded by the prolonged courtship display known from several *Cardiocondyla* species (Mercier et al., 2007). Whether queens rejected mating with these males because they were not yet receptive or already mated or whether this reflects female choice remains unclear. However, the courtship display with antennation, mandible contact, stridulation and the male rocking back and forth on the female's back (Mercier et al., 2007) might contain signals that allow for female choice. Unlike in ant species that mate in aerial swarms or at least exhibit some kind of pre-copulatory dispersal by males, mating inside the nest in *Cardiocondyla* species provides no selection by flying abilities and general vigor. While differences between males available for female sexuals inside the nest may be small due to the usually high relatedness, and male fighting may pre-select for "good quality" males, the idiosyncratic courtship behavior in the genus could provide virgin queens with information on male quality before entering a "commitment for life". In contrast to several other *Cardiocondyla* species with usually only a single ergatoid male surviving per colony (Oettler and Heinze, 2010) and only occasionally additional winged males (e.g., Cremer and Heinze, 2003; Heinze et al., 2004), female sexuals of *C. venustula* can potentially choose between several males.

On the contrary, as described in Chapter 3, males apparently were non-selective concerning their mates. Partners for attempts by males included dealate queens, workers, other males or even corpses, and males often realized their "mistake" only late in the process. This indicates that virgin females do not signal their receptivity, thus mating attempts with non-receptive female sexuals may be an explanation for rejected matings. By dissecting young queens that had eclosed in a small colony with a male present one to eight days after eclosion, I was able to determine that female sexuals become receptive between the second and fourth day (unpublished data). Mated queens apparently started to shed their wings almost instantly. Thus, it cannot be ruled out that rejected matings fall in the period before receptivity or after successful mating, and further investigation is needed to clarify whether female choice plays a role in this respect. However, as the copulation per se is usually short and inconspicuous in comparison to the lengthy courtship ritual, it is fairly difficult to conclude from observation alone whether a mating was successful.

5.2 The difficulty of species determination in the *Cardiocondyla venustula* group

Early in the population genetic analysis of *C. venustula*, it became obvious, that the colonies collected in South Africa in 2011 and 2013 belonged to several distant genetic lineages. With low dispersal ability other than from accidental transport by humans, populations may easily become isolated after an introduction event and genetic drift is expected to be strong. Thus, the large genetic distance between the populations in Rietvlei and a part of the populations in Hlalanathi, over 300 kilometers apart was according to our expectations. Surprisingly, one part of the population in Hlalanathi was genetically extremely close to the population in Rietvlei, but distinct from the neighboring population that inhabited spots in only 200 to 400 m distance. This may be the result of human transport, since the sample sites in Hlalanathi were in or close by a tourist resort. MtDNA sequences of most samples of both populations revealed a close genetic relatedness concordant with passive long-distance dispersal by queens.

Interestingly, the brachypterous males already described in (Heinze et al., 2013) were only found in a subset of the population in Hlalanathi, whereas none of the colonies from Rietvlei produced this type of males. In Hlalanathi, brachypterous males were found both in the field and subsequently in the colonies transferred to the lab. Except from the production of brachypterous males in some of the colonies from Hlalanathi, *C. venustula* from both sample sites did not show discernible morphological differences. This was similar for a few colonies that were found to differ profoundly from the other samples both in mtDNA (COI/COII) and microsatellite analysis. Several of these samples appeared to be more closely related to *C. venustula* found in Puerto Rico and *C. shuckardi* from Madagascar, leading to a paraphyletic tree (Supplementary chapter II). Unfortunately, the lack of sufficiently variable nuclear markers other than microsatellites did not allow for the creation of a nuclear phylogeny of the *C. shuckardi* species group. Standard nuclear markers, including a set of markers that were described to be highly variable across apocritan Hymenoptera (Hartig et al., 2012), revealed hardly any variability.

While mtDNA sequences such as the COI/COII genes are a regularly used and valuable tool in phylogeny, phylogenetic trees based solely on mtDNA may lead to erroneous conclusions due to incomplete lineage sorting or hybridization between species (Rubinoff and Holland, 2005). MtDNA trees can, for example, indicate paraphyly where nuclear DNA or morphological parameters suggest monophyletic taxa (e.g., Seifert, 2009). Furthermore, the bacterial symbiont *Wolbachia* found widespread across arthropod taxa may lead to significant mtDNA diversification (e.g., Shoemaker et al., 2003; Xiao et al., 2012). These effects can thus not be fully ruled out in the case of *C. venustula* and *C. shuckardi*, where mtDNA phylogenetic trees apparently deviate from the species' morphological taxonomy (Seifert, 2003; Heinze, 2020). Unfortunately, closely related *Cardiocondyla* from several species groups are far from being distinguishable from each other by eye, and rather require sophisticated morphometric examination (Heinze, 2017). However, the concordance of mtDNA and microsatellite analysis in several of the "aberrant" samples from South Africa point to the existence of sympatric genetically differentiated lineages. Male and female sexuals from South Africa were successfully crossbred with *C. venustula* from Puerto Rico and *C. shuckardi* from Madagascar in the lab and produced viable worker and sexual offspring. On the one hand, this result indicates that hybridization between different lineages or species may be possible, on the other hand, it suggests that the different lineages of *C. venustula* might be separated by a reproductive barrier in the field or be the consequence of recent introductions. It therefore remains exciting, whether the combination of genetic and morphological data will finally solve the challenging taxonomy of the *C. shuckardi* species group.

5.3 Population genetics in *Cardiocondyla venustula*

Morphology, ecology and behavior have been described for a number of *Cardiocondyla* species, while research on population genetics of the genus is rather scarce. This seems striking because population genetics could be used to analyze the diverse mating systems of the genus, e.g., to detect polyandry, or to measure dispersal ability or reproductive success of individuals and colonies (e.g., Jaffé, 2014; Ingram et al., 2013). Unfortunately, population genetic analysis of *Cardiocondyla* species using standard molecular genetic tools like microsatellites is restrained by the notoriously low variability of these markers (e.g., Heinze et al., 2014). In animals, the number of microsatellites is usually positively correlated with genome size (Hancock, 1996; Primmer et al., 1997; Schlötterer and Harr, 2000). With the genome of *C. obsurior* being the smallest genome found in an ant species to date (Schrader et al., 2014), numbers of microsatellites are thus expected to be low. High levels of inbreeding due to a high percentage of matings between siblings further reduce genetic diversity within populations (e.g., Heinze et al., 2014; Schrempf et al., 2005b). A number of markers described to be various across different ant species (Butler et al., 2014) were successfully amplified in *C. venustula*, but none of these showed sufficient polymorphisms even between distant populations (unpublished data). Therefore, in most *Cardiocondyla* species, microsatellites can contribute to a rough analysis of population structure and relatedness, but more detailed research on paternity or reproductive success of individual queens is difficult, if not impossible. Population genetic analysis of *C. venustula* revealed high levels of inbreeding with over 80% of matings between siblings (Chapter 4). Table 5.1 gives a comparison of the population genetic parameters and colony structure of *C. venustula* with the other *Cardiocondyla* species analyzed so far.

While we found genetic differences between the sampled subpopulations, indicated by significant FST values, the correlation between geographic and genetic distance was weak in the intensively sampled subpopulation in a $40 \times 40\,\mathrm{m}^2$ grid, and it was absent on a larger scale. This suggests that on a small scale, budding is the prevailing mode of dispersal in *C. venustula*, albeit the existence of more than one mtDNA haplotype in some colonies indicates alternative dispersal modes, for example, adoption of foreign queens into nests or potentially cooperative founding queens. In the lab, groups of ant queens were able to cooperatively found a new

Table 5.1: Comparison of levels of inbreeding, relatedness and colony structure of different *Cardiocondyla* species. m (sib): percentage of mating between siblings, F: inbreeding coefficient, r: mean nestmate relatedness (not corrected for inbreeding), mf: effective mating frequency of queens, n (q): number of queens per colony

species	m (sib)	F	r	mf	n (q)	source
C. batesii	> 80	0.55	0.66	1.49	1	Schrempf et al. (2005b)
C. elegans	70	0.37	0.49	4.52	1	Lenoir et al. (2007)
C. nigra	> 66	0.34	0.58	3.84	1	Schrempf (2014)
C. obscurior	62	0.29	0.58	1	1-15	Heinze and Delabie (2005)
C. shuckardi	> 80	0.69	0.77	1	1-7	Heinze et al. (2014)
C. venustula	**> 80**	**0.52**	**0.81**	**1**	**1-7**	**Chapter 4**

colony (Supplementary chapter I). On a larger scale, dispersal appears to be mostly by passive transport rather than by active motion. As a tramp ant inhabiting anthropogenically disturbed habitats, *C. venustula* is presumably easily carried to new locations by accident if solitary queens or colony fragments are transported with soil, plants or garbage.

The level of inbreeding in *C. venustula* was shown to be similarly high as in the closely related species *C. shuckardi* (Heinze et al., 2014). The extent to which inbreeding affects the viability of populations differs within and between species (Hedrick and Garcia-Dorado, 2016). However, reduced genetic variability as a consequence of inbreeding nearly inevitably leads to reduced adaptability to changing environments, higher infection risks or decreased performance of offspring (e.g., Hansson and Westerberg, 2002). The loss of genetic diversity may reduce the efficiency of nestmate recognition, thus promoting the formation of supercolonies in introduced populations of a species (e.g., Tsutsui and Case, 2001; Tsutsui and Suarez, 2003; Helanterä et al., 2009). Yet, supercolonies are not known in *Cardiocondyla* to date. In haplodiploid social insects with single-locus complementary sex determination, the occurrence of diploid, sterile males is probably the most detrimental effect of high rates of inbreeding (Harpur et al., 2013). Beyond this effect, studies revealed inbreeding depression with reduced female fitness in haplodiploid parasitoids (Henter, 2003) and some ants (Haag-Liautard et al., 2009; Schrempf et al., 2006), whereas no effects were visible in other ant species (Kureck et al., 2012). Many ant species have evolved reproductive strategies that reduce the adverse effects of inbreeding. The most common may be that male and/or female sexuals disperse and mate away from their natal nest (e.g., Hölldobler and Wilson, 1990). Polygyny and polyandry have been discussed as strategies to increase a colony's genetic diversity, however, the impact of multiple mating on genetic diversity and colony fitness remains inconclusive (e.g., Boomsma et al., 2005; Crozier and Fjerdingstad, 2001; Fjerdingstad, 2012; Hughes et al., 2008b). Similarly to other invasive ant species (e.g., Eyer et al., 2018), the high levels of inbreeding and the apparently resulting high tolerance to inbreeding found in several *Cardiocondyla* may be an important factor leading to their success as tramp ants around the world.

Adding to its intriguing mating biology, the genus *Cardiocondyla* has also evolved fascinating strategies to reduce detrimental effects of inbreeding. The most deleterious effect of inbreeding in haplodiploids, the production of diploid males, is prevented by an alternative mechanism of sex determination: in an early study, Schrempf et al. (2006) found that prolonged inbreeding over ten generations had an impact on fitness, but colonies did not produce diploid males, thus excluding a single-locus mechanism of sex determination. Instead, sex-specific splicing of the *transformer* gene induced by a yet unknown mechanism leads to the development of males from unfertilized eggs and females – workers or queens – from fertilized eggs (Klein et al., 2016). Furthermore, accumulation of transposable elements (TE) in some areas of the genome that were found in *C. obscurior* facilitates rapid adaptation despite high rates of inbreeding leading to low genetic diversity, particularly after invasion events (Schrader et al., 2014). The findings of genetic and genomic analyses thus reveal the fascinating and unique adaptations in the genus *Cardiocondyla*.

5.4 Conclusions

The genus *Cardiocondyla*, characterized by several peculiar features such as intranidal mating and ergatoid males, represents an excellent model for the study of the evolution of reproductive tactics and sexual selection in social insects. For research on male tactics, *C. venustula* and other species of the *C. shuckardi* group are of particular interest. These species likely mark the transition from fighting to mutually peaceful males. Intriguingly, the intermediate phylogenetic position between species with fighting males and the Palearctic clade with mutually peaceful males is in fact mirrored in the males' behavior. With several unique features only recently discovered, the genus *Cardiocondyla* may hold many more secrets yet to be uncovered. Advances in research on the genus, such as the now available *C. obscurior* genome, will support new approaches to the study of the genus using genetic and genomic data across species.

5.5 Summary

The ant genus *Cardiocondyla* presumably comprises more than 100 species exhibiting a wide range of life-history strategies. The most striking feature is the existence of wingless, ergatoid (=worker-like) males in addition to or instead of the "standard" winged ant males. In several species, ergatoid males engage in lethal fighting to monopolize access to receptive female sexuals. Male morphology and mating strategies vary largely between species, as do traits like number of queens or colony size. Unlike in most other ant genera, mating takes place predominantly in the nest. Many *Cardiocondyla* species mate readily in the lab and therefore offer the opportunity to study several aspects of mating behavior in detail. The genus thus represents a fascinating model for the analysis of life history evolution, particularly the evolution of male morphology and male-male competition.

Chapter 2 of this thesis reviews the current knowledge of male-male competition across ant species. The extent and nature of male competition varies with several factors, such as mating syndrome/location (male aggregation in aerial swarms vs. female calling with mating on the ground or intranidal mating) sex ratio, or number of mates per queen. While pre-copulatory competition has been studied for over a century, post-copulatory competition such as mating plugs or sperm competition in species with multiply mating queens has only come into focus in the course of the last decades. Pre-copulatory competition between ant males reaches from scramble competition in swarm-mating species to interference with other males' matings in species that mate on the ground, to lethal fighting in species of the genera *Cardiocondyla* and *Hypoponera* with intranidal mating.

Within the genus *Cardiocondyla*, males of the study species of this thesis, *C. venustula*, revealed a competitive behavior yet undescribed for social hymenoptera: wingless males spread out in the nest and defend small territories. This presumably marks a transition between males of species from tropics and the monophyletic Palearctic clade. Males of the former usually engage in often lethal fighting, while males of the latter are mutually peaceful. In **Chapter 3**, this peculiar behavior, which was first observed in one colony from the introduced population in Hawai'i, is described in detail. In 26 colonies from two populations in South Africa and one population from Puerto Rico, males were observed spacing out in their nests and killing freshly eclosing rivals in the surrounding brood pile, but usually only engaging briefly in fights

with other adult males. This corroborated the observation from the Hawai'ian colony. Male behavior was highly variable both within and between colonies, with territorial (stationary) males defending small spaces in the nest and other males floating freely through the colony. While reproductive success could not be estimated directly, the study showed that territorial males were in contact with alate female sexuals more frequently on average. We hypothesize that the defense of small territories inside the nest is an adaptation to the seasonal production of numerous sexuals and the spread of colonies over several small subterranean chambers prohibiting males from defending a "nest-wide harem" similarly to males in other tropical *Cardiocondyla* species.

Chapter 4 provides insights into the population structure of *C. venustula.* Field observations, laboratory experiments and the genetic analysis of an intensively sampled population in Rietvlei, South Africa, suggest that *C. venustula* colonies are facultatively polygynous, containing one or several single-mated queens. Similar to other tropical *Cardiocondyla* species, colonies appear to be primarily founded by budding, but the existence of multiple mtDNA haplotypes in some of the colonies evidence that dispersing queens may occasionally be adopted into foreign nests or colonies may be founded co-operatively by more than one queen. Overall, levels of inbreeding and nestmate relatedness were extraordinarily high, indicating that most intranidal matings involve siblings. Furthermore, the peculiar behavior of *C. venustula* males, which defend small areas in the nest and thus are able to monopolize mating with several female sexuals, is considered to add to the high inbreeding levels. The weak isolation by distance between populations despite the apparently limited dispersal ability of queens and especially budding colonies indicate that long distance dispersal may be facilitated by human activity. Small colony fragments transported with soil, for example in potted plants, are thus able to colonize new habitats. This ability has also led to the spread of *C. venustula* to formerly uninhabited regions of the tropics and subtropics as a tramp ant.

5.6 Zusammenfassung

Die Ameisengattung *Cardiocondyla* umfasst vermutlich mehr als 100 Arten mit einer großen Spannbreite an Lebenszyklusstrategien. Die auffälligste Eigenschaft der Gattung ist das Vorkommen flügelloser, sogenannter ergatoider, also arbeiterinnenähnlicher Männchen, die alleine oder in Kombination mit den "gewöhnlichen" geflügelten Ameisenmännchen auftreten. In einigen der Arten führen die Männchen tödliche Kämpfe, um sich alleinigen Zugang zu paarungsbereiten Jungköniginnen zu verschaffen. Die Morphologie der Männchen sowie ihre Paarungsstrategien unterscheiden sich deutlich zwischen den verschiedenen Arten, ebenso variieren etwa die Zahl der Königinnen pro Kolonie oder die Koloniegröße. Anders als in den meisten bekannten Ameisengattungen verpaaren sich Ameisen der Gattung *Cardiocondyla* überwiegend innerhalb des Nests. Viele der Arten lassen sich leicht im Labor vermehren, so dass verschiedene Aspekte des Paarungsverhaltens im Detail beobachtet werden können. Die Gattung ist daher ein faszinierendes Forschungsobjekt für die Untersuchung von Lebenszyklusstrategien, insbesondere der Evolution der Morphologie von Männchen sowie ihres Konkurrenzverhaltens.

In **Kapitel 2** wird ein Überblick über den aktuellen wissenschaftlichen Kenntnisstand zu Konkurrenzverhalten zwischen Ameisenmännchen gegeben. Das Ausmaß und die Ausprägung von Konkurrenz zwischen Ameisenmännchen variiert in Abhängigkeit von verschiedenen Faktoren wie der Verpaarungsstrategie bzw. dem Ort der Verpaarung (Ansammlungen von Männchen in großen Schwärmen, bodengebundene Paarung oder sogar Verpaarung innerhalb des Nests), dem Geschlechterverhältnis oder Einfach- oder Mehrfachverpaarung der Königinnen. Konkurrenzverhalten vor der Verpaarung ist bereits seit über einem Jahrhundert Gegenstand der Forschung, während Männchenkonkurrenz nach der Verpaarung (etwa durch Spermienkonkurrenz oder Behinderung der Mehrfachverpaarung von Weibchen) erst in den letzten Jahrzehnten in den Fokus der Wissenschaft gerückt ist. Die einfachste Form des Konkurrenzverhaltens bei Ameisenmännchen wird bei Arten angetroffen, die zur Paarung große Schwärme bilden: hier kommt es im Gerangel einer Vielzahl Männchen um die begrenzte Anzahl paarungsbereiter Jungköniginnen nur auf allgemeine Robustheit an, ohne dass direkte Konkurrenzkämpfe entstehen. Bei Arten, bei denen paarungsbereite Jungköniginnen Männchen anlocken und die Paarung bodennah erfolgt, können Ameisenmännchen sich gegenseitig bei der Paarung behindern. Die offensichtlichste Form des Konkurrenzverhaltens wurde bei Männchen der Gattungen *Hypoponera* und *Cardicondyla* beobachtet, die ihre Rivalen in Kämpfen zu töten versuchen.

Innerhalb der Gattung *Cardicondyla* wurde für die Männchen der Art *C. venustula*, die Gegenstand dieser Arbeit ist, ein Verhalten beschrieben, das für soziale Hautflügler bisher unbekannt war: die Männchen verteilen sich innerhalb des Nests und verteidigen kleine Territorien gegen Konkurrenten. Dieses Verhalten könnte einen Übergang zwischen den Männchen der tropischen *Cardicondyla*-Arten und der palearktischen Klade der Gattung darstellen: während die ersteren tödliche Kämpfe ausfechten, koexistieren die letzteren friedlich innerhalb des Nests.

Kapitel 3 behandelt dieses außergewöhnliche Verhalten, das zuerst an einer Kolonie aus Hawaii beschrieben wurde, detailliert. In 26 experimentellen Kolonien aus zwei Populationen in Südafrika und einer Population in Puerto Rico wurde beobachtet, dass sich die Männchen auf die einzelnen Kammern des Nests verteilen und dort zwar frisch geschlüpfte Männchen töten, mit anderen adulten Männchen aber überwiegend nur kurze aggressive Interaktionen zeigen. Das Verhalten der Männchen variierte stark, sowohl innerhalb einer Kolonie als auch zwischen verschiedenen Kolonien. Hierbei war zwischen territorialen Männchen, die über längere Zeit einen Teil des Nests gegen Rivalen verteidigten, und sich frei in den Nestern bewegenden Männchen zu unterscheiden. Der Reproduktionserfolg der Männchen konnte nicht exakt ermittelt werden, allerdings wurde gezeigt, dass territoriale Männchen häufiger Kontakt zu Jungköniginnen und somit vermutlich höhere Paarungschancen hatten. Es ist anzunehmen, dass die Verteidigung kleiner Bereiche des Nests eine Anpassung an die saisonale Produktion großer Zahlen an Jungköniginnen und Männchen sowie die Neststruktur mit vielen kleinen Kammern ist. Diese Faktoren verhindern, dass die Männchen der Art wie die Männchen anderer tropischer *Cardiocondyla*-Arten das ganze Nest gegen Rivalen verteidigen können.

Kapitel 4 gibt einen Einblick in die Populationsgenetik der Art *C. venustula*. Aus Freilandbeobachtungen, Laborstudien und genetischen Analysen an einer detailliert erfassten Population in Rietvlei, Südafrika lässt sich schließen, dass die Kolonien der Art fakultativ polygyn sind, also eine oder mehrere Königinnen enthalten, die sich jedoch jeweils nur mit einem Männchen verpaaren. Ähnlich wie andere tropische *Cardiocondyla*-Arten scheint sich *C. venustula* vor allem durch Teilung bestehender Kolonien zu vermehren. Allerdings wurden in einigen Kolonien mehrere unterschiedliche mtDNA-Haplotypen gefunden, was darauf hinweist, dass vereinzelt junge Königinnen in fremde Kolonien aufgenommen werden oder mehrere Königinnen gemeinsam Kolonien gründen. Insgesamt wurde eine starke Inzucht und ein hoher Verwandtschaftsgrad der Individuen einer Kolonie ermittelt. Dies deutet darauf hin, dass Paarungen vorwiegend unter Geschwistern erfolgen. Darüber hinaus kann auch das außergewöhnliche Verhalten der Männchen, die kleine Bereiche des Nests gegen Rivalen verteidigen und sich so mit einer größeren Anzahl an Jungköniginnen verpaaren, zu den hohen Inzuchtkoeffizienten beitragen. Die Korrelation der genetischen Divergenz mit der geographischen Entfernung von Populationen war trotz der nur geringen Ausbreitungsfähigkeiten von Königinnen und insbesondere auch von Arbeiterinnen bei der Verbreitung durch Teilung von Kolonien gering. Eine Erklärung hierfür ist eine Ausbreitung durch den Menschen. Kleine Koloniefragmente, die mit Erde, beispielsweise mit Topfpflanzen, transportiert werden, können neue, weit entfernte Habitate besiedeln. Diese Fähigkeit hat auch dazu geführt, dass *C. venustula* sich in einigen zuvor nicht durch die Art besiedelten Teilen der Tropen und Subtropen ausbreiten konnte.

Supplementary chapter I: Colony founding in *Cardiocondyla venustula*

Introduction

Among ant species, the mode of colony founding varies greatly. In most monogynous species, queens disperse to found colonies independently (independent colony founding, ICF). Various polygynous species re-adopt daughter queens and found new colonies by budding or colony fission, thus dependent on the mother colony (dependent colony founding, DCF) (Hölldobler and Wilson, 1977). In ICF, colonies can be started by single queens (haplometrosis) or groups of (usually unrelated) queens (pleometrosis). During the founding phase, these queens either completely rely on body reserves (claustral founding) or occasionally forage (semi-claustral founding) (Peeters and Ito, 2001). In most species with pleometrotic colony founding, queen number is reduced to one after the first workers emerge, either by queen-queen or by worker-queen aggression (Heinze, 1993). Few species have been reported to date to found polygynous colonies through pleometrosis (primary polygyny) (e.g., Eriksson et al., 2019). Pleometrosis enhances chances for successful colony founding and thus outweighs the reduction or even complete loss of individual reproductive success. In other species, pleometrosis may also contribute to mitigation of inbreeding effects (e.g., described for *Solenopsis geminata* by Lenancker et al., 2019).

Dependent colony founding by budding is presumably a common strategy in *C. venustula*, as it is in other tropical polygynous *Cardiocondyla* species (e.g., Heinze and Delabie, 2005). One or several mated queens and a few workers with some brood disperse from their natal colony as a small propagule to found new colonies nearby. However, lab and field observations suggest that reproduction in *C. venustula* is more seasonal than in other tropical *Cardiocondyla* species and colonies are usually small, containing 10 to 20, occasionally up to 100 workers. Thus, before budding, these colonies would either need to undergo a cycle of worker production by old and young queens to provide buds with sufficient workers (described for *C. obscurior*, e.g., in Schrempf and Heinze, 2008; Heinze and Delabie, 2005), produce buds with very few workers or exhibit ICF as an alternative strategy. While colonies in species of the palearctic clade are founded semi-claustrally by single queens (Schrempf and Heinze, 2007), single queens of other *Cardiocondyla* species including *C. venustula* usually fail at starting a colony in the lab (own observations, Schrempf and Heinze, 2008). However, mated queens appear to disperse in several species (Heinze, 2017) and groups of multiple queens were shown to successfully rear worker offspring in *C. obscurior* in the lab (Schrempf and Heinze, 2008; Heinze and Koller, 2020), suggesting the existence of alternative founding modes. Furthermore, genetic analysis in *C. venustula* revealed that colonies occasionally consist of individuals with more than one mtDNA haplotype (Heinze et al., 2013). This suggests that either foreign young queens are adopted into established nests or multiple queens from more than one colony co-found a new colony (pleometrosis).

In *C. venustula* with mating inside the nest and usually very limited dispersal, co-founding of related individuals may occur by joint dispersal or by incidental meeting of several related or unrelated queens in free cavities surrounding the natal nest. This study investigates whether *C. venustula* queens are able to found colonies co-operatively and compares founding success between independently founding queens and queens in small buds.

Material and methods

The ability of successful colony founding was tested in a pilot study by setting up experimental nests with different combinations of nestmate young queens ($n = 150$) and nestmate workers from larger stock colonies. Both independent, pleometrotic founding and dependent founding with different bud sizes was analyzed. For each setup, five experimental nests were established: groups of four or eight young queens, two young queens assisted by four workers, four young queens assisted by four or eight workers, eight young queens assisted by sixteen workers. Young queens had access to males in their stock colony and were only selected for the experiment after they had

Table SI.1: Colony setup for founding experiments

Setup	**number of**	
	queens	workers
4	4	-
8	8	-
2+4	2	4
4+4	4	4
4+8	4	8
8+16	8	16

shed their wings, indicating that they were already mated. Colonies were housed in plaster nests and supplied with honey, cockroach pieces and water. The experimental colonies were checked every two to three days and the number of queens, workers, eggs, larvae and pupae was counted. Additionally, the location of queen, brood and workers in the nest was recorded. During the inspection, conspicuous behaviors, such as aggression between workers and queens or among queens, or foraging activity of queens, were noted. Dead individuals were removed upon detection. Colonies were considered as successful in founding when the first young workers emerged.

Statistical analysis was performed using R (R Core Team, 2008). Survival curves were drawn with the package 'survminer' (Kassambara et al., 2020). To test for influence of initial queen number and presence or absence of workers, a cox regression model was used.

Results

Of the 30 experimental colonies, eleven successfully reared worker offspring. According to expectations, the colonies that contained higher numbers of workers were more successful on average. While none of the five colonies consisting of four queens alone was successful, two of five groups of eight queens successfully raised workers (Tab. SI.2). Occasional aggression between queens was observed in four of the 30 colonies (two with workers, two without workers). Queens spread their mandibles and attempted to bite, in one colony queens struggled with each other for a number of eggs. These fights apparently did not inflict injuries in most cases. Two queens were found injured, however, both originated from colonies with workers, thus it

Table SI.2: Results of founding experiments

Setup	**number of colonies**			**mean latency (days) to first appearance of**		
	producing worker	exhibiting queen-queen aggression	producing male	egg	worker	male
4	0	1	0	8	–	–
8	2	1	1	2	40	41
2+4	1	1	0	2	36	–
4+4	2	0	1	3	34	39
4+8	3	0	1	1	34	34
8+16	3	1	2	1	39	31

cannot be concluded whether workers or other queens were involved. Albeit queens were occasionally observed foraging, eggs were obviously used for feeding in almost all of the colonies. In both groups with and without workers, eggs disappeared without evolving into larvae. Males were produced in five colonies, both with and without workers and both with four and with eight queens. Except for one colony, in which the first male was produced nearly two weeks before the first worker and one colony, which only produced males, males eclosed at the same time as ($n = 1$) or two days after ($n = 2$) the first new worker.

Queen and worker mortality was high in all of the colonies. Three of the successful colonies went through a short workerless phase (maximum time span 5 days) before the first new workers emerged. Queen survival was higher in colonies with workers (Fig. SI.1, $p = 0.0002$), while the initial number of queens did not influence survival time significantly (Fig. SI.2). In experimental colonies with four queens, eggs were only laid after four to fifteen days, whereas eggs were found on day one in most of the colonies of the other setups. The latency to the emergence of the first worker varied within groups, but was similar in the successful colonies with ($n = 9$) and without ($n = 2$) workers.

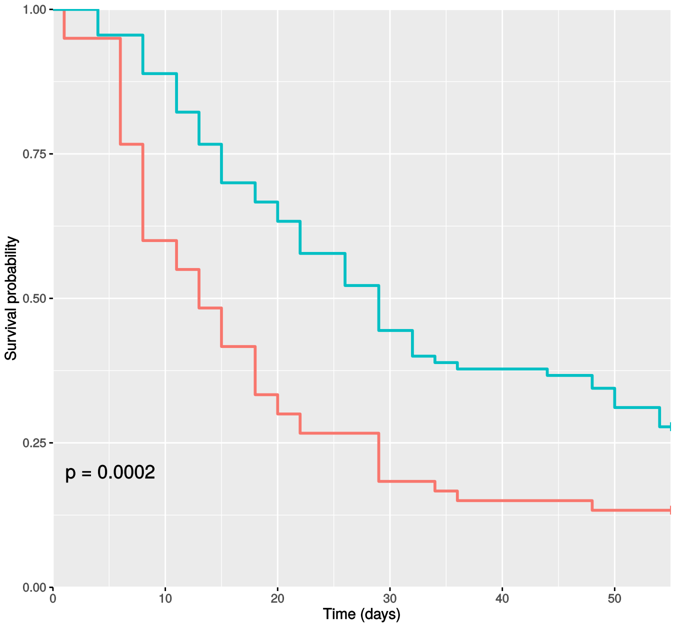


Figure SI.1: Queen survival in experimental colonies with (blue) and without (red) workers

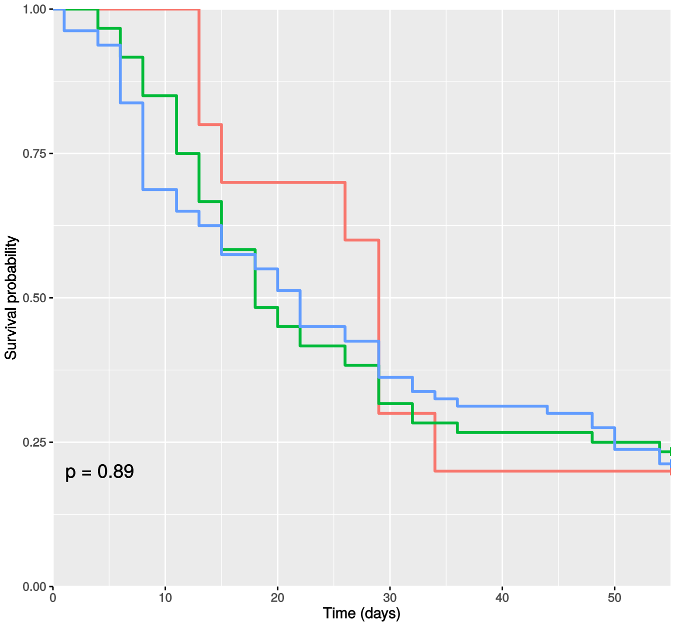


Figure SI.2: Queen survival in experimental colonies with different initial numbers of queens: two queens (red), four queens (green) and eight queens (blue)

Discussion

In *C. venustula*, groups of several queens were able to successfully found new colonies. The young queens foraged and engaged in brood care. Colony founding by semi-claustral pleometrosis may thus be an alternative strategy to the prevailing founding by budding. While it cannot be concluded whether colony founding by multiple queens does occur under natural conditions, the observation of dealate queens dispersing by foot in the field in combination with the experimental results suggest possible alternative founding strategies in *C. venustula.* Mixed strategies are known for several ant species (e.g., Espadaler and Rey, 2001; Cronin et al., 2013; Eriksson et al., 2019) and may also exist in other *Cardiocondyla* species (e.g., Heinze and Delabie, 2005; Heinze and Koller, 2020). The mode of colony founding may vary with environmental factors. However, as the young queens in this experiment stemmed from the same colony, it remains to be investigated whether multiple non-nestmate queens are able to found new colonies.

Surprisingly, queen-queen aggression was observed in four of the experimental colonies. In (functionally) monogynous species with pleometrotic colony founding, aggression is common and usually starts with the emergence of the first workers. Queen-queen aggression then leads to the reduction of queen numbers to one, to a dominance hierarchy with only the dominant queen reproducing or at least to a high reproductive skew (e.g., Heinze, 1993; Bernasconi and Keller, 1996). On the contrary, queen-queen aggression is scarce in polygynous species (e.g., Johnson, 2004) in general and has not been observed in polygynous *Cardiocondyla* species to date. In *Cardiocondyla*, aggression between queens has been observed in (functionally) monogynous species from the *"C. argentea"* group. Queens were observed to establish dominance hierarchies by antennal boxing and biting (Yamauchi et al., 2007) or fight to death over colony inheritance (Heinze and Weber, 2011), while in *C. "argyrotricha"*, the first emerging queen monopolizes reproduction without overt violence (Schmidt et al., 2017). In *C. obscurior*, queens apparently show division of labor between co-founding queens, which was however not determined by overt aggression (Heinze and Koller, 2020). Queen-queen aggression over reproduction is expected to arise after the emergence of the first workers or - in this case - in colonies containing workers. In contrast, aggression over the division of labor is expected to prevail in workerless groups of queens which require foraging by queens. Overall, aggressive behavior was scarce in the experimental colonies of *C. venustula*. However, of the four colonies with observed queen-queen aggression, two were workerless and two contained workers. Thus, the function of queen-queen aggression in *C. venustula* remains unclear. With queen-queen aggression arising predominantly in failing experimental colonies (three of four colonies), it may reflect undertaking behavior aimed at removing infected or unfit queens, similar to corpse removal described by Pull and Cremer (2017) or nonadaptive behavior due to infection of the aggressing queens.

Usually, queens of social insects only start producing sexuals after the emergence of a sufficient number of workers. On the contrary, queens in polygynous *Cardiocondyla* colonies with fighter males have been found to start male production early as an expression of queen-queen competition (Yamauchi et al., 2006; Suefuji et al., 2008). In species such as *C. obscurior* with males engaging in lethal fighting, the first eclosing male be at an advantage in fighting with younger brothers (Cremer et al., 2012), thus monopolizing matings with the first female sexuals. While males in larger colonies of *C. venustula* defend small territories rather than killing all rivals, they are able to find and kill emerging males in small colonies (Chapter 3, own observations). Thus, the observation of males produced prior to or shortly after the eclosion of the first new worker even in experimental nests without workers in *C. venustula* is in accordance with studies in other *Cardiocondyla* species. As expected, colonies with larger initial size were more successful in rearing workers, but some of the smaller colonies were also able to produce new workers. Under natural conditions, buds may in addition contain brood (Heinze and Delabie, 2005) and thus probably be even more successful despite small propagule sizes. As a conclusion, the results suggest that pleometrosis represents an alternative mode of colony founding in *C. venustula*. While budding is likely to be the prevailing mode of colony founding and new colonies can emerge from buds containing only a few individuals, the experiments revealed that groups of young queens are able to co-operatively found new colonies.

Supplementary chapter II: Phylogeny of *Cardiocondyla venustula*: Insights from mitochondrial and microsatellite markers

Introduction

The myrmicine genus *Cardiocondyla* comprises an estimated number of over 100 species, several of which have spread throughout the ant-inhabited continents as tramp ants (Heinze et al., 2006; Heinze, 2017). While the internal phylogeny has been resolved by analysis of mtDNA and nuclear DNA sequences in concordance with morphologically defined species or species groups, both the wider picture - the position of the genus within the ant subfamily Myrmicinae (Ward et al., 2015) - and the details - the separation of species within species groups (e.g., Heinze, 2020) - remain difficult. The separation of *Cardiocondyla* species of several species groups has been under discussion in the course of the last decades. Several species are hardly distinguishable by standard morphological approaches, requiring sophisticated morphometric analysis. Thus, these species are easily confused in the field (e.g., Heinze, 2017). Results of mtDNA sequence analysis have been discovered to contradict findings from morphological taxonomy in some of the species groups (e.g., Okita et al., 2015; Heinze, 2020). Species of the *C. shuckardi* group, including *C. venustula*, strongly resemble each other morphologically. Seifert (2003) revised the *C. shuckardi* group and synonymized several taxa with *C. venustula* and *C. shuckardi*. However, results from mtDNA were inconclusive with regard to species delimitation and variation of mtDNA sequences was high even within samples from one population (e.g., Heinze et al., 2013; Heinze, 2020). This study compares results from the analysis of mitochondrial and microsatellite markers in both *C. venustula* from its native and introduced range and *C. shuckardi* from Madagascar.

Material and methods

Samples used for DNA analysis were collected from Puerto Rico (2012), Madagascar (2012) and two sample sites in South Africa (2011 and 2013). Colonies were either transferred into a tube with 100% ethanol p.a. upon sampling or colonies were taken to the laboratory alive and some individuals were collected from each colony and transferred to tubes with 100% ethanol. DNA extraction and amplification of microsatellites markers and mtDNA sequences were conducted as described in Chapter 3. Due to amplification problems, we used additional primers described in Heinze et al. (2005) to obtain mtDNA sequences of sufficient length for some of the samples. A phylogenetic tree was calculated using the software "MrBayes" (Ronquist et al., 2012). The optimal evolutionary model was selected after analyzing the data in jModeltest 2.1 (Darriba et al., 2012). FigTree v1.4.4 (Rambaut, 2018) was used to visualize the trees obtained by MrBayes. Based on the results of jModeltest, a HKY + G model was selected for analysis in MrBayes. The analysis was performed with 3,000,000 generations and sampling after every 1,000 generations. The default of 25% of the samples were discarded as burn-in. Microsatellite genotypes were analyzed with the software STRUCTURE (Pritchard et al., 2000). The optimal number of clusters was determined by analyzing the data with STRUCTURE harvester (Earl and von Holdt, 2012). The results from multiple STRUCTURE runs were aggregated with the software CLUMPP (Jakobsson and Rosenberg, 2007). Additionally, the microsatellite data was analyzed with a discriminant analysis of principal components (DAPC) as implemented in the package "adegenet" (Jombart, 2008) in R (R Core Team, 2008).

Results

We obtained microsatellite genotypes for individuals from 208 colonies, 194 from South Africa, 11 from Puerto Rico and 3 of *C. shuckardi* from Madagascar. A

1,038 bp COI/COII sequence was available for 98 samples: 74 from South Africa, 17 from Puerto Rico and 7 for *C. shuckardi* from Madagascar. The mtDNA tree suggested paraphyly for the putative *C. venustula* samples (Fig. SII.1). Of the two well supported branches, one contains most *C. venustula* samples from Rietvlei and Hlalanati, the other contains a number of the samples of *C. venustula* from South Africa and other African countries, the samples from introduced populations in Puerto Rico and Hawai'i together with *C. shuckardi* populations from Madagascar. A small group of *C. venustula* samples from South Africa formed a branch separate from all other samples, however, this branch was relatively unstable. The STRUCTURE analysis suggested K=2 as the optimum (Fig. SII.2a). However, as STRUCTURE analysis is known to potentially underestimate number of clusters when proposing $K = 2$ (Janes et al., 2017) and LnProb was relatively low for $K = 2$ (Fig. SII.2b), the relative optimum of $K = 4$ was included in further analysis as well. The BIC-values obtained from the "find.cluster" did not reveal a distinct optimum. With delta K as a criterion, $K = 2$ was proposed as the optimal number of clusters. To compare results from STRUCTURE and DAPC, analysis was run with K set to 2 and 4, respectively. Assignment to clusters was identical in STRUCTURE and DAPC except for six individuals. For $K = 2$, the highly homogeneous population from Rietvlei, including some of the nearly identical samples from Hlalanati, formed one cluster, while all of the remaining individuals were assigned to a second cluster. Setting $K = 4$ resulted in three clusters containing most of the South African samples and one cluster containing the individuals from Puerto Rico, a few individuals from Hlalanathi and the *C. shuckardi* samples (Figure SII.3). The results of the microsatellite analysis largely concur with the lineages revealed by the mtDNA tree. All of the *C. venustula* samples that were assigned to the mtDNA lineage containing samples from the introduced populations in Puerto Rico and Hawai'i as well as *C. shuckardi* from Madagascar, but none of the samples assigned to the other lineages, were found in one STRUCTURE cluster. Albeit the distant microsatellite cluster contained the genetically distant mtDNA lineages of individuals in Hlalanathi, it also included samples from the main South African clade. The high differentiation between the population from Rietvlei and one of the populations in Hlalanathi, that was found by microsatellite analysis, was thus not visible in mtDNA samples.

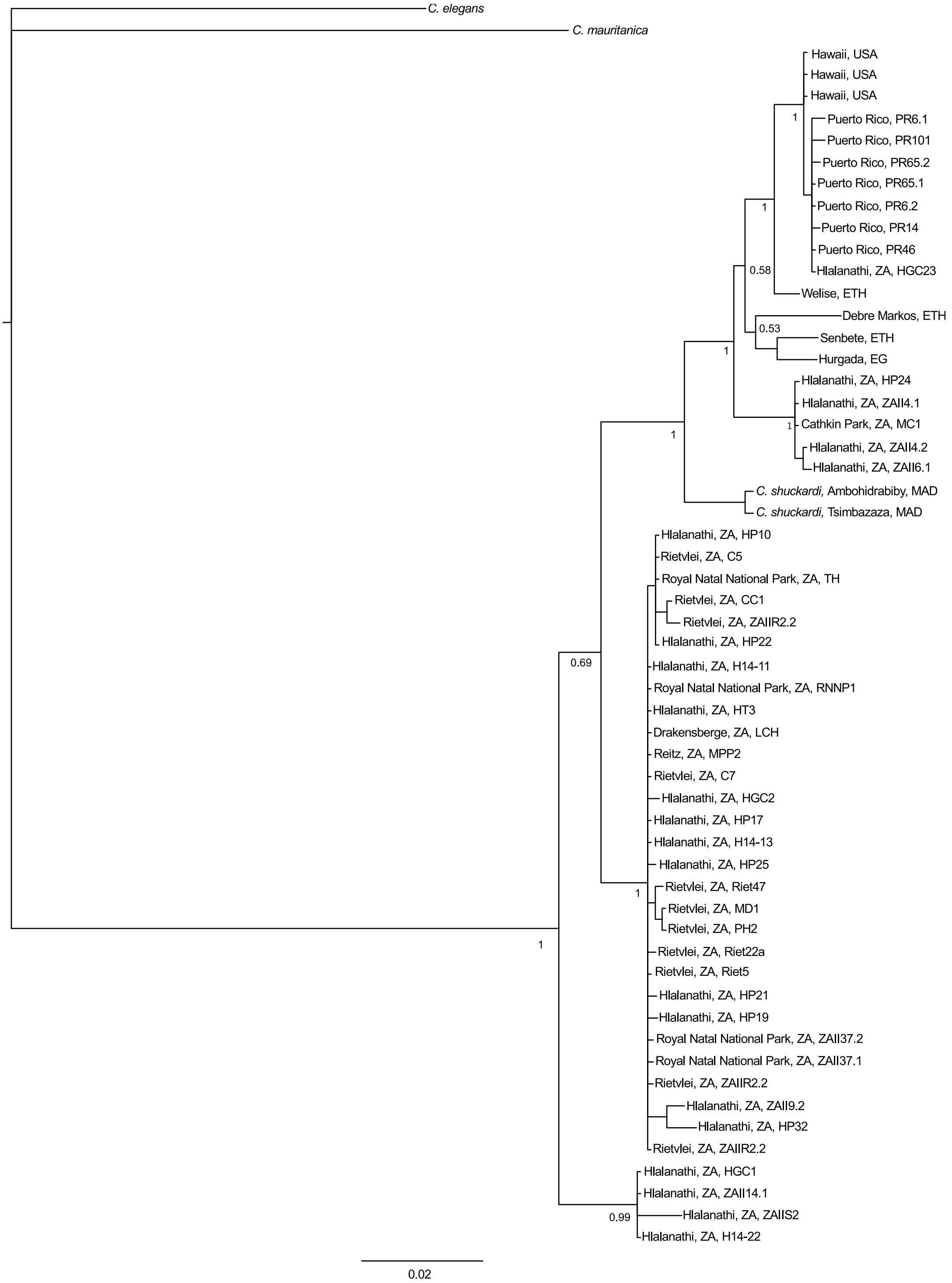
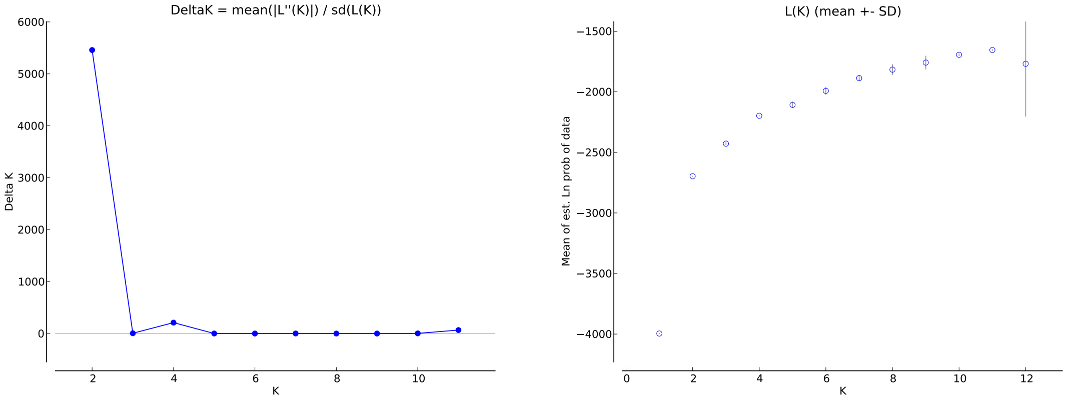


Figure SII.1: Phylogenetic tree of *Cardiocondyla venustula*. Bayesian posterior probabilities are indicated at major nodes.



(a) Delta K **(b)** L(K)

Figure SII.2: Estimation of K in STRUCTURE: Delta K – estimator of the number of optimal clusters (K) and L(K) – average likelihood of runs with different number of clusters, as calculated by STRUCTURE harvester

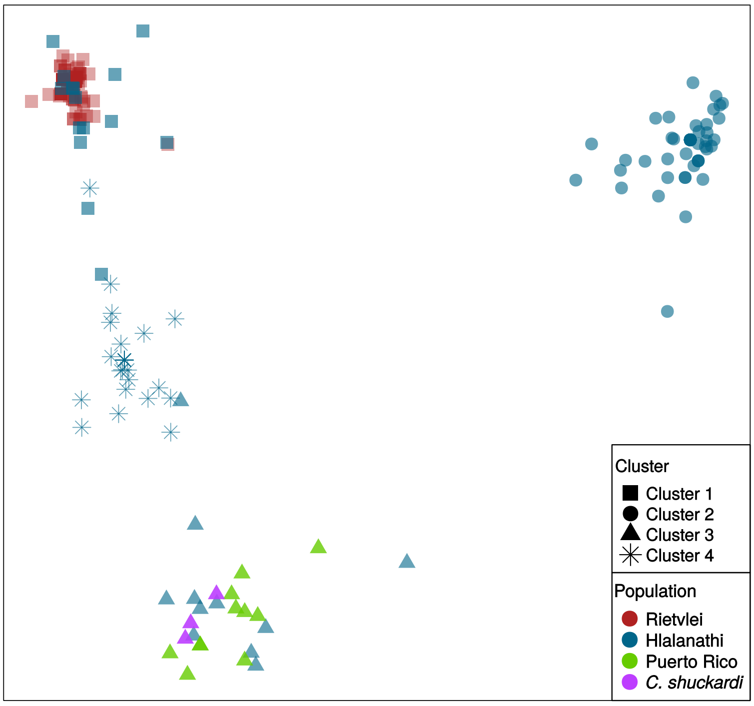


Figure SII.3: DAPC scatterplot of *Cardiocondyla venustula* populations for $K = 4$. Different symbols represent the clusters assigned by DAPC, different colors represent the populations of origin

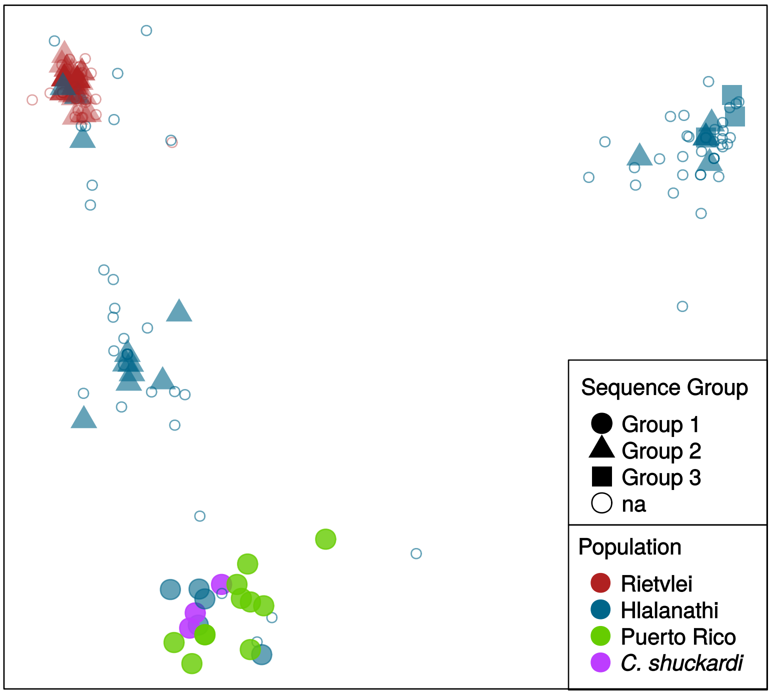


Figure SII.4: Comparison of mtDNA and microsatellite data: DAPC scatterplot of *Cardiocondyla venustula* populations for $K = 4$. Different symbols represent the three main phylogenetic branches of the mtDNA tree, different colors represent the populations of origin

Discussion

The comparative analysis of the mtDNA tree and clusters inferred from microsatellite data revealed the existence of distinct genetic lineages within *C. venustula*. Albeit microsatellite and sequence data showed differing patterns for some of the South African populations, both marker types assigned a number of samples from the population in Hlalanathi, South Africa to a group consisting of *C. venustula* from Puerto Rico, Hawai'i and northern Africa, but also *C. shuckardi* samples from Madagascar. These results contradict morphological data, by which the two species can be distinguished (Seifert, 2003), but are in accordance with the results of Heinze (2020) that include several additional samples from West Africa. MtDNA sequences, in particular the COI/COII genes are a regularly used and valuable tool in phylogeny. However, phylogenetic trees based on mtDNA sequences have been found to deviate from morphological and nuclear phylogenies (e.g., Rubinoff and Holland, 2005; Seifert, 2009). Due to incomplete lineage sorting or hybridization between species, mtDNA trees can, for example, indicate paraphyly where nuclear DNA or morphological parameters suggest monophyletic taxa . Indeed, male and female sexuals from the main South African cluster/lineage were successfully crossbred with *C. venustula* from Puerto Rico and *C. shuckardi* from Madagascar in the lab and produced viable worker and sexual offspring (unpublished data). This suggests that hybridization between different lineages or species may be possible, exacerbating the construction of phylogenetic trees. Furthermore, the bacterial symbiont *Wolbachia* found widespread across arthropod taxa may lead to significant mtDNA diversification (e.g., Shoemaker et al., 2003; Hurst and Jiggins, 2005; Xiao et al., 2012). These effects can thus not be ruled out in the case of *C. venustula* and may account for the highly differentiated lineage in the Hlalanathi population. Unfortunately, the lack of sufficiently variable nuclear markers both in coding and non-coding DNA sequences did not allow for the creation of a nuclear phylogeny of the *C. shuckardi* species group. Standard nuclear markers, including a set of markers that were described to be highly variable across apocritan Hymenoptera (Hartig et al., 2012), revealed only low variability. Thus, the more variable microsatellites were used as nuclear markers. While there are caveats about using microsatellites to infer phylogenetic relationships, they can provide an additional source of information for the analysis of closely related species (Zhu et al., 2000; Takezaki and Nei, 2008; Knaden et al., 2005; Ueda et al., 2015). However, microsatellite and mitochondrial markers revealed congruent results for the *C. venustula* samples from South Africa and Puerto Rico assigned to the group containing *C. shuckardi*, thus indicating that the *C. shuckardi* species group may need another thorough revision. Unfortunately, morphological differences between closely related *Cardiocondyla* species are often minute and species delimitation requires sophisticated morphometric analysis and computations (Heinze, 2020). It therefore remains exciting, whether the combination of genetic and morphological data will finally solve the challenging taxonomy of the *C. shuckardi* species group.

Supplementary Chapter III: Raw Data – Chapter 4

Table SIII.1: Microsatellite Genotypes Rietvlei

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**		**Haplotype**
Riet1	84	84	107	107	194	194	132	132	121	127	131	131	80	82	
Riet2	84	84	107	107	194	194	132	132	121	121	131	131	82	82	1
Riet3	84	84	107	107	194	194	132	132	121	121	125	125	82	82	2
Riet4	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
Riet5	84	84	107	107	194	194	132	132	121	121	131	135	80	84	
Riet6	84	84	107	107	194	194	134	134	121	121	135	135	80	80	6
Riet7	84	84	107	107	194	194	134	134	121	121	135	135	80	80	
Riet9	84	84	107	107	194	194	132	134	121	121	131	135	80	82	
Riet10	84	84	107	107	194	194	134	134	121	121	135	135	80	80	
Riet11	84	84	107	107	194	194	132	132	121	121	131	131	82	82	1
Riet12	84	84	107	107	194	194	132	132	127	127	123	125	80	82	
Riet13	84	84	107	107	194	194	132	132	121	121	131	131	80	82	
Riet15	84	84	107	107	194	194	132	132	127	127	123	125	80	80	
Riet16	84	84	107	107	194	194	132	132	127	127	123	135	80	80	
Riet18	84	84	107	107	194	194	132	132	121	121	131	131	80	80	1
Riet19	84	84	107	107	194	194	132	132	127	127	123	125	80	80	
Riet21	84	84	107	107	194	194	0	0	127	127	121	125	80	80	
Riet22a	84	84	107	107	194	194	132	132	121	121	131	133	82	82	4
Riet24	84	84	107	107	194	194	132	132	121	121	131	131	80	80	1
Riet26	84	84	107	107	194	194	132	132	121	127	131	133	80	82	
Riet27	84	84	107	107	194	194	132	132	121	121	131	133	80	80	
Riet30a	84	84	107	107	194	194	132	132	121	121	131	133	80	82	4

Table SIII.1: Microsatellite Genotypes Rietvlei (continued)

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**		**Haplotype**
Riet32	84	84	107	107	194	194	132	132	121	121	131	133	80	82	
Riet33	84	84	107	107	194	194	132	132	121	121	131	131	80	84	3
Riet34	84	84	107	107	194	194	132	132	121	127	131	131	80	80	
Riet35	84	84	107	107	194	194	132	134	127	127	123	125	80	80	
Riet37	84	84	107	107	194	194	132	132	121	121	131	131	80	80	1
Riet38	84	84	107	107	194	194	132	132	127	127	131	131	80	80	
Riet39	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
Riet41	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
Riet45	84	84	107	107	194	194	132	132	121	121	129	129	80	80	
Riet46	84	84	107	107	194	194	132	134	121	121	129	129	80	82	
Riet47	84	84	107	107	194	194	132	132	121	121	129	129	80	80	3
Riet51	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
Riet53	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
Riet60	84	84	107	107	194	194	132	132	121	127	131	131	80	80	
Riet61	84	84	107	107	194	194	132	132	121	127	129	131	80	80	
Riet62	84	84	107	107	194	194	132	132	0	0	131	131	80	82	
Riet63	84	84	107	107	194	194	134	134	121	121	129	129	80	80	
Riet64	84	84	107	107	194	194	132	132	121	127	131	131	80	80	
Riet65	84	84	107	107	194	194	132	132	121	127	131	131	80	82	
Riet66	84	84	107	107	194	194	132	132	121	121	133	133	82	82	
Riet67	84	84	107	107	194	194	132	132	121	121	131	131	80	80	1
Riet68	84	84	107	107	194	194	132	132	121	121	131	131	80	80	1

Table SIII.1: Microsatellite Genotypes Rietvlei (continued)

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**		**Haplotype**
Riet69	84	84	107	107	194	194	132	132	121	121	131	131	80	80	1
Riet70	84	84	107	107	194	194	132	132	121	121	131	133	80	82	1
Riet71	84	84	107	107	194	194	134	134	121	121	129	129	80	82	3
Riet73	84	84	107	107	194	194	132	134	121	121	129	131	80	82	
Riet73a	84	84	107	107	194	194	132	134	121	121	129	131	80	82	
Riet74	84	84	107	107	194	194	132	134	121	121	129	131	80	80	3
Riet75	84	84	107	107	194	194	132	132	121	121	139	139	80	80	5
Riet76	84	84	107	107	194	194	132	132	121	121	131	131	80	82	
Riet76a	84	84	107	107	194	194	132	132	121	121	139	139	80	80	
Riet77	84	84	107	107	194	194	132	132	127	127	129	139	80	80	
Riet79	84	84	107	107	194	194	132	132	121	127	129	129	80	80	1
Riet81	84	84	107	107	194	194	132	132	121	121	131	131	82	82	1
Riet82	84	84	107	107	194	194	132	132	121	121	133	135	80	84	
PH1	84	84	107	107	194	194	132	132	127	127	131	131	80	80	1
PH2	84	84	107	107	194	194	134	134	121	121	131	131	84	84	
MD1	84	84	107	107	194	194	132	132	121	121	139	139	80	80	
MD2	84	84	107	107	194	194	132	132	121	121	133	133	80	82	5
MD3	84	84	107	107	194	194	132	132	121	121	139	139	80	80	
MD4	84	84	107	107	194	194	0	0	121	121	139	139	80	80	
MD5	84	84	107	107	194	194	132	132	121	121	139	139	80	80	5
MD6	84	84	107	107	194	194	132	132	121	121	139	139	80	80	5

Table SIII.1: Microsatellite Genotypes Rietvlei (continued)

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**		**Haplotype**
MD8	84	90	107	107	194	194	132	132	121	127	123	133	80	80	
MD9	84	84	107	107	194	194	132	132	121	121	139	139	80	80	
C1	82	84	107	107	194	194	132	132	121	121	131	133	76	80	
C2	84	92	107	107	194	194	132	132	121	127	133	133	80	80	
C3	84	92	107	107	194	194	132	132	121	127	123	123	80	80	
C4	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
C5	84	84	107	107	194	194	132	134	121	121	131	131	82	82	1
C6	84	84	107	107	194	194	132	132	121	121	131	131	80	80	2
C7	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
C8	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
C9	84	84	107	107	194	194	132	132	121	121	131	131	80	80	
C10	84	84	107	107	194	194	132	132	127	127	0	0	82	82	
C11	84	84	107	107	194	194	132	132	121	121	127	127	82	82	
C12	84	84	107	107	194	194	134	134	121	121	123	129	80	82	
C13	84	84	107	107	194	194	132	132	121	127	127	127	82	82	
IV1	84	84	107	107	194	194	134	134	121	121	131	131	82	82	1
IV3	84	84	107	107	194	194	134	134	121	121	131	131	82	82	1
IV4	84	84	107	107	194	194	134	134	121	121	131	131	82	82	1
IV5	84	84	107	107	194	194	134	134	121	121	131	131	82	82	1
IV6	84	84	107	107	194	194	134	134	121	121	131	131	82	82	
IV7	84	84	107	107	194	194	134	134	121	121	131	131	82	82	

Table SIII.1: Microsatellite Genotypes Rietvlei (continued)

Sample	Ce2-3a		Ce2-4e		Ce2-5d		Ce2-12d		Card8		Cobs3		Cobs13		Haplotype
IV10	84	84	107	107	194	194	134	134	121	121	131	131	82	82	
IV11	84	84	107	107	194	194	134	134	121	121	131	131	82	82	
CC1	84	84	107	107	194	194	134	134	121	121	139	139	82	82	7
CC2	84	84	107	107	194	194	134	134	121	121	131	131	82	82	7
CC4	84	84	107	107	194	194	132	134	121	121	139	139	82	82	7
CC5	84	84	107	107	194	194	132	134	121	121	139	139	82	82	7

Table SIII.2: Microsatellite Genotypes Rietvlei Colonies

Sample	**Card8**		**Cobs3**		**Cobs13**		**Ce2-3a**		**Ce2-12d**		**Haplotype**
Riet18_o	121	121	131	131	80	80	84	84	132	132	
Riet18_1	121	121	131	133	80	82	84	84	132	132	
Riet18_2	121	121	131	133	80	80	84	84	132	132	
Riet18_3	121	121	131	133	80	82	84	84	132	132	
Riet18_4	121	121	133	133	82	82	84	84	132	132	
Riet18_5	121	121	133	133	82	82	84	84	132	132	
Riet18_6	121	121	133	133	80	82	84	84	132	132	
Riet18_7	121	121	131	133	80	82	84	84	132	132	
Riet18_8	121	121	131	133	80	82	84	84	132	132	
Riet18_9	121	121	131	133	80	80	84	84	132	132	
Riet18_10	121	121	131	133	82	82	84	84	132	132	
Riet18_11	121	121	131	133	80	82	84	84	132	132	
Riet18_12	121	121	131	133	80	82	84	84	132	132	
Riet18_13	121	121	131	133	80	82	84	84	132	132	
Riet18_14	121	121	131	133	80	82	84	84	132	132	
Riet18_15	121	121	131	133	80	82	84	84	132	132	
Riet18_16	121	121	133	133	80	80	84	84	132	132	
Riet68_o	121	121	131	131	80	80	84	84	132	132	1
Riet68_A1	121	121	131	131	82	82	84	84	132	132	
Riet68_A2	121	121	131	131	80	82	84	84	132	132	1
Riet68_A3	121	121	131	131	80	82	84	84	132	132	
Riet68_A4	121	121	131	131	80	82	84	84	132	132	
Riet68_A5	121	121	131	131	80	82	84	84	132	132	
Riet68_A6	121	121	131	131	80	80	84	84	132	132	1
Riet68_A7	121	121	131	131	82	82	84	84	132	132	1
Riet68_A8	121	121	131	131	82	82	84	84	132	132	
Riet68_A9	121	121	131	131	80	82	84	84	132	132	
Riet68_A10	121	121	131	131	80	80	84	84	132	132	
Riet68_P1	121	121	131	131	80	80	84	84	132	132	
Riet68_P2	121	121	131	131	80	80	84	84	132	132	
Riet68_A11	121	121	131	131	80	80	84	84	132	132	
Riet68_A12	121	121	131	131	80	80	84	84	132	132	

Table SIII.2: Microsatellite Genotypes Rietvlei Colonies (continued)

Sample	Card8		Cobs3		Cobs13		Ce2-3a		Ce2-12d		Haplotype
Riet68_A13	121	121	131	131	80	80	84	84	132	132	
Riet68_A14	121	121	131	131	80	80	84	84	132	132	
Riet68_A15	121	121	131	131	80	80	84	84	132	132	
Riet68_A16	121	121	131	131	80	80	84	84	132	132	
Riet68_A17	121	121	131	131	80	80	84	84	132	132	
Riet68_A18	121	121	131	131	80	80	84	84	132	132	
Riet68_A19	121	121	131	131	80	80	84	84	132	132	
Riet68_A20	121	121	131	131	80	80	84	84	132	132	
C1_A1	121	121	131	133	76	80	84	84	132	132	
C1_A2	121	121	131	133	80	80	82	84	132	132	
C1_A3	121	121	131	133	76	80	84	84	132	132	
C1_A4	121	121	131	133	76	80	82	84	132	132	
C1_A5	121	121	131	133	76	80	84	84	132	132	
C1_A6	121	121	131	133	0	0	84	84	132	132	
C1_A7	121	121	131	133	76	80	84	84	132	132	
C1_A8	121	121	131	133	76	80	84	84	132	132	
C1_A9	121	121	131	133	76	80	84	84	132	132	
C1_A10	121	121	131	133	76	80	84	84	132	132	
MD1_o	121	121	139	139	80	80	84	84	132	132	
MD1_1	121	121	139	139	80	80	84	84	132	132	
MD1_2	121	121	139	139	80	80	84	84	132	132	
MD1_3	121	121	139	139	80	80	84	84	132	132	
MD1_4	121	121	139	139	80	80	84	84	132	132	
MD1_5	121	121	139	139	80	80	84	84	132	132	
MD1_6	121	121	139	139	80	80	84	84	132	132	
MD1_7	121	121	139	139	80	80	84	84	132	132	
MD1_8	121	121	139	139	80	80	84	84	132	132	
MD1_9	121	121	139	139	80	80	84	84	132	132	
MD1_10	121	121	139	139	80	80	84	84	132	132	
C11_o	121	121	127	127	82	82	84	84	132	132	
C11_1	121	121	127	127	82	82	84	84	132	132	

Table SIII.2: Microsatellite Genotypes Rietvlei Colonies (continued)

Sample	**Card8**		**Cobs3**		**Cobs13**		**Ce2-3a**		**Ce2-12d**		**Haplotype**
C11_2	121	121	127	127	82	82	84	84	132	132	
C11_3	121	121	127	127	82	82	84	84	132	132	
C11_4	121	121	127	127	82	82	84	84	132	132	
C11_5	121	121	127	127	82	82	84	84	132	132	
C11_6	121	121	127	127	82	82	84	84	132	132	
C11_7	121	121	127	127	82	82	84	84	132	132	
C11_8	121	121	127	127	82	82	84	84	132	132	
C11_9	121	121	127	127	82	82	84	84	132	132	
C11_10	121	121	127	127	82	82	84	84	132	132	
IV4_o	121	121	131	131	82	82	84	84	134	134	
IV4_1	121	121	131	131	82	82	84	84	134	134	
IV4_2	121	121	131	131	82	82	84	84	134	134	
IV4_3	121	121	131	131	82	82	84	84	134	134	
IV4_4	121	121	131	131	82	82	84	84	134	134	
IV4_5	121	121	131	131	82	82	84	84	134	134	
IV4_6	121	121	131	131	82	82	84	84	134	134	
IV4_7	121	121	131	131	82	82	84	84	134	134	
IV4_8	121	121	131	131	82	82	84	84	134	134	
IV4_9	121	121	131	131	82	82	84	84	134	134	
IV4_10	121	121	131	131	82	82	84	84	134	134	
Riet2_o	121	121	131	131	82	82	84	84	132	132	
Riet2_1	121	121	131	131	80	82	84	84	132	132	
Riet2_2	121	121	131	133	82	82	84	84	132	132	
Riet2_3	121	121	131	131	82	82	84	84	132	132	
Riet2_4	121	121	131	133	82	82	84	84	132	132	
Riet2_5	121	121	131	131	82	82	84	84	132	132	
Riet2_6	121	121	131	133	82	82	84	84	132	132	
Riet2_7	121	121	131	131	82	82	84	84	132	132	
Riet2_8	121	121	131	131	80	82	84	84	132	132	
Riet2_9	121	121	131	131	82	82	84	84	132	132	
Riet2_10	121	121	131	131	82	82	84	84	132	132	

Table SIII.2: Microsatellite Genotypes Rietvlei Colonies (continued)

Sample	Card8		Cobs3		Cobs13		Ce2-3a		Ce2-12d		Haplotype
Riet71_o	121	121	129	129	80	82	84	84	134	134	
Riet71_1	121	121	129	129	82	82	84	84	134	134	
Riet71_2	121	121	129	129	82	82	84	84	134	134	
Riet71_3	121	121	129	129	82	82	84	84	134	134	
Riet71_4	121	121	129	129	82	82	84	84	134	134	
Riet71_5	121	121	129	129	82	82	84	84	134	134	
Riet71_6	121	121	129	129	82	82	84	84	134	134	
Riet71_7	121	121	129	129	82	82	84	84	134	134	
Riet71_8	121	121	129	129	82	82	84	84	134	134	
Riet71_9	121	121	129	129	82	82	84	84	134	134	
Riet71_10	121	121	129	129	82	82	84	84	134	134	
Riet74_o	121	121	129	131	80	80	84	84	132	134	3
Riet74_1	121	121	129	131	80	82	84	84	132	134	
Riet74_2	121	121	129	131	80	82	84	84	132	134	1
Riet74_3	121	121	129	131	80	82	84	84	132	134	
Riet74_4	121	121	129	129	80	82	84	84	132	134	
Riet74_5	121	121	129	131	80	82	84	84	132	134	
Riet74_6	121	121	129	131	80	80	84	84	132	134	1
Riet74_7	121	121	129	129	80	82	84	84	132	134	
Riet74_8	121	121	131	131	80	80	84	84	132	134	
Riet74_9	121	121	129	129	82	82	84	84	132	134	
Riet74_10	121	121	129	131	80	82	84	84	132	134	
CC1_o	121	121	139	139	82	82	84	84	134	134	
CC1_1	121	121	139	139	82	82	84	84	134	134	
CC1_2	121	121	139	139	82	82	84	84	134	134	
CC1_3	121	121	139	139	82	82	84	84	134	134	
CC1_4	121	121	139	139	82	82	84	84	134	134	
CC1_5	121	121	139	139	82	82	84	84	134	134	
CC1_6	121	121	139	139	82	82	84	84	134	134	
CC1_7	121	121	139	139	82	82	84	84	134	134	
CC1_8	121	121	139	139	82	82	84	84	134	134	
CC1_9	121	121	139	139	82	82	84	84	134	134	

Table SIII.3: Microsatellite Genotypes Hlalanathi

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**	
HP2	84	84	101	107	194	194	138	138	121	121	131	135	76	76
HP4	84	84	101	101	194	194	138	138	121	121	135	135	76	76
HP6	84	84	107	107	194	194	134	134	121	121	129	129	76	76
HP9	84	84	107	107	194	194	138	138	121	121	133	135	76	76
HP7	84	84	107	107	194	196	132	132	121	121	131	131	86	88
HP10	84	84	107	107	194	194	136	136	121	121	125	125	76	76
HP11	84	84	107	107	194	194	136	136	121	121	125	125	76	76
HP12	84	84	107	107	194	194	132	132	121	121	131	131	82	82
HP14	84	84	107	107	194	194	136	136	121	121	125	125	76	76
HP15	84	96	107	107	194	194	136	136	121	121	125	125	76	76
HP16	84	84	107	107	194	194	136	136	121	121	125	125	76	76
HP17	84	84	101	107	194	194	136	136	121	121	133	133	76	76
HP19	84	84	0	0	194	194	138	138	121	121	133	133	76	76
HP21	84	84	101	101	194	194	136	136	121	121	129	135	76	76
HP22	84	84	107	107	194	194	136	136	121	121	125	125	76	76
HP23	84	84	107	107	194	194	136	136	121	121	125	125	76	76
HP25	84	84	107	107	194	194	134	134	121	121	127	127	76	80
HP26	84	84	107	107	194	194	136	136	121	121	125	125	76	76
HP27	86	86	107	107	194	194	138	138	121	121	135	135	76	76
HP28	92	92	107	107	194	194	136	136	121	121	125	133	76	76
HP29	84	84	107	107	194	194	136	136	121	121	133	133	76	76
HP30	92	92	107	107	194	194	134	138	121	121	131	133	80	80

Table SIII.3: Microsatellite Genotypes Hlalanathi (continued)

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**	
HP32	92	92	107	107	194	194	136	136	121	121	127	127	80	80
HP33	92	92	107	107	194	194	136	138	121	121	127	139	80	80
CV1	84	84	101	101	194	194	136	136	121	121	131	131	80	80
CV2	84	84	107	107	194	194	136	136	121	121	125	127	80	80
CV3	84	84	0	0	194	194	136	136	121	121	127	127	80	80
CV4	0	0	101	107	194	194	134	136	121	127	125	127	80	80
CV5	84	84	107	107	194	194	136	136	121	121	125	125	80	80
H14-1	90	90	107	107	194	194	174	174	129	129	103	103	74	74
H14-3	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-4	90	90	101	101	194	194	174	174	129	129	103	125	74	74
H14-6	90	90	101	101	194	194	168	174	129	129	103	103	74	74
H14-7	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-8	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-9	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-11	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-12	90	92	101	101	194	196	174	182	129	133	103	103	72	74
H14-13	92	94	101	101	194	198	174	182	127	127	103	125	74	74
H14-14	90	92	101	101	194	198	174	182	129	133	103	103	74	74
H14-15	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-16	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-17	90	90	101	101	194	194	174	174	129	129	103	103	74	74

Table SIII.3: Microsatellite Genotypes Hlalanathi (continued)

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**	
H14-18	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-19	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-20	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-21	90	92	101	101	194	198	172	182	129	129	103	103	74	74
H14-22	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-23	90	96	101	101	194	194	174	174	129	129	103	103	74	74
H14-25	90	92	101	101	198	198	174	182	129	129	103	103	74	74
H14-26	90	92	101	101	198	198	174	182	129	129	103	103	74	74
H14-27	90	90	101	101	194	194	174	174	129	129	103	103	74	74
H14-28	92	92	101	101	198	198	182	182	129	129	103	103	74	74
H14-29	90	92	101	101	198	198	174	182	129	129	103	103	74	74
H14-30	90	92	101	101	194	194	172	182	129	133	103	103	74	74
H14-31	90	92	101	101	198	198	172	182	129	129	103	103	74	74
H14-33	88	90	101	101	194	198	172	172	129	133	103	105	74	74
H14-35	92	92	101	101	194	198	182	182	129	129	103	103	74	74
H14-36	92	92	101	101	194	194	178	182	129	133	103	103	74	74
H14-37	90	90	101	101	194	194	174	174	129	129	103	103	74	74
HGC1	88	92	101	101	194	196	202	202	133	133	103	125	74	74
HGC4	80	92	101	101	194	194	154	202	133	133	103	125	74	74
HGC5	92	92	101	101	194	194	204	206	133	133	103	103	74	74
HGC6	92	92	101	101	194	196	164	164	133	133	103	107	74	74
HGC8	92	92	101	101	194	194	202	202	123	133	103	103	74	74

Table SIII.3: Microsatellite Genotypes Hlalanathi (continued)

Sample	**Ce2-3a**		**Ce2-4e**		**Ce2-5d**		**Ce2-12d**		**Card8**		**Cobs3**		**Cobs13**	
HGC9	82	82	101	101	194	194	152	152	129	133	103	103	74	74
HGC10	92	92	101	101	194	194	202	202	123	133	103	103	74	74
HGC11	84	84	101	101	194	194	152	152	133	133	103	103	74	74
HGC13	84	84	101	101	194	194	202	202	123	133	103	103	74	74
HGC14	92	92	101	101	194	196	202	202	123	133	103	103	74	74
HGC15	84	102	101	101	194	194	202	202	123	129	103	103	74	74
HGC16	84	84	101	101	194	196	152	152	129	129	103	103	74	74
HGC17	82	82	101	101	194	194	202	202	129	133	107	125	74	74
HGC18	84	84	101	101	194	194	202	202	133	133	125	125	74	74
HGC19	84	84	101	101	194	194	152	200	123	129	103	103	74	74
HGC20	92	92	101	101	194	196	152	152	133	133	103	103	74	74
HGC21	84	84	101	101	192	192	168	168	125	125	103	103	76	76
HGC22	92	92	101	101	194	196	200	202	133	133	103	103	74	74
HGC23	94	94	101	101	194	194	202	202	133	133	125	125	74	74
HT1	92	92	0	0	194	194	174	174	123	123	107	107	74	74
HT2	82	84	101	107	192	194	164	164	125	125	103	139	76	82

Table SIII.4: Microsatellite Genotypes Hlalanathi Colonies

Sample	**Card8**		**Cobs3**		**Cobs13**		**Ce2-3a**		**Ce2-12d**	
HP17_A1	121	121	133	133	76	76	84	84	136	136
HP17_A2	121	121	133	133	76	76	84	84	136	136
HP17_A3	121	121	133	133	76	76	84	84	136	136
HP17_A4	121	121	133	133	76	76	84	84	136	136
HP17_A5	121	121	133	133	76	76	84	84	136	136
HP17_A6	121	121	133	133	76	76	84	84	136	136
HP17_A7	121	121	133	133	76	76	84	84	136	136
HP17_A8	121	121	133	133	76	76	84	84	136	136
HP17_A9	121	121	133	133	76	76	84	84	136	136
HP17_A10	121	121	133	133	76	76	84	84	136	136
HP17_A11	121	121	133	133	76	76	84	84	136	136
HP17_A12	121	121	133	133	76	76	84	84	136	136
HP17_L1	121	121	133	133	76	76	84	84	136	136
HP17_L2	121	121	133	133	76	76	84	84	136	136
HP17_L3	121	121	133	133	76	76	84	84	136	136
HP17_P1	121	121	133	133	76	76	84	84	136	136
HP17_P2	121	121	133	133	76	76	84	84	136	136
HP17_P3	121	121	133	133	76	76	84	84	136	136
HP17_P4	121	121	133	133	76	76	84	84	136	136
HP17_P5	121	121	133	133	76	76	84	84	136	136
HP17_P6	121	121	133	133	76	76	84	84	136	136
HP17_P7	121	121	133	133	76	76	84	84	136	136
HP17_P8	121	121	133	133	76	76	84	84	136	136
HP16_1	121	121	125	125	76	76	84	84	136	136
HP16_2	121	121	125	125	76	76	84	84	136	136
HP16_3	121	121	125	125	76	76	84	84	136	136
HP16_4	121	121	125	125	76	76	84	84	136	136
HP16_5	121	121	125	125	76	76	84	84	136	136
HP16_6	121	121	125	125	76	76	84	84	136	136
HP16_7	121	121	125	125	76	76	84	84	136	136
HP16_8	121	121	125	125	76	76	84	84	136	136
HP16_9	121	121	125	125	76	76	84	84	136	136

Table SIII.4: Microsatellite Genotypes Hlalanathi Colonies (continued)

Sample	**Card8**		**Cobs3**		**Cobs13**		**Ce2-3a**		**Ce2-12d**	
HP16_10	121	121	125	125	76	76	84	84	136	136
HP21_1	121	121	127	135	76	80	84	84	136	138
HP21_2	121	121	129	135	76	76	84	84	136	136
HP21_3	121	121	130	136	76	80	84	84	136	138
HP21_4	121	121	131	137	76	80	84	84	136	138
HP21_5	121	121	132	138	76	80	84	84	134	138
HP21_6	121	121	133	139	76	76	84	84	136	138
HP21_7	121	121	134	140	76	76	84	84	136	138
HP21_8	121	121	135	141	76	80	84	84	136	138
HP21_9	121	121	127	135	76	80	84	84	136	138
HP25_1	121	121	127	127	76	76	84	84	134	134
HP25_2	121	121	127	127	76	80	84	84	134	134
HP25_3	121	121	127	127	76	80	84	84	134	134
HP25_4	121	121	127	127	80	80	84	84	134	134
HP25_5	121	121	127	127	76	80	84	84	134	134
HP25_6	121	121	127	127	80	80	84	84	134	134
HP25_7	121	121	127	127	80	80	84	84	134	134
HP25_8	121	121	127	127	76	80	84	84	134	134
HP25_9	121	121	127	127	80	80	84	84	134	134
HP25_10	121	121	127	127	76	80	84	84	134	134
HP27_1	121	121	135	135	76	76	84	84	138	138
HP27_2	121	121	135	135	76	76	84	84	138	138
HP27_3	121	121	135	135	76	76	84	84	138	138
HP27_4	121	121	135	135	76	76	84	84	138	138
HP27_5	121	121	135	135	76	76	84	84	138	138
HP27_6	121	121	135	135	76	76	84	84	138	138
HP27_7	121	121	135	135	76	76	84	84	138	138
HP27_8	121	121	135	135	76	76	84	84	138	138
HP27_9	121	121	135	135	76	76	84	84	138	138
HP27_10	121	121	135	135	76	76	84	84	138	138
CV2_1	121	121	125	127	80	80	84	84	136	136

Table SIII.4: Microsatellite Genotypes Hlalanathi Colonies (continued)

Sample	**Card8**		**Cobs3**		**Cobs13**		**Ce2-3a**		**Ce2-12d**	
CV2_2	121	121	125	127	80	80	84	84	136	136
CV2_3	121	121	125	127	80	80	84	84	136	136
CV2_5	121	121	125	127	80	80	84	84	136	136
CV2_7	121	121	125	127	80	80	84	84	136	136
CV2_8	121	121	125	127	80	80	84	84	136	136
CV2_9	121	121	127	127	80	80	84	84	136	136
CV2_10	121	121	125	125	80	80	84	84	136	136
HGC10_A1	123	133	103	103	74	74	92	92	202	202
HGC10_A2	123	133	103	103	74	74	92	92	202	202
HGC10_A3	123	133	103	103	74	74	92	94	152	202
HGC10_A4	123	133	103	103	74	74	92	94	202	202
HGC10_P1	123	133	103	103	74	74	92	92	202	202
HGC10_P2	123	133	103	103	74	74	92	94	152	202
HGC10_P3	123	133	103	103	74	74	92	94	152	202
HGC10_Q1	133	133	103	103	74	74	94	94	202	202
HGC23_A1	133	133	125	125	74	74	94	94	202	202
HGC23_A2	133	133	125	125	74	74	92	92	202	202
HGC23_A3	133	133	125	125	74	74	92	94	202	202
HGC23_A4	133	133	125	125	74	74	92	92	202	202
HGC23_A5	133	133	103	125	74	74	92	94	202	202
HGC23_A6	133	133	125	125	74	74	92	92	202	202
HGC23_P1	133	133	125	125	74	74	92	92	202	202
HGC23_JK	133	133	125	125	74	74	92	92	202	202
H14-23_A1	129	129	103	103	74	74	90	96	174	174
H14-23_A2	129	129	103	103	74	74	90	90	174	174
H14-23_A3	129	129	103	103	74	74	90	90	174	174
H14-23_A4	129	129	103	103	74	76	90	96	174	174
H14-23_JK1	129	129	103	103	74	74	90	90	174	174
H14-23_JK2	129	133	103	105	74	74	90	90	172	182
H14-23_JK3	129	129	103	103	74	74	90	96	174	174
H14-23_JK4	129	129	105	105	74	78	90	90	170	170

Table SIII.4: Microsatellite Genotypes Hlalanathi Colonies (continued)

Sample	**Card8**		**Cobs3**		**Cobs13**		**Ce2-3a**		**Ce2-12d**	
H14-11_1	129	129	103	103	74	74	90	90	174	174
H14-11_2	129	129	103	103	74	74	90	90	174	174
H14-11_3	129	129	103	103	74	74	90	90	174	174
H14-11_4	129	129	103	103	74	74	90	90	174	174
H14-11_5	129	129	103	103	74	74	90	90	174	174
H14-11_6	129	129	103	103	74	74	90	90	174	174
H14-11_7	129	129	103	103	74	74	90	90	174	174
H14-11_8	129	129	103	103	74	74	90	90	174	174
H14-16_1	129	129	103	103	74	74	90	90	174	174
H14-16_2	129	129	103	103	74	74	90	90	174	174
H14-16_3	129	129	103	103	74	74	90	90	174	174
H14-16_4	129	129	103	103	74	74	90	90	174	174
H14-16_5	129	129	103	103	74	74	90	90	174	174
H14-16_6	129	129	103	103	74	74	90	90	174	174
H14-16_7	129	129	103	103	74	74	90	90	174	174
H14-16_8	129	129	103	103	74	74	90	90	174	174
H14-16_9	129	129	103	103	74	74	90	90	174	174
H14-16_10	129	129	103	103	74	74	90	90	174	174
HGC1_1	133	133	125	125	74	74	92	92	202	202
HGC1_2	133	133	103	125	74	74	84	84	202	202
HGC1_3	133	133	125	125	74	74	84	84	202	202
HGC1_4	133	133	103	103	74	74	84	92	202	202
HGC1_5	133	133	103	125	74	74	84	84	202	202
HGC1_6	133	133	103	125	74	74	84	92	202	202
HGC1_7	133	133	103	125	74	74	84	84	154	202
HGC1_8	133	133	103	125	74	74	84	92	154	202
HGC1_9	133	133	103	125	74	74	92	92	154	202
HGC1_10	133	133	125	125	74	74	92	92	202	202

Table SIII.5: Relatedness Rietvlei

Sample	**Q+G jacknife**	**SE**
Riet18	0.6976	0.1407
Riet68	0.7442	0.2666
Riet2	0.8401	0.0629
Riet71	0.9833	0.0176
Riet74	0.5677	0.1752
C1	0.8376	0.1242
C11	1	0
MD1	1	0
IV4	1	0
CC1	1	0
Mean	0.8671	0.0787

Table SIII.6: Microsatellite Statistics Rietvlei

Collection site	**He**	**Ho**	**f**
Riet	0.335167	0.183333	0.455216
PH	0.4	0	1
MD	0.16	0.1	0.391304
C	0.389552	0.171795	0.568981
IV	0	0	0
CC	0.171429	0.1	0.454545
Mean	0.242691	0.092521	0.608394

Table SIII.7: Relatedness Hlalanathi

Sample	Q+G jacknife	SE
HP16	0.9602	0.031
HP17	1	0
HP21	0.637	0.1446
HP25	0.9243	0.0938
HP27	1	0
CV2	0.6684	0.1633
H14-11	1	0
H14-16	1	0
H14-23	0.0296	0.2906
HGC1	0.5557	0.1985
HGC10	0.7211	0.071
HGC23	0.8779	0.1231
HGC4	0.847	0.1353
Mean	0.7862	0.0962

Table SIII.8: Microsatellite Statistics Hlalanathi

Collection site	He	Ho	f
H14	0.241444	0.170507	0.297246
HGC	0.395651	0.180451	0.550702
HT	0.761905	0.428571	0.636364
HP	0.355162	0.08619	0.761118
CV	0.194444	0.15	0.252822
Mean	0.389721	0.203144	0.550621

Table SIII.9: Haplotypes

	Haplotyp 1	Haplotyp 2	Haplotyp 3	Haplotyp 4	Haplotyp 5	Haplotyp 6	Haplotyp 7
Acc. Number	MK138575	MK138576	MK138574	MK138578	MK138577	MK138579	MK138580
	Riet79	C7	Riet47	Riet22a	Riet75	Riet5	CC2
	IV1	Riet3	Riet33	Riet30a	MD2		CC1
	IV3		Riet71		MD5		CC4
	IV4		Riet74		MD6		CC5
	IV5				Riet6		
	C5						
	PH1						
	Riet2						
	Riet11						
	Riet18						
	Riet24						
	Riet37						
	Riet67						
	Riet68						
	Riet69						
	Riet70						
	Riet81						

Acknowledgements

First of all, I want to express my gratitude to Jürgen Heinze for the opportunity to work on this fascinating ant species. I am truly grateful for his thorough support and guidance, for the opportunity to visit several conferences, deepening my knowledge on social insects and their evolution and broadening my horizon.

I thank Prof. Dr. Heike Feldhaar for taking the time to review my thesis and Prof. Dr. Christoph Oberprieler for agreeing to be part of the commitee. Moreover, I want to thank Dr. Alexandra Schrempf and Prof. Dr. Sylvia Cremer for mentoring me over the course of my PhD. Thank you Alex for your support, your advice, your encouraging words!

I'd like to express my thanks to all the members of the Heinze department for sharing a great time in Regensburg. It was a pleasure to meet you all. Thank you Antonia, Julia, Marion, Tina for your friendship that reached beyond University! Thanks to the technical assistants Andi, Tina, Helena, Agnes, Birgit, Maria, Doris and Stefan, and to Sonja for help and advice whenever needed.

Joachim, thank you for all your support during the last years, for encouraging me, for always believing in me. I'm looking forward to the times lying ahead of us!

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