Morphological, Genetic and Behavioural Aspects of Reproduction in the Ant Genus *Cardiocondyla*

DISSERTATION ZUR ERLANGUNG DES

DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.)

DER FAKULTÄT FÜR BIOLOGIE UND VORKLINISCHE MEDIZIN

DER UNIVERSITÄT REGENSBURG

vorgelegt von
Christine V. Schmidt, geb. Beißwenger
aus
Göppingen
im Jahr
2017

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Unterschrift:

„In der Natur fühlen wir uns so wohl, weil sie kein Urteil über uns hat.“

Friedrich Wilhelm Nietzsche

Manuscripts that contributed to this Thesis

Chapter 1:

Schmidt CV, Heinze J (2017) Genital morphology of winged and wingless males in the ant genus *Cardiocondyla* (Formicidae, Myrmicinae). Insect Systematics and Evolution 48 (2).

Authors' contribution: CVS and JH designed the study; CVS conducted preparation, morphometric measurements, scanning electron microscopy; CVS analysed the data; CVS and JH wrote the manuscript

Chapter 2:

Schmidt CV, Heinze J (manuscript) Mandible morphology reflects the type of male antagonism in the ant genus *Cardiocondyla*.

Authors' contribution: CVS and JH designed the study; CVS conducted preparation, morphometric measurements, scanning electron microscopy; CVS analysed the data; CVS and JH wrote the manuscript.

Chapter 3:

Schmidt CV, Trindl A, Schrempf A, Heinze J (2016) Microsatellite markers for the tramp ant, *Cardiocondyla obscurior* (Formicidae: Myrmicinae). Journal of Genetics 95: e1-e4.

Authors' contribution: CVS, AS, JH designed the study; CVS, AS, AT developed microsatellite primers; CVS, AS tested microsatellite primers; CVS conducted population genetic analysis, CVS, AS analysed the data; CVS, AS and JH wrote the manuscript.

Chapter 4:

Schmidt CV, Frohschammer S, Schrempf A, Heinze J (2014) Virgin ant queens mate with their own sons to avoid failure at colony foundation. Naturwissenschaften 101: 69-72.

Authors' contribution: SF discovered mother-son mating; SF, JH designed the study; CVS, SF conducted experiments; JH analysed the data; CVS, AS, JH wrote the manuscript.

Chapter 5:

Schmidt CV, Heimbucher A, Bernadou A, Heinze J (2017) First come, first served: the first-emerging queen monopolizes reproduction in the ant *Cardiocondyla "argyrotricha"*. Journal of Ethology 35: 21-27.

Authors' contribution: CVS, AH, JH designed the study; AH performed the experiments; CVS, AB analysed the data; CVS, JH wrote the manuscript.

In addition, I contributed to the following paper:

Heinze J, Schmidt CV, Nugroho H, Seifert B (2010) Wingless fighter males in the Wallacean ant *Cardiocondyla nigrocerea* (Insecta: Formicidae). The Raffles Bulletin of Zoology 58: 323–328.

The paper reports on the morphology and fighting behaviour of the as yet unknown wingless male of *Cardiocondyla nigrocerea*.

Author's contribution: CVS performed behavioural experiments.

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General Introduction

Social hymenopteran species are generally assumed to exhibit relatively uniform and simple ways of reproduction. In most ants and other social hymenopterans, shortly after emergence winged reproductives typically disperse and mate with foreign individuals away from the natal nest. During this brief period of time, queens may mate once or multiply, but do not remate after this mating event. Males die soon after mating, and mated queens seek to start a new colony either solely or together with other newly mated queens. They start to lay fertilized eggs to build up a worker force and, later, also produce sexual offspring (Wilson 1971).

However, some members of the social Hymenoptera may exhibit remarkable diverse reproductive life cycles, including alternative reproductive tactics of males (reviewed e.g. for ants: Heinze and Tsuji 1995; for bees: Paxton 2005) and female reproductives (Heinze and Tsuji 1995, Rüppell and Heinze 1999, Heinze and Keller 2000), linked to diverse behaviours, morphologies and physiologies.

The ant genus *Cardiocondyla* Emery, 1869 is extraordinary, as its species deviate in many aspects from the typical reproductive life-history of the social Hymenoptera (Heinze 2017). Instead of dispersing and mating away from the nest, mating regularly takes place inside the natal nest and between closely related individuals (e.g. Kinomura and Yamauchi 1987, Heinze *et al.* 2006). Besides the occurrence of winged males, which are present in some of the species, all species produce non-dispersing, long-lived males with a superficial worker-like morphology and continuous spermatogenesis (Heinze and Hölldobler 1993). These ergatoid males display an enormous variation in reproductive behaviours across different species (Jacobs and Schrempf 2017). In several species ergatoid males locally compete and may monopolize mating with available females over months by eliminating all male rivals (e.g. Kinomura and Yamauchi 1987, Heinze *et al.* 1998). In addition to diverse male mating tactics, number of queens and queen mating frequencies are diverse across species of *Cardiocondyla* (Heinze 2017).

The myrmicine genus *Cardiocondyla* is widely distributed in the Old World, and several of its species are cosmopolitan tramp ants, which have also invaded the New World (Fisher and Bolton 2016). To date, 70 valid species are identified (Bolton 2017), assigned to at least twelve species groups (Seifert 2003 and pers. comm., Heinze *et al.* 2010, Heinze 2017) and belonging to two clades (Oettler *et al.* 2010). This thesis presents data based on studies of thirteen different species (Fig. 1) belonging to eight species groups. Colonies of *Cardiocondyla* species may be easily reared in the lab and are ideal for examining a variety of different aspects of the life cycle in the context of reproduction, such as colony founding, mating systems, number of reproductive queens, nest inheritance, consequences of inbreeding and sex determination system. Furthermore, as a model system for sexual selection, studies may retrace the evolution of male traits such as morphology and reproductive tactics. The following paragraphs highlight the peculiarities of different aspects of the life cycle with regard to reproduction in *Cardiocondyla*, which are focused on in the individual chapters of this thesis.

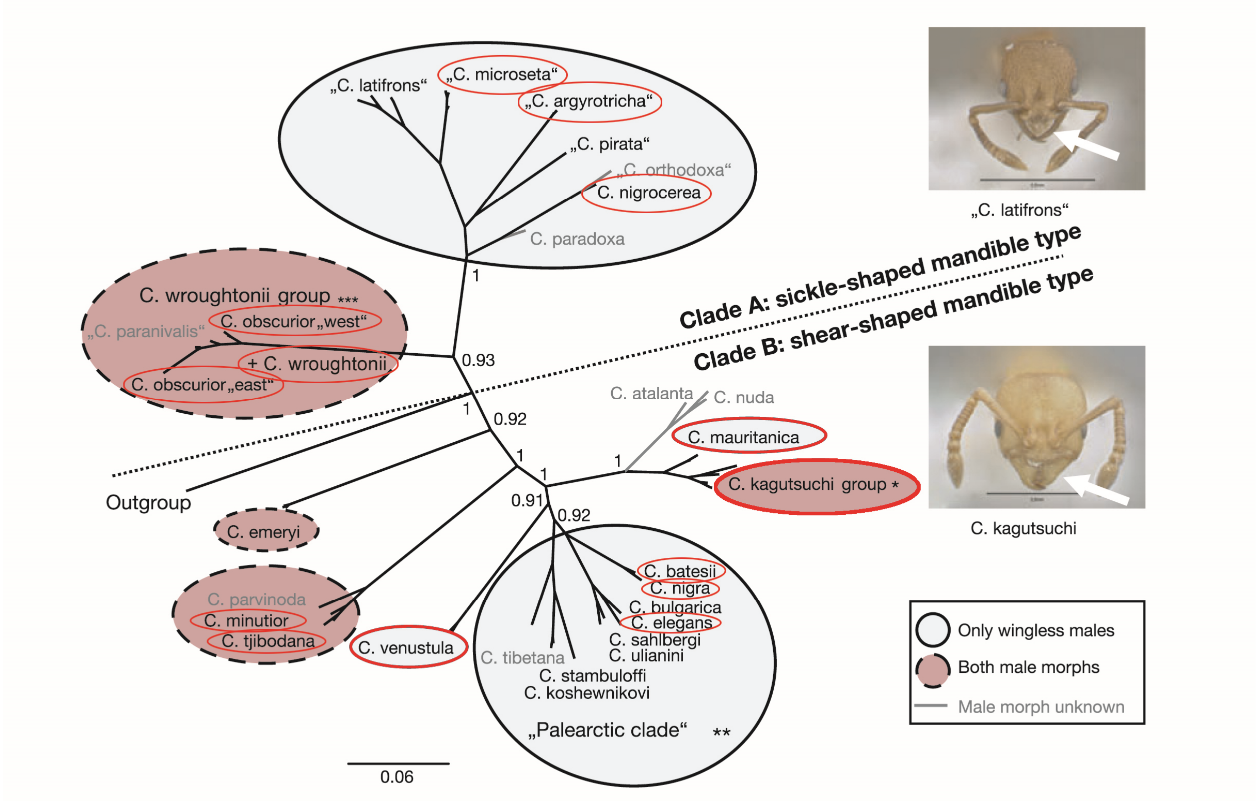

Fig. 1: Phylogenetic tree of the ant genus *Cardiocondyla* (modified after Oettler *et al.* 2010). Red circles indicate species examined in this thesis.

Males in Social Hymenoptera

Social hymenopteran males typically die within a few hours after the mating event. After death, the male persists in the colony only as sperm, which is stored in the queen’s spermatheca (Hölldobler and Bartz 1985). The queen uses this sperm throughout her life to fertilize eggs, resulting in life-long pair-bonding even years after the male's death (Hölldobler and Wilson 1990). Males of the social Hymenoptera are typically short-lived and appear to play a minor role in a colony's life cycle. Equipped with wings, large eyes, long antennae and only a limited amount of sperm, as spermatogenesis ceases before or shortly after emergence, they are generally considered to exhibit traits only adapted to disperse, locate and mate with female reproductives. Only few genera exhibit males that differ from the typical ant males described above, e.g. *Cardiocondyla* (e.g. Heinze and Hölldobler 1993, Heinze *et al.* 1998), *Formicoxenus* (Loiselle and Francoeur 1988), *Hypoponera* (Yamauchi *et al.* 1996, Foitzik *et al.* 2002), and *Technomyrmex* (Yamauchi *et al.* 1991, Pech and Bezděk 2016). Species of these genera produce wingless, so-called ergatoid males with a morphology superficially resembling workers. The loss of wings is most probably an adaptation to staying in the maternal nest and intranidal mating (Hölldobler and Wilson 1990).

In contrast to males of e.g. many mammalian taxa, social hymenopteran males typically do not have the opportunity to monopolize females, because mating takes place only during a brief period and typically occurs in large swarms (Wilson 1971), with low chances to prevent male competitors from mating. Thus, alternative reproductive tactics, which are often associated with aggressive interactions and the development of morphological traits effective as weapons, have only rarely evolved, and competition among males is mainly present in the form of scramble competition (Boomsma *et al.* 2005). However, in *Hypoponera punctatissima* and several species of *Cardiocondyla*, as a consequence of the intranidal life cycle, ergatoid males compete with each other (local mate competition, Hamilton 1967) in order to monopolize mating with female reproductives (Hamilton 1979, Stuart *et al.* 1987, Yamauchi and Kinomura 1987, Heinze and Hölldobler 1993, Heinze *et al.* 1993).

Males in the Ant Genus *Cardiocondyla*: Alternative Reproductive Tactics

The genus *Cardiocondyla* is characterized by a striking male diphenism with two male phenotypes reflected in different reproductive behaviours. Winged males are produced under environmentally stressful conditions (Cremer and Heinze 2003), they behave docile, and, before dispersing and mating with foreign virgin queens, they mate within the nest (Kinomura and Yamauchi 1987, Cremer *et al.* 2002). Besides the occurrence of these winged disperser males (Fig. 2A) in about one quarter of the species (Heinze 2017), all species produce worker-like, ergatoid males, which stay in the maternal nest throughout their lives. This non-dispersing, but wholly intranidal life cycle is not only reflected in their morphology, but also in the physiology and behaviour, i.e. in the expression of alternative reproductive tactics. Ergatoid males are wingless, lack ocelli and have only small eyes (Fig. 2B). They may be relatively long-lived (up to one year, Yamauchi *et al.* 2007), and exhibit life-long spermatogenesis, a trait unique among the social Hymenoptera (Heinze and Hölldobler 1993). The presence of both winged and wingless males is the ancestral condition of the genus, with ergatoid males engaging in lethal fights to increase their reproductive success by monopolizing mating with available female sexuals (Heinze and Hölldobler 1993), which are present year-round in species of the tropics and subtropics. Different fighting techniques occur in the genus: ergatoid males of several species use their robust "shear-shaped" mandibles to crush the weakly sclerotized cuticle of young male competitors (Heinze *et al.* 1993, 1998, Frohschammer and Heinze 2009), while the long and narrow "sickle-shaped" mandibles of ergatoid males of other species are used to both pierce the not yet fully sclerotized cuticle of emerging males (Kinomura and Yamauchi 1987; Stuart *et al.* 1987), and to clasp around the rival's body and besmear it with a hindgut secretion, which elicits worker aggression and leads to the elimination of the rival by workers (e.g. Kinomura and Yamauchi 1987; Stuart *et al.* 1987; Yamauchi and Kawase 1992; Yamauchi *et al.* 2006). In contrast, male fighting has been lost in several species occurring in Palearctic regions, as ergatoid males are tolerant of each other's presence (e.g. Schrempf *et al.* 2005; Lenoir *et al.* 2007). Male territoriality revealed in one species of *Cardiocondyla* (*C. venustula*; Frohschammer and Heinze 2009, Jacobs and Heinze 2016) presumably represents an intermediate condition between fighting and tolerance.

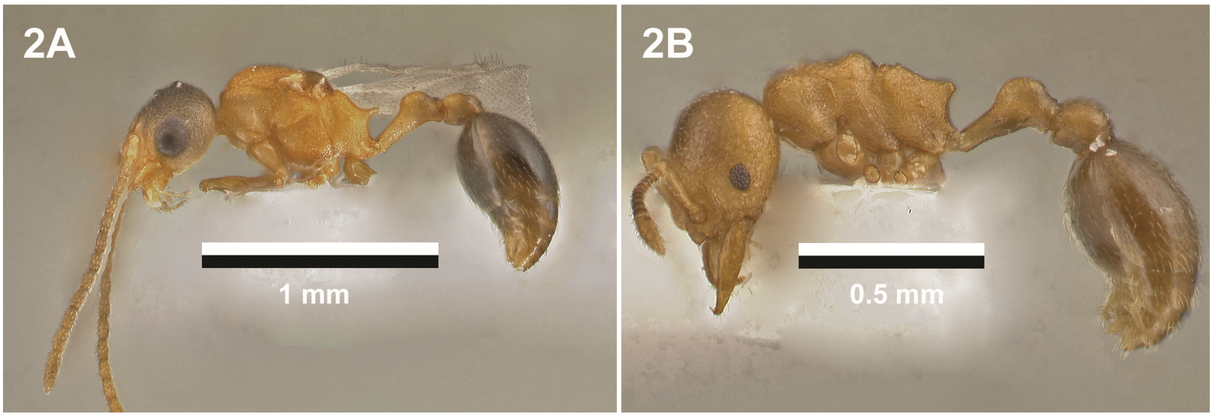

Fig. 2: Males of *C. obscurior*, lateral view. A: winged male, B: ergatoid male. Images by S. Frohschammer.

Lack of Data on Male Traits

Information on the biology of males of the social Hymenoptera is generally scarce. This is presumably due to a) the limited availability due to the short time spans during which males are produced and their limited lifespan, and, probably as a consequence, b) because of the erroneous belief that ant and other social hymenopteran males are “ephemeral sperm-delivery vessels” (but see Boomsma *et al.* 2005, Shik *et al.* 2012, 2013), only adapted to disperse, locate virgin queens and subsequently mate with them.

The vast majority of studies examining the morphology, physiology and behaviour of ants are thus focused on female colony members, i.e. workers and female sexuals. Males are unknown for several genera, and described for only about a quarter of the approximately 12.800 valid ant species (Boudinot 2015). The current taxonomy of the Formicidae is largely based on worker morphology (but see e.g. Yoshimura and Onoyama 2002, Yoshimura and Fisher 2007, 2009, and 2012, Boudinot 2015). However, it turned out that male morphological characters are important for taxonomy (e.g. Eguchi *et al.* 2006, Yoshimura and Fisher 2012) and systematics (LaPolla *et al.* 2012). In *Cardiocondyla*, the morphology of ergatoid male mandibles is useful for discriminating between the two clades of the genus (Oettler *et al.* 2010). Characters of male ant external genitalia, too, may allow e.g. for the identification of species (Gotzek *et al.* 2012), as well as for distinguishing genera (Yoshimura and Fisher 2012) and subfamilies (Yoshimura and Fisher 2011). In chapter 1 a detailed morphological description of the male external genitalia of 13 species of *Cardiocondyla* (Fig. 3) is given. Several characters turned out being useful for infrageneric taxonomy and phylogeny. Moreover, one character is potentially useful for resolving the phylogenetic relationships among the subfamily Crematogastrini, and may reveal the phylogenetic position of *Cardiocondyla*, which is unclear to date (Ward *et al.* 2015). Furthermore, the study is a valuable contribution to the study of sexual selection, as it also compares the morphology of genitalia between ergatoid and winged male phenotypes, and, in addition, examines the potential influence of different levels of sperm competition in ergatoid males having different reproductive tactics, which may lead to specific morphological adaptations of the male genital apparatus. While fighter males should have low levels of sperm competition, as only a single male is able to mate, tolerant ergatoid males of several Palearctic species are assumed to exhibit high sperm competition levels. As a result, elaborate genitalic structures, such as e.g. claspers, may have evolved to increase male mating success under highly competitive situations (Eberhard 1985).

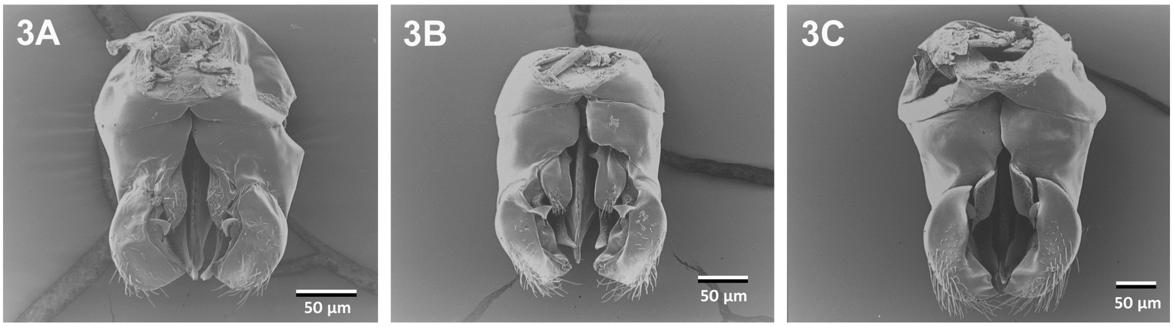

Fig. 3: External genitalia of males of *Cardiocondyla*, ventral view. A: *C. obscurior*, winged male; B: *C. tjibodana*, winged male; C: *C. elegans*, ergatoid male.

Because of the above mentioned elusive nature of ant males, only few studies focused on the evolution of ant male biology and examined adaptations of male traits (exceptions e.g. Boomsma *et al.* 2005, Shik *et al.* 2013). These studies, however, revealed that male ant biology is much more diverse than previously thought. Several traits appear to be correlated with mating success (male size and mandible length: Abell *et al.* 1999), mating syndrome (eye and mandible size with female calling syndrome, Shik *et al.* 2013) and mating opportunities (male lifespan: Heinze 2016). The morphology of wingless males of *Cardiocondyla* is assumed to be generally adapted to fighting behaviour (“fighter-phenotype”, Seifert 2003), however, morphometric data is available primarily of workers of about 50 species of the genus (e.g. Seifert 2003, Heinze *et al.* 2010, Seifert and Frohschammer 2013). Chapter 2 therefore presents detailed measurement data of several morphological characters of ergatoid males and workers and compares it both between species having different reproductive tactics, and between ergatoid males and workers of the respective species.

Furthermore, this study examines the morphology of ergatoid male mandibles. In general, ant mandibles are the tools serving to manipulate the environment, and they have to fulfil a variety of different tasks (Hölldobler and Wilson 1990). Morphological characters of ant mandibles have been proven useful for ant classification and may help to reveal phylogenetic trends especially within species groups (e.g. Ettershank 1966, Gotwald 1969, Boudinot 2015). Recently, it has been shown that ecological factors such as prey size (Ohkawara *et al.* 2016) or food quality (Camargo *et al.* 2015) may influence mandible morphology of ants. Workers of many species possess derived mandibles serving e.g. as weapons in order to defend the colony (e.g. genus *Eciton*) or possessing a morphology adapted to cutting leafs (e.g. genus *Atta*) (Hölldobler and Wilson, 1990). In contrast, male mandibles are typically described as ‘reduced’ and ‘often without function’ (Ettershank 1966, Gotwald 1969), and functional adaptations of male mandibles remain obscure, although it has been reported that males of several species use their mandibles to grasp the female during copulation (e.g. Nagel and Rettenmeier 1973, Abell *et al.* 1999, Mercier *et al.* 2007). Mandible morphology of ergatoid males (Fig. 4) is associated with the different ways of killing male competitors in the two clades of *Cardiocondyla*, and it has been indicated that minor shape differences may be linked to the specific reproductive tactic of ergatoid males of the respective species (e.g. Heinze *et al.* 2016). Despite of this, mandibles of ergatoid males of *Cardiocondyla* have been studied only superficially (e.g. Kugler 1983, Terayama 1999) and never been quantified. Chapter 2 thus provides a detailed analysis of the morphological differences of mandibles among ergatoid males of the genus *Cardiocondyla* having different reproductive life histories to reveal possible correlations between mandible morphology and reproductive tactics of ergatoid males of *Cardiocondyla*.

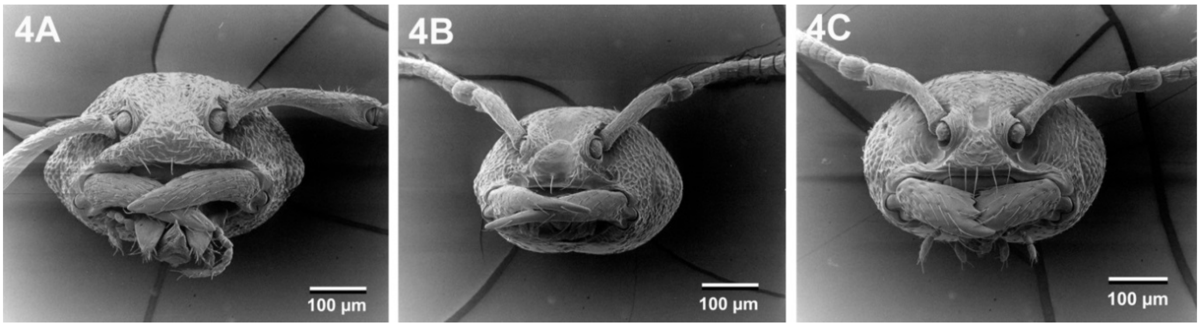

Fig. 4: Head capsules of ergatoid males of *Cardiocondyla*, full-face view.
A: *C. “argyrotricha”* (preliminary name for taxon currently being described by B. Seifert),
B: *C. obscurior*, C: *C. minutior*.

Inbreeding and Sex Determination

In the social Hymenoptera, typically both sexes leave the maternal nest, often at different times, and mate with foreign individuals (Hölldobler and Wilson 1990). This pre-mating dispersal is the primary mechanism to avoid inbreeding in social hymenopteran species. The recognition of close kin through olfactory signals is another mechanism to prevent mating between closely related individuals (Smith 1983).

Organisms generally avoid inbreeding, as mating of closely related individuals abolishes the advantages of sexual reproduction (reviewed in Crow 1994), arising from the production of genetically variable offspring. Instead, mating of close relatives produces offspring with increased levels of homozygosity, and, due to the expression of recessive deleterious mutations (Charlesworth and Charlesworth 1999), continuous inbreeding is associated with detrimental effects, which reduce fitness (inbreeding depression, Keller and Waller 2002). Inbreeding is believed to be less detrimental in haplodiploid organisms, as such deleterious recessive mutations are exposed to selection in haploid males, and thus are effectively expelled from the population (Werren 1993). However, inbreeding in the haplodiploid Hymenoptera nevertheless is often associated with negative effects. Most hymenopteran species reproduce via arrhenotokous parthenogenesis, in which fertilized, diploid eggs develop as females, and haploid, male offspring results from unfertilized eggs. The common underlying genetic mechanism is single locus complementary sex determination (sl-CSD), which is assumed to represent the ancestral condition in Hymenoptera (Cook 1993, Heimpel and de Boer 2008). Under sl-CSD multiple alleles at a single locus are determining sex, with heterozygotes at the sex locus developing as females, while hemizygotes and homozygotes develop as males. Inbreeding may lead to homozygosity at the sex determining locus, resulting in the production of diploid males, which are typically sterile or not viable (Stouthamer *et al.* 1992, Cook and Crozier 1995; but see functionally reproductive diploid males in the solitary vespid wasp *Euodynerus foraminatus*, Cowan and Stahlhut 2004, and in the gregarious endoparasitoid wasp *Cotesia glomerata*, Elias *et al.* 2009). In social Hymenoptera, diploid male production at the expense of workers or female reproductives is particularly disadvantageous with respect to colony growth and survival (e.g. Plowright and Pallett 1979). Consequently, sl-CSD is especially detrimental under inbreeding (van Wilgenburg *et al.* 2006), and members of the social Hymenoptera thus typically avoid mating between close relatives.

Nevertheless, some species of socially parasitic ants exhibit regular mating between closely related individuals (e.g. Winter and Buschinger 1983, Buschinger and Linksvayer 2004), and it is assumed that mechanisms other than sl-CSD are responsible for sex determination in these species (Buschinger 1989). However, in contrast to non-social Hymenoptera (e.g. Beukeboom *et al.* 2000), the absence of sl-CSD has only rarely been proven in social hymenopteran species (Schrempf *et al.* 2006). Alternative mechanisms of sex determination are for instance multiple locus CSD (e.g. de Boer *et al.* 2008) and genomic imprinting (e.g. Beukeboom 1995).

Intranidal Mating and Inbreeding in *Cardiocondyla*

The risk of inbreeding is particularly high if mating occurs inside the nest. Mating between close relatives may be nevertheless avoided by e.g. male dispersal prior to mating (Peeters and Crewe 1986) or adoption of alien males (Passera and Keller 1994). In addition, in some species, different colonies are characterized by the exclusive production of either male or female reproductives (Boomsma and Grafen 1990). Despite being exposed to potential mating with close sib, intranidal mating minimizes the risks for a young queen associated with dispersal and independent colony founding.

In *Cardiocondyla*, mating both takes place in the nest and regularly involves close sib. Colonies of several species of the genus, especially single-queened ones, are thus highly inbred (Schrempf *et al.* 2005, Lenoir *et al.* 2007, Schrempf 2014). In the polygynous tramp ant *Cardiocondyla obscurior* no negative effects of inbreeding have been revealed in natural populations (Schrempf *et al.* 2006). After several generations with constant inbreeding, some experimental colonies showed negative effects, such as reduced queen lifespan, reduced male fecundity and higher brood mortality. Only 1.5% of males in nine examined colonies of *C. obscurior* were diploid, which sharply contrasts the high levels of diploid male production under sl-CSD, revealing that sex is determined by another mechanism in *C. obscurior*, and the same presumably holds true for all species of *Cardiocondyla*. It has already been pointed out, that absence of sl-CSD may be advantageous especially for tramp species (Heinze *et al.* 2006), as genetic loss due to founder effect may otherwise lead to diploid male production, which is often associated with failure at colony foundation (Ross and Fletcher 1986).

To avoid negative effects of constant inbreeding after several generations, colonies of *C. obscurior* are assumed to regularly outbreed by e.g. mating with seasonally produced, winged disperser males (Heinze *et al.* 2006). Other mechanisms of outbreeding have been observed in species of *Cardiocondyla*, such as dispersal of ergatoid males (Bolton 1982) or transfer of uninseminated queens into alien nests (Lenoir *et al.* 2007). In addition, the adoption of alien queens may lower genetic relatedness in polygynous species such as *C. obscurior*. As nothing was known about the genetic structure of this unobtrusive tramp ant, which has successfully spread from its presumed origin in Southeast Asia to the tropics and subtropics of all continents, six polymorphic microsatellite markers were established, and both relatedness and the level of inbreeding in a Japanese population of *C. obscurior* was analysed (chapter 3). By doing so, the mechanisms by which negative effects of inbreeding are avoided, and the characteristics, which make the species so effective in establishing in new habitats, may be revealed.

Mother-Son Inbreeding

Due to restricted mating sites under intranidal mating, local mate competition may arise (Hamilton 1967). In several species of *Cardiocondyla*, ergatoid males fight against each other in order to monopolize mating with available virgin queens (Anderson *et al.* 2003). As a consequence, queens adjust sex-ratio to an extreme female-bias and produce only enough males to ensure insemination of available virgin queens (e.g. Cremer and Heinze 2002, Schrempf *et al.* 2005). Therefore, especially in monogynous species, young emerging queens may face the risk to end up without mating partner, in particular when emergence takes place after their mother's death. Uninseminated queens are capable of laying unfertilized, haploid eggs, yet, this results in male offspring only. Without the possibility to produce a large worker force (diploid offspring), such unmated queens will fail to establish a colony. The only option to raise worker offspring is to mate with the queen's own son to produce diploid offspring. However, mating between the mother and her own son is practically inexistent in the social Hymenoptera because of a) the detrimental effects of inbreeding under sl-CSD, and b) due to the fact that mating usually occurs only during a single event early in a queen's life and no remating takes place after the start of egg production (Wilson 1971). Indeed, only two cases of mother-son mating have been reported in social Hymenoptera (Garófalo 1973, Liebig *et al.* 1998), which sharply contrasts the situation in solitary haplodiploid taxa (e.g. Ueda 1997, Hardy *et al.* 1999) and diploid social insects, i.e. termites (Kobayashi *et al.* 2013). Chapter 4 documents, however, that young, uninseminated queens of *C. "argyrotricha"* (Fig. 5) may avoid colony failure by first producing haploid male offspring, mating with one of their own emerging sons several weeks later, and subsequently raising workers and female sexual offspring resulting from fertilized eggs. This indicates that sl-CSD is absent in *C. "argyrotricha"*, too. The study highlights the striking plasticity of social insect life-histories by demonstrating how alternative female reproductive tactics as well as sex determination systems may evolve under certain constraints.

Queen Number and Nest Inheritance

Queens of the social Hymenoptera exhibit a variety of different reproductive tactics (Heinze and Keller 2000), similarly to the surprisingly flexible traits of males. Queen number appears to be highly plastic within ants, and is variable even within some species (Gill *et al.* 2009). Monogyny (a single reproductive queen) seems to represent the ancestral state in ants (Ross and Carpenter 1991). While polygyny (several reproductive queens per nest) typically is associated with dependent colony founding by budding, young queens emerging in monogynous colonies typically disperse, mate away from the nest and found a new colony after finding an appropriate nesting site (independent colony founding). Polygyny presumably has evolved as a response to the high costs of solitary colony founding e.g. in patchy habitats, with queens selected for seeking adoption in the natal nest (Bourke and Heinze 1994). A reversal from polygyny to monogyny is for example found in slave-making ants (Beibl *et al.* 2005), but seems to be rare, as queens of polygynous colonies often do not exhibit morphological adaptations necessary for independent colony founding, such as a high fat content, reflected in a bulky thorax. Polygyny represents the ancestral state in the genus *Cardiocondyla* (Oettler *et al.* 2010), while several Palearctic species (*C. elegans*, *C. batesii*, *C. nigra*) show a reversal to single queening (Heinze *et al.* 2002, Lenoir *et al.* 2007, Schrempf and Heinze 2007). In chapter 5 we document a second reversal from polygyny to monogyny in the Southeast Asian species *C. "argyrotricha"*.

Fig. 5: Wingless queen of *C. "argyrotricha"*.

Staying in the natal nest is often beneficial if compared to independent colony founding, as dispersal is associated with a high risk of failure due to greater mortality before colony establishment and the risk of not finding a suitable nesting site. Theory predicts a trade-off between dispersal and staying in the nest, i.e. instead of dispersing and facing the risks of independent colony foundation in order to reproduce, a potential reproductive may stay in the nest in order to inherit this resource in the future and subsequently reproduce (Myles 1988). A stable nest with a worker force thus constitutes an especially valuable heritage for queens of the social Hymenoptera. However, fights over nest inheritance are surprisingly uncommon, and the colony’s lifespan is typically bound to the lifespan of the single reproducing queen, with the colony quickly declining after her death. Few studies indicate that group members compete for inheritance of the nest. For example, virgin queens of the honey bee *Apis mellifera* are known to show fatal fighting over nest inheritance (Gilley 2001), and conflict over reproduction also occurs among subordinates in a monogynous queenless ant (Monnin and Peeters 1998).

Chapter 5 describes nest inheritance in monogynous colonies of the ant *Cardiocondyla “argyrotricha”*. In contrast to two polygynous species of the same species-group, which are known to establish rank hierarchies and to engage in lethal fights over nest inheritance (Yamauchi *et al.* 2007, Heinze and Weber 2011), analysis of queen location and aggressive interaction between colony members in small experimental colonies, containing several consecutively emerging queens, shows that aggression occurs on a low level only. Instead, it appears that mainly the order of emergence of queens determines which of them will become reproductive and inherit the nest. The status of the reproductive queen is reflected in the location in the nest, as the reproductive queen sits on the brood pile, while non-reproducing queens spend most of their time in the peripheral areas.

Aims of this Thesis

In species of the myrmicine ant genus *Cardiocondyla* several aspects of reproduction deviate from the typical reproductive life cycle of the social Hymenoptera. Intranidal mating, regular sib-mating linked to high levels of inbreeding, and the occurrence of worker-like males, which stay in the maternal nest, is associated with a variety of different life-history strategies, which have evolved in species of *Cardiocondyla*. Both male and female sexuals of *Cardiocondyla* exhibit a wide range of reproductive tactics, which are accompanied by correlated morphological traits. This thesis focusses on morphological, genetic and behavioural aspects concerning reproduction in the ant genus *Cardiocondyla*, in order to provide data helpful for gaining insights into the evolutionary pathways of the different reproductive tactics and correlated traits in social insects.

Chapter 1

Genital Morphology of Winged and Wingless Males in the Ant Genus *Cardiocondyla* (Formicidae, Myrmicinae)

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Key words: *Cardiocondyla*, ergatoid males, morphology, external genitalia, male diphenism, Myrmicinae

Abstract

The myrmicine ant genus *Cardiocondyla* is characterized by a peculiar male diphenism with winged disperser males and wingless, “ergatoid” fighter males. Here we describe and illustrate the morphology of the male external genitalia of 13 species of this genus. Several characters, especially the parossiculus of the volsella and the medial face of the paramere, vary between different species groups and might be useful to clarify the infrageneric taxonomy of *Cardiocondyla*. External genitalia between different phenotypes of a species differ primarily in size. All examined males, both winged and ergatoid, have a parameral hook that resembles a structure described in *Nesomyrmex* males but which is apparently absent in other studied myrmicine ants. The presence and absence of this hook might be useful for future studies on the relationships within the myrmicine tribe Crematogastrini.

Introduction

The current taxonomy of the Formicidae is largely based on worker morphology, and data on the morphology of ant males generally is scarce (Bolton 2003). This lack of information is probably due to the limited availability of ant males, which in most taxa are produced only during a short period of the year (e.g., Boomsma *et al.* 2005). Nevertheless, recent studies have revealed the value of morphological characters of males for taxonomy (e.g., Eguchi *et al.* 2006, Yoshimura & Fisher 2012) and systematics (LaPolla *et al.* 2012). Among other morphological features, male external genitalia provide useful characters for species identification (Gotzek *et al.* 2012), infrageneric taxonomy (Eguchi 2003), distinguishing genera (Yoshimura & Fisher 2012) and subfamilies (Yoshimura & Fisher 2011), as well as for identifying new genera (Francoeur & Loiselle 1988). Male characters can offer new information for the phylogenetic analysis of unresolved clades. Detailed descriptions of the genital apparatus across ant taxa are therefore critically needed.

The ant genus *Cardiocondyla* Emery, 1869 is part of the myrmicine tribe Crematogastrini (sensu Ward *et al.* 2015) and contains 70 valid species (Bolton 2016) assigned to at least twelve species groups (Seifert 2003 and pers. comm., Heinze *et al.* 2010, Heinze 2017). It is characterized by large variation in reproductive tactics of both sexes and an ancestral male diphenism with winged disperser males and wingless males (ergatoid males), which engage in lethal fighting for access to female sexuals in their natal nests (Kugler 1983, Seifert 2003, Oettler *et al.* 2010, Heinze 2017). Ergatoid males have a very aberrant morphology and originally were mistaken as ergatoid queens of two new genera of ants parasitizing *Cardiocondyla* (*Emeryia* Forel 1890 and *Xenometra* Emery 1917). While the position of *Cardiocondyla* within the diverse ant subfamily Myrmicinae remains unclear (Ward *et al.* 2015), its infrageneric taxonomy (Seifert 2003) and phylogenetic relationships (Heinze *et al.* 2005, Oettler *et al.* 2010) have largely been resolved based on external morphology of both males and workers and on molecular data. According to a molecular phylogeny, all presently studied species groups of *Cardiocondyla* belong to one of two basal clades. This dichotomy is also reflected in the mandibular morphology of ergatoid males, which are sickle-shaped or shear-shaped (see Oettler *et al.* 2010). Ergatoid males occur in all species of the genus *Cardiocondyla*, but winged males have been lost convergently in several species groups. Furthermore, in several species lethal fighting among ergatoid males has been replaced by complete tolerance or intranidal territoriality, resulting in different queen mating frequencies and different levels of sperm competition. An examination of male genitalia therefore is of interest not only for taxonomy and systematics but also in the context of sexual selection.

Males are currently known for 24 species of *Cardiocondyla* and their morphology has been described in more or less detail for 16 species (*C.* "*argentea*" group, *C. pirata* Seifert & Frohschammer, 2013: Frohschammer and Seifert 2013; *C. batesii* group, C. *batesii* Forel, 1894: Santschi 1907; *C. elegans* group, *C. elegans* Emery, 1869: Minozzi 1918, Kugler 1983; *C. emeryi* group, *C. emeryi* Forel, 1881: André 1881; *C. minutior* group: *C. minutior*, as "*C. tsukuyomi,"* Terayama 1999; *C. nuda* group, *C. kagutsuchi* Terayama 1999; Terayama 1999; *C. mauritanica* Forel, 1890: Forel 1904, and *C. nuda* Mayr, 1866: Emery 1897, Terayama 1999; *C. paradoxa* group, *C. thoracica* Smith, 1859: Heinze *et al.* 2016; *C. "Prosopidris"* group, *C. papuana* Reiskind, 1965: Reiskind 1965; *C. shuckardi* group, *C. shuckardi* Forel, 1891: Heinze *et al.* 2014 and *C. venustula* Wheeler 1908: Heinze *et al.* 2013; *C. stambuloffii* group, *C. stambuloffii* Forel, 1892: Forel 1892; *C. wheeleri* group, *C. nigrocerea* Karavaiev, 1935: Heinze *et al.* 2010; *C. wroughtonii* group: *C. wroughtonii* Forel, 1890: Kugler 1983 and, as "*C. yamauchii,"* Terayama 1999). Male external genitalia have only been described and/or illustrated in five of these species (*C. elegans*, *C. emeryi*, *C. papuana*, *C. stambuloffii*, *C. wroughtonii*). Here we examine the male external genitalia of a total of 13 species of *Cardiocondyla* and determine their value as genus-level identification tools and for the identification of species groups. Our study extends the knowledge of male morphological characters in ants in general and in the future may help to clarify phylogenetic relationships of the Crematogastrini (sensu Ward *et al.* 2015).

Material and Methods

Examined species

We examined external male genitalia of 13 different species of *Cardiocondyla* belonging to different species groups (Seifert 2003), including four species with both ergatoid and winged males (table 1). Several specimens of each species and, if present, both male phenotypes, were studied. The length of penisvalvae could be measured in only seven of these species (see asterisk in table 1), as only few individuals were available for some of the species, and many preparations failed because of the difficult preparation of the minute structures.

No material was available from two species groups, which originally were considered to belong to separate (sub-) genera but later were synonymized with *Cardiocondyla* (Bolton 1982). While Reiskind (1965) provided a careful analysis of the genitalia of the male of *C. papuana* (as *Prosopidris papuana*), *C. monardi* (described as subgenus *Loncyda*, Santschi 1930) is known only from type material. *Monomorium cristatum* Santschi 1912, transferred by Santschi (1930) into a new subgenus *Cardiocondyla* (*Dyclona*), apparently does not belong to *Cardiocondyla* (B. Seifert, pers. comm.).

Table 1: Examined species of different species groups, collection sites and occurrence of male phenotypes.

	species group	species	collection site	male phenotype
clade A: sickle shaped mandible type	*C. wroughtonii* group	*C. obscurior**	Bahia, Brazil	ergatoid, winged
		C. wroughtonii	Limay, Bataan, Philippines	ergatoid, winged
	C. wheeleri group	*C. nigrocerea**	Pulau Obi, Maluku Utara, Indonesia	ergatoid
	C. “*argentea*” group[1]	*C.* “*argyrotricha*”*	Los Baños, Philippines	ergatoid
		C. “*microseta*”	Pulong Polillo, Philippines	ergatoid
clade B: shear shaped mandible type	*C. minutior* group	*C. minutior*	Okinawa, Japan	ergatoid, winged
		*C. tjibodana**	Gua Londron, Indonesia	ergatoid, winged
	C. nuda group	*C. kagutsuchi**	Okinawa, Japan	ergatoid
		C. mauritanica	Porto Moniz, Madeira, Portugal	ergatoid
	C. shuckardi group	*C. venustula* *	Kaua‘i, Hawaii	ergatoid
	C. batesii group	*C. batesii* *	Padul, Spain	ergatoid
		C. nigra	Limassol, Cyprus	ergatoid
	C. elegans group	*C. elegans*	River Allier, Chemilly, Allier department, France	ergatoid

* measurement of penisvalve length

[1] *C.* “*argentea*”, *C.* “*argyrotricha*”, and *C.* "*microseta*” are the preliminary names for taxa currently being described by B. Seifert

Sample preparation and assessment of morphological characters

Ants were collected either in the field or from laboratory colonies and kept in 70% ethanol in small plastic flacons. Whole specimens were dehydrated in an ascending alcohol series (80%, 90%, 99.6% ethanol).

Male external genitalia were removed from dehydrated specimens to allow qualitative description using a scanning electron microscope. The male gaster was dissected and the external genitalia were mounted in different positions on metal stubs with double-sided adhesive carbon pads and air-dried for at least 48 h. In addition, we dissected genitalia to examine the morphology of external genitalia. After this procedure, samples were coated with a mixture of gold-palladium with the use of a sputter-coater (*Fisons Instruments, UK*) before being examined in a DSM 950 Zeiss Scanning Electron Microscope. Three elements of *Cardiocondyla* male genitalia were studied: the paired valves of the parameres, volsellae, and penisvalvae. The terminology follows Boudinot (2013) and Schulmeister (2003).

To measure the size of male penisvalvae, the gaster of males of several dehydrated individuals (see Table 1) was removed and subsequently soaked in 10% potassium hydroxide for 12-24 hours at room temperature. Dissections were carried out under a Stemi 2000-C stereomicroscope (Carl Zeiss Microscopy GmbH, Germany). The penisvalvae were transferred to glass slides and embedded in Depex mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA). An Axiophot microscope (Carl Zeiss Microscopy GmbH, Germany) was used to create digital color pictures of the penisvalvae. Pictures were imported in AxioVision and subsequently the length of the penisvalve (straight-line distance between the basalmost (anteriormost) point of the ventral margin and the apex (posteriormost point) of the penisvalve, see Fig. 1J) was measured. Mean measurement error based on three measurements was 0.1 µm. Only penisvalvae, which completely were in the focal plane, were measured. In addition, we counted the number of teeth on the penisvalvae.

Cephalic length (CL) and width (CW) of respective individual lacking the gaster were measured using a WildM10 stereomicroscope (Leica Microsystems, Germany) with the aid of an ocular micrometer. Definition of numeric characters was (according to Seifert, 2003): CL: maximum cephalic length in median line, excavations of occiput and/or clypeus reduce CL; CW: cephalic width; across and including the eyes. Cephalic size was used as an indicator of body size of individuals (see Seifert 2003) and calculated by (CL+CW)/2. A mean measurement error of ± 1.4 µm was calculated based on three measurements for CL and CW. To allow for comparison within and between species, penisvalve length was divided by CS.

Statistical analyses were carried out using the software IBM SPSS Statistics 23 (IBM Corporation).

Results

Description of male external genitalia of Cardiocondyla

All thirteen examined species of *Cardiocondyla* share the following characteristics:

Parameres: The paired parameres meet both basiventrally and basidorsally (Fig. 1A and B). In profile these outer valves are subtriangular, the dorsal face is bent ventrally, the apex is rounded (Fig. 1C). The parameres are the largest valves encompassing the volsellae and the penisvalvae. The parameres are composed of the basimere and the distal telomere. In ventral view, the telomere is laterally convex and its apex bears setae externally. A ventrolaterally oriented, curved hook is present in all species studied (arrows in Fig. 1A). This hook originates at the medial wall of the telomere (Fig. 1D). The medial parameral face protrudes close to the ventromedial margin, creating a cavity in several species (arrow points to cavity in Fig. 1E). Medial face of paramere antero-laterally adjacent to the parameral hook either smooth or with microsculpture (Fig. 1F). Furthermore, the medial face of the paramere develops deep depressions or furrows in some of the species examined (Fig. 1G).

Volsellae: The paired middle valves are heavily sclerotized. The volsellae consist of the parossiculus and the digitus (gonossiculus). The basivolsella is attached to the median basiventral part of the paramere. The parossiculus shows much variation among different species groups of *Cardiocondyla*. In some species, the ventral surface of the parossiculus is concave, while in others a short, slender projection originating from the ventral surface of the parossiculus is present (basivolsellar projection, Fig. 1H). The ventral surface of the parossiculus bears at least a few setae and pits. The digitus is laterally compressed, in profile hook-shaped, curved ventrad, gradually tapering towards the apex. The lateral surface of digitus bears very short setae.

Penisvalvae: These paired inner valves are laterally compressed, in profile longer than high, subtriangular (Fig. 1J). The penisvalvae consist of the blade-like valviceps and a basal apodeme termed valvura, which extends basidorsolaterally. The ventral margin is serrate with little tooth-like projections, teeth are absent apicoventrally. In profile, pits are found beneath the ventral margin. Penisvalvae are rather similar in the examined species.

The general morphology of external genitalia does not show differences within a phenotype. In the four diphenic species, ergatoid and winged males do not differ in the general morphological characteristics of the genital apparatus, but show differences in the size of genitalic valves (i.e., penisvalvae) and the number of teeth on the ventral margin of the penisvalvae (Table 2). Winged males of *Cardiocondyla* are larger than ergatoid ones, and the same holds for their penisvalvae (Table 2). The length of penisvalvae is positively correlated with body size (CS) in winged males of the two diphenic species in which it was studied, while there is no such correlation in ergatoid males of examined *Cardiocondyla* species. The coefficient of variation (CV) of penisvalvae length is higher in ergatoid than winged males. Number of teeth is not correlated to length of penisvalvae within a phenotype. However, winged males tend to have more teeth on the ventral margin of the penisvalve.

Table 2: Measurement data of males of seven different *Cardiocondyla* species. CS: cephalic size, CV: coefficient of variation

Species	**male phenotype**	**length of penisvalve [µm]**	**Pearson's correlation (length of penisvalve - CS)**	**length of penisvalve [µm]/CS**	**CV of penisvalve length**	**number of teeth**	**Pearson's correlation (number of teeth - length of penisvalve)**
C. nigrocerea	ergatoid [N = 5]	134 ± 3	0.386	0.327	0.021	8-9	0.302
C. „argyrotricha“	ergatoid [N = 6]	121 ± 3	- 0.157	0.252	0.027	9-11	0.494
C. obscurior	ergatoid [N = 7]	109 ± 4	0.747	0.285	0.040	7-10	-0.193
	winged [N = 6]	129 ± 3	0.958**	0.278	0.024	9-12	0.676
C. tjibodana	ergatoid [N = 6]	119 ± 4	0.013	0.290	0.030	9-10	0.132
	winged [N = 6]	136 ± 2	0.849*	0.295	0.015	10-11	0.571
C. kagutsuchi	ergatoid [N = 7]	149 ± 2	0,245	0.286	0.016	11-14	0.221
C. venustula	ergatoid [N = 4]	196 ± 6	0.648	0.317	0.028	11	0.859
C. batesii	ergatoid [N = 6]	181 ± 5	-0.185	0.354	0.025	9-11	-0.102

* 0.05 significance level
** 0.01 significance level

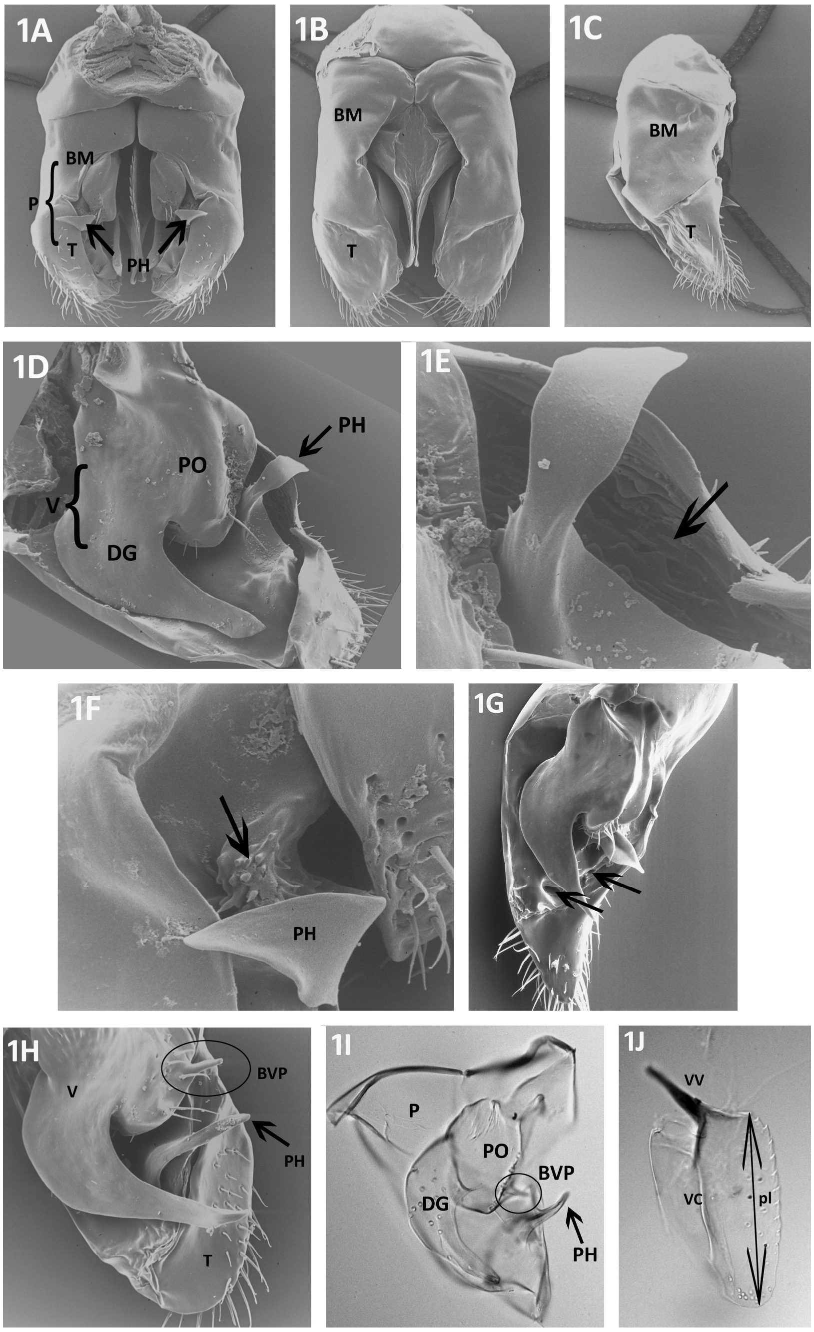

Fig. 1: Male external genitalia of different species of *Cardiocondyla*. A: *C. minutior*, ventral view (arrows point to the parameral hook), B: ventral view, *C. minutior*, C: lateral view, *C. minutior*, D: apico-medial-view showing volsella and paramere (arrow points to parameral hook), *C. nigra*, E: detail of paramere, showing parameral hook and excavation of parameral wall, *C. nigra*, F: ventral view showing microsculpture (arrow) adjacent to parameral hook, *C. tjibodana*, G: medial view of volsella and paramere (arrows point to depressions in medial face of paramere), *C. tjibodana*, H: apico-medial-view of volsella and paramere, *C. "argyrotricha"*, I: mesial view of volsella and paramere, *C. "argyrotricha"*, J: lateral view of penisvalve (pl: length of penisvalve), *C. "argyrotricha"*. BM: basimere, BVP: basivolsellar projection (indicated by circle), DG: digitus, P: paramere, PH: parameral hook (indicated by arrow), PO: parossiculus, T: telomere, V: volsella, VC: valviceps, VV: valvura.

Morphology of male external genitalia of 13 different species of Cardiocondyla

Morphology of male external genitalia in the ant genus *Cardiocondyla* revealed minor differences between closely related species. However, several morphological characters are well-suited for distinguishing several of the species groups.

C. wheeleri and ***C. "argentea"*** **groups:** *C. nigrocerea* (Fig. 2), *C. "argyrotricha"* (Fig. 3), *C. "microseta"* (Fig. 4) (preliminary names for taxa currently being described by B. Seifert)

Medial face of paramere laterally adjacent to the parameral hook without microsculpture (Fig. 2B, 3A, 4A). Volsella (parossiculus) producing a short slender projection directed ventrally (basivolsellar projection, Fig. 2B, 3B, 4B). Parossiculus with several setae. Digitus in situ shorter than, or as long as penisvalve.

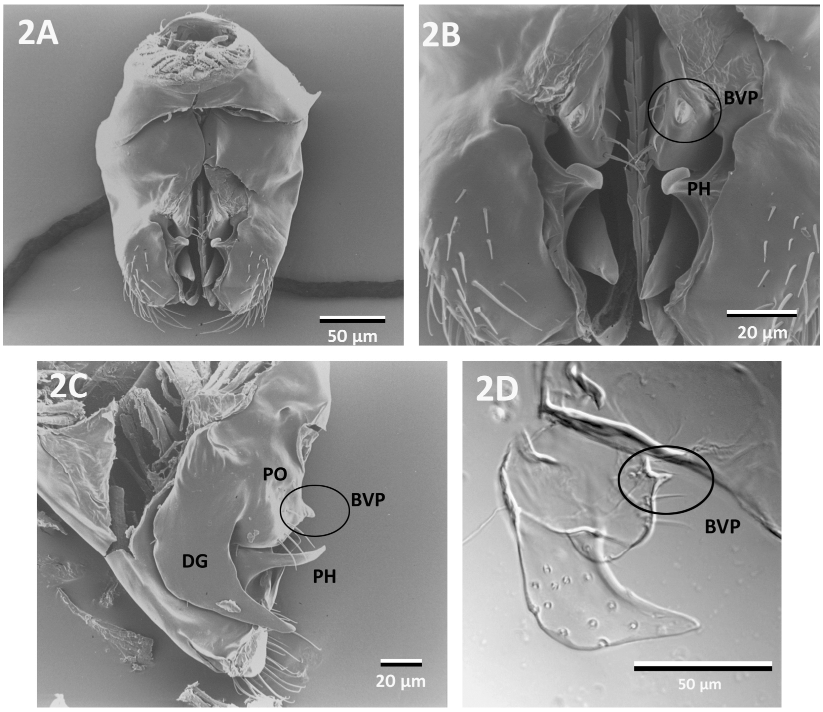

Fig. 2: *C. nigrocerea*, ergatoid male: A, B: ventral view of male external genitalia (circle indicates basivolsellar projection), C: medial view of volsella and paramere, D: lateral view of volsella. BVP: basivolsellar projection (indicated by circle), DG: digitus, PH: parameral hook, PO: parossiculus.

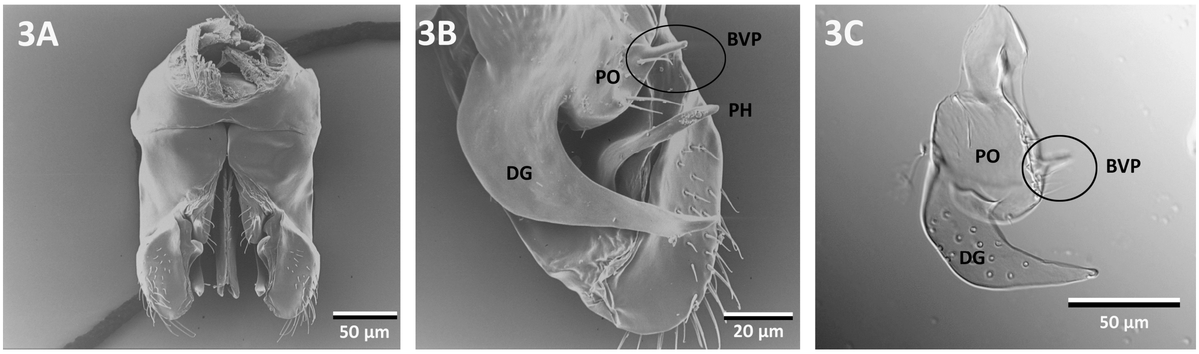

Fig. 3: *C. “argyrotricha”*, ergatoid male: A: ventral view of male external genitalia, B: medial view of volsella and paramere, C: medial view of volsella. BVP: basivolsellar projection (indicated by circle), DG: digitus, PH: parameral hook, PO: parossiculus.

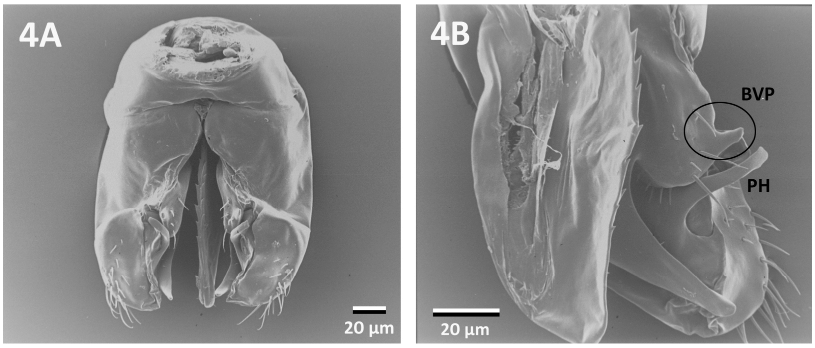

Fig. 4: *C. “microseta”*, ergatoid male: A: ventral view of male external genitalia, B: medial view of penisvalve, volsella and paramere. BVP: basivolsellar projection (indicated by circle), PH: parameral hook.

C. wroughtonii* group*: *C. obscurior* Wheeler, 1929 (Fig. 5) and *C. wroughtonii* Forel, 1890 (Fig. 6)

Medial face of paramere antero-laterally adjacent to the parameral hook without microsculpture. Paramere with approximately dorso-ventrally oriented rugae on medial face (Fig. 5B, 6C). Volsella without projection. Apical part of parossiculus in profile well-rounded (Fig. 5E, 6B). Parossiculus covered with setae ventrally and apically, setae having distinct sockets. Digitus with approximately basi-apically oriented rugae on lateral face (Fig. 5C, 6B). Digitus in situ longer than penisvalve.

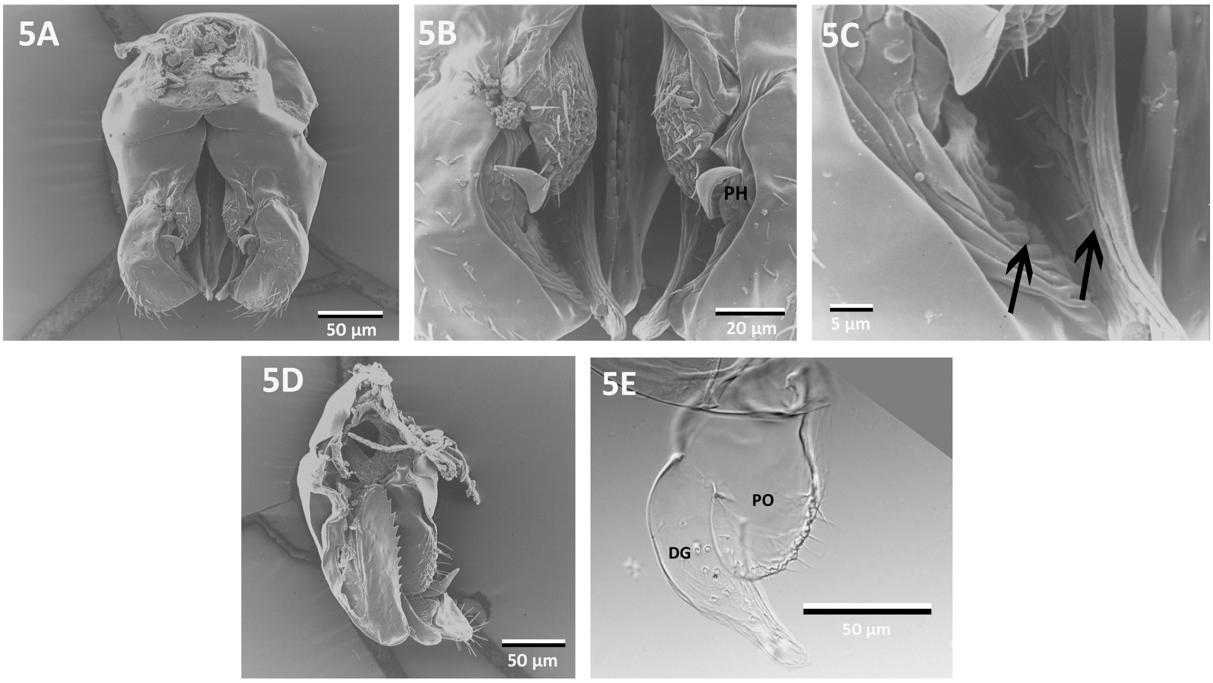

Fig. 5: *C. obscurior*, winged male: A, B: ventral view of male external genitalia of *C. obscurior*, C: rugae on medial wall of paramere and digitus (arrows), D: medial view of penisvalve, volsella and paramere, E: lateral view of volsella. DG: digitus, PH: parameral hook, PO: parossiculus.

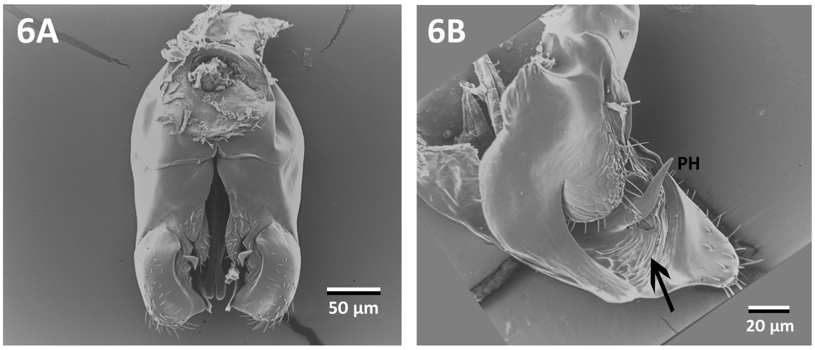

Fig. 6: *C. wroughtonii*, winged male: A: ventral view of male external genitalia, B: medial view of volsella and paramere with rugae on medial face of paramere (arrow). PH: parameral hook.

***C. minutior* group:** *C. minutior* Forel, 1899 (Fig. 7), *C. tjibodana* Karavaiev, 1935 (Fig. 8)

Medial face of paramere antero-laterally adjacent to the parameral hook with microsculpture (arrows in Fig. 7B). Two deep depressions on the medial face of the paramere, one apically adjacent to parameral hook, and one near apex (arrows in Fig. 7C, 8B and D). Volsella without projection. Basi-lateral part of parossiculus and medial margin slightly bulged, ventral surface slightly concave. Ventral surface of parossiculus with several pits and few setae. Digitus in situ shorter than penisvalve.

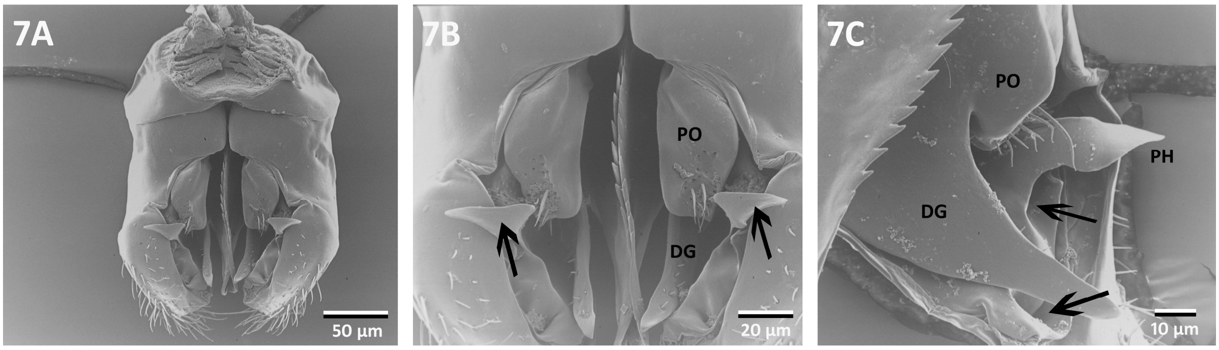

Fig. 7: *C. minutior*, winged male: A, B: ventral view of male external genitalia (arrows point to microsculpture), C: medial view of penisvalvae, volsella, and paramere (deep depressions indicated by arrows). DG: digitus, PH: parameral hook, PO: parossiculus.

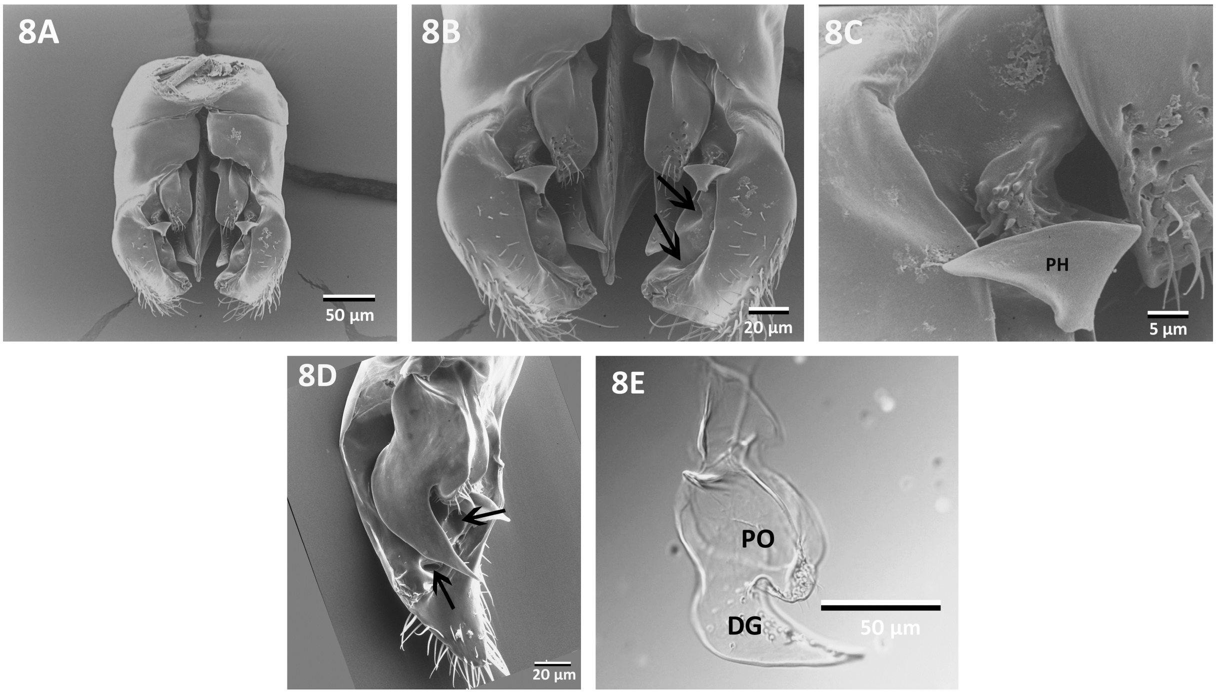

Fig. 8: *C. tjibodana*, winged male: A, B, and C: ventral view of male external genitalia (deep depressions of the paramere indicated by arrows), D: medial view of volsella and paramere (arrows point to deep depressions in medial face of the paramere), E: medial view of volsella. DG: digitus, PH: parameral hook, PO: parossiculus.

***C. nuda* group:** *C. kagutsuchi* Terayama, 1999 (Fig. 9), *C. mauritanica* Forel, 1890 (Fig. 10)

Medial face of paramere laterally adjacent to parameral hook with microsculpture (arrows in Fig. 9B, 10B). Large excavation between parameral hook and medial wall of paramere present. Medial wall of paramere producing a narrow furrow starting at the base of the parameral hook and running towards parameral apex (Fig. 9B, 10D). No projection present on volsella. Basi-lateral part of parossiculus and medial margin slightly bulged, ventral surface concave (Fig. 9C, 10D). Parossiculus in profile tapering apico-ventrally, dorsal and ventral margin meeting at an acute angle (9C, 10C). Ventral surface of parossiculus with several pits and a single seta. Digitus in situ shorter than penisvalve.

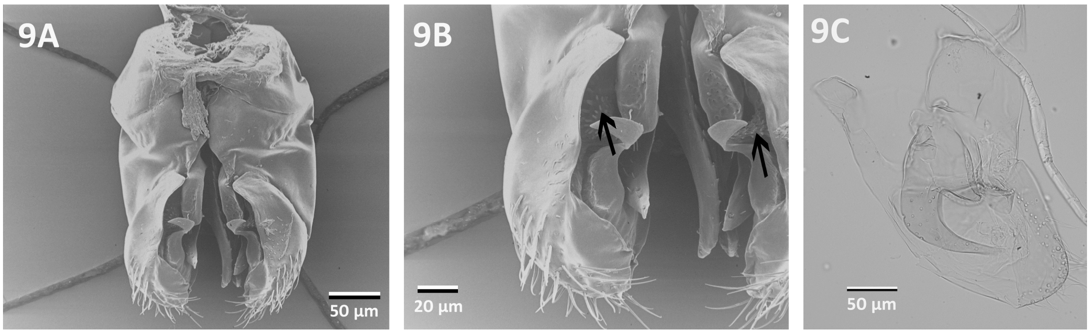

Fig. 9: *C. kagutsuchi*, ergatoid male: A and B: ventral view of male external genitalia (arrows point to microsculpture), C: medial view of volsella and paramere.

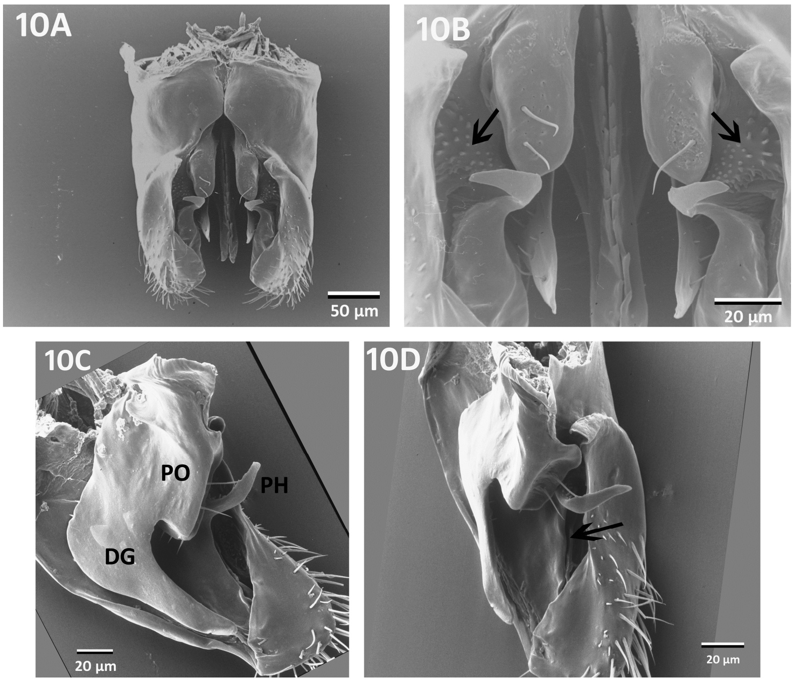

Fig. 10: *C. mauritanica*, ergatoid male: A and B: ventral view of male external genitalia (arrows point to microsculpture), C: medial view of volsella and paramere, D: antero-ventral view of volsella and paramere showing furrow (arrow). DG: digitus, PH: parameral hook, PO: parossiculus.

***C. shuckardi* group**: *C. venustula* Wheeler, 1908 (Fig. 11)

Medial face of paramere laterally adjacent to the parameral hook with microsculpture (Fig. 11B, C). Large excavation between parameral hook and medial wall of paramere present (Fig. 11B). Medial wall of paramere producing a broad furrow running from base of the parameral hook and running towards apex. Volsella without projection. Basi-lateral part of parossiculus protruding latero-ventrally, medial margin slightly bulged, ventral surface concave. Ventral surface of parossiculus covered with numerous large pits (Fig. 11B). Few setae present. Digitus in situ shorter than penisvalve (Fig. 11A).

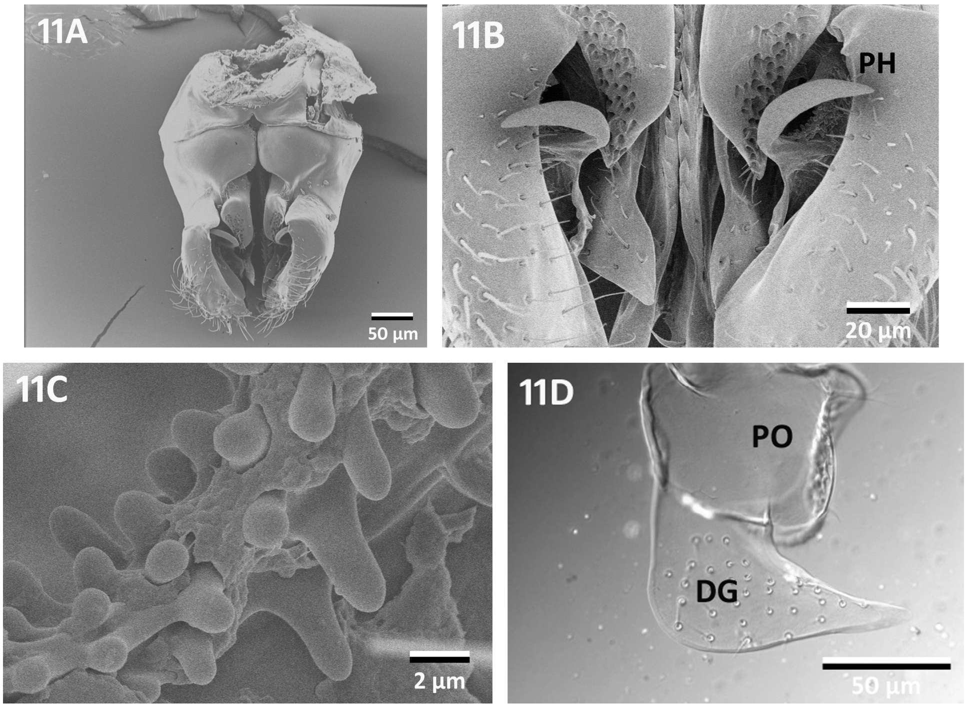

Fig. 11: *C. venustula*, ergatoid male: A, B, and C: ventral views of male external genitalia with detail of microsculpture antero-laterally adjacent to parameral hook (C), D: medial view of volsella. DG: digitus, PH: parameral hook, PO: parossiculus.

***C. batesii* group:** *C. batesii* Forel, 1894 (Fig. 12), *C. nigra* Forel, 1905 (Fig. 13)

Medial face of paramere antero-laterally adjacent to the parameral hook with microsculpture. Large excavation between parameral hook and medial wall of paramere present. Narrow furrow present on the medial face of the paramere running from base of parameral hook towards apex. No projection present on volsella. Basi-lateral margin of parossiculus with a distinct latero-ventral extension, and medial margin slightly bulged, ventral surface concave (arrow in Fig 12C, 13C). Ventral surface of parossiculus with several small pits and a single seta. Digitus in situ shorter than penisvalve.

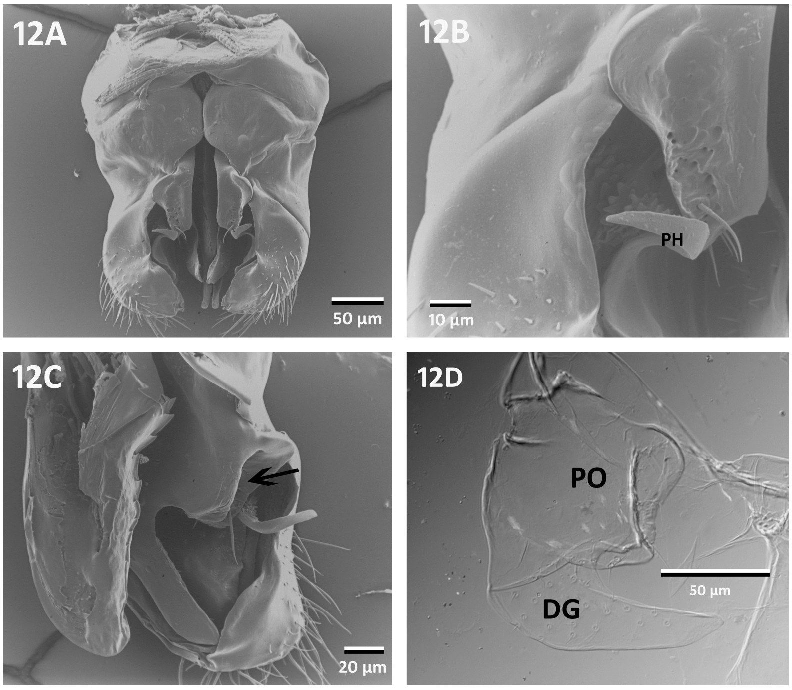

Fig. 12: *C. batesii*, ergatoid male: A: ventral view of male external genitalia, B latero-ventral view of male external genitalia, C: medial view of volsella and paramere (arrow points to concave ventral surface of parossiculus). D: medial view of volsella. DG: digitus, PH: parameral hook, PO: parossiculus.

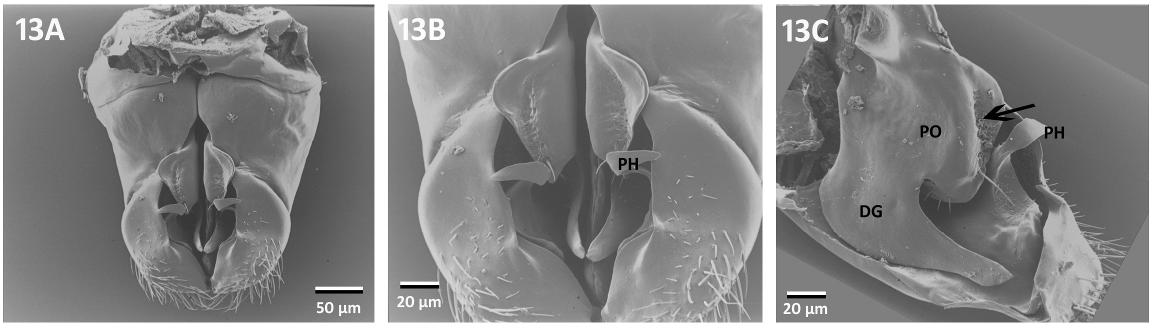

Fig. 13: *C. nigra*, ergatoid male: A, B: ventral view of male external genitalia, C: medial view of volsella and paramere (arrow points to concave ventral surface of parossiculus). DG: digitus, PH: parameral hook, PO: parossiculus.

***C. elegans* group:** *C. elegans* Emery, 1869 (Fig. 14)

Medial face of paramere laterally adjacent to the parameral hook with microsculpture. Large excavation between parameral hook and medial wall of paramere present (arrow in Fig. 14B). Narrow furrow present on the medial face of the paramere, running from base of parameral hook towards apex (arrow in Fig. 14C). Volsella without projection. Basi-lateral margin with a distinct ventrally oriented extension (arrow in Fig. 14A), and medial margin bulged, ventral surface concave. Ventral surface of volsella with covered numerous small pits and a single seta. Digitus in situ shorter than penisvalve.

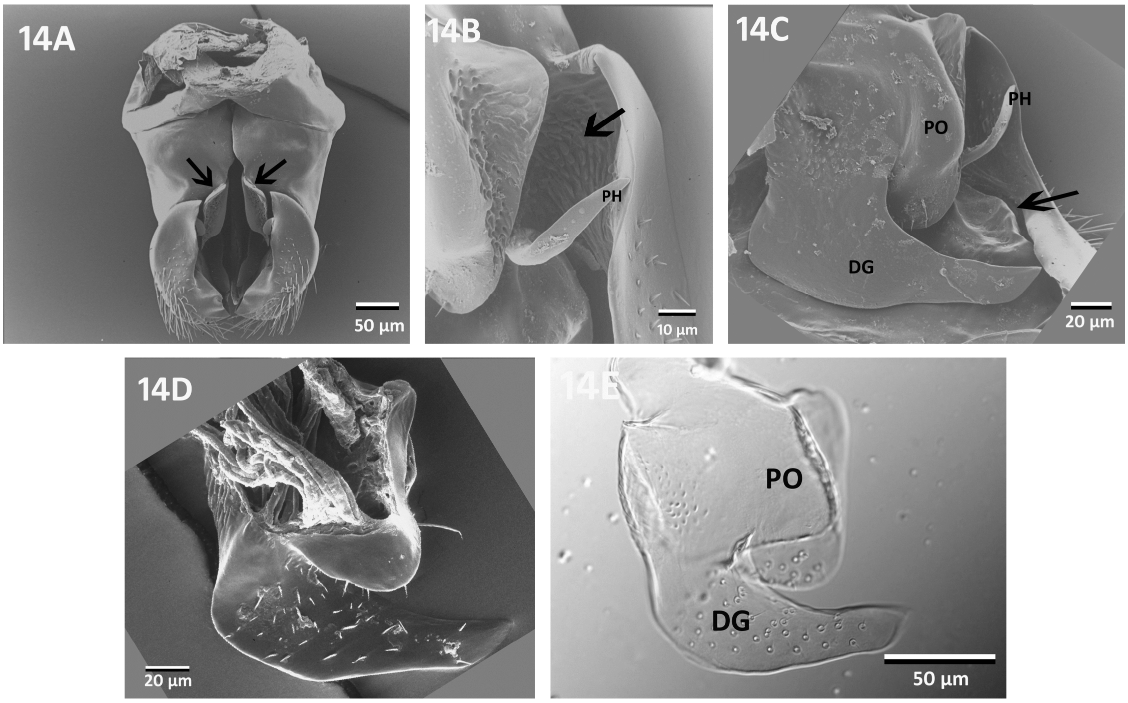

Fig. 14: *C. elegans*, ergatoid male: A: ventral view of male external genitalia (distinct ventrally oriented extension of parossiculus indicated by arrow), B: medial view of volsella, parameral hook and microsculpture on paramere (arrow points to large excavation between parameral hook and medial wall of paramere), C: medial view of volsella and paramere (arrow points to furrow in medial parameral wall), D: lateral view of volsella, E: medial view of volsella. DG: digitus, PH: parameral hook, PO: parossiculus.

Table 3. Character matrix for male external genitalia of *Cardiocondyla*, showing intra-generic variations in six characters, making them useful for distinguishing different clades and species groups.

1: microsculpture adjacent to parameral hook absent (0); present (1)

2: medial face of paramere (0) without depressions of furrows; (1) with deep depressions apically adjacent to parameral hook and near apex; (2) with a furrow running from base of parameral hook towards apex

3: rugae on medial face of paramere and lateral face of digitus (gonossiculus) absent (0); present (1)

4: basivolsellar process absent (0); present (1)

5: ventral surface of parossiculus not concave (0), concave (1)

6: digitus (gonossiculus) in situ shorter or as long as penisvalve (0); longer than penisvalve (1)

Clade	species group	Species/Character	1	2	3	4	5	6
clade A: sickle-shaped mandible type	*C. wheeleri* group	*C. nigrocerea*	0	0	0	1	0	0
	C. “argentea” group	*C. “argyrotricha”*	0	0	0	1	0	0
	C. “argentea” group	*C. “microseta”*	0	0	0	1	0	0
	C. wroughtonii group	*C. obscurior*	0	0	1	0	0	1
	C. wroughtonii group	*C. wroughtonii*	0	0	1	0	0	1
clade B: shear-shaped mandible type	*C. minutior* group	*C. minutior*	1	1	0	0	1	0
	C. minutior group	*C. tjibodana*	1	1	0	0	1	0
	C. nuda group	*C. kagutsuchi*	1	2	0	0	1	0
	C. nuda group	*C. mauritanica*	1	2	0	0	1	0
	C. shuckardi group	*C. venustula*	1	2	0	0	1	0
	C. batesii group	*C. batesii*	1	2	0	0	1	0
	C. *batesii* group	*C. nigra*	1	2	0	0	1	0
	C. *elegans* group	*C. elegans*	1	2	0	0	1	0

Discussion

Male external genitalia of the genus *Cardiocondyla* correspond to the general pattern found in the ant subfamily Myrmicinae as described by Ogata (1991). With regard to the volsella, the identity of the elements that constitute the parossiculus of *Cardiocondyla* is unclear. According to Schulmeister (2003) basivolsella and distivolsella (cuspis) are not separate elements but one contiguous sclerite, and it has been difficult to discriminate between these two parts of the parossiculus in other species (Yoshimura & Fisher 2012, Boudinot 2015). Furthermore, the homology of the lobe developed by the lateral apicoventral part of the volsella in the genus *Pheidole* Westwood, 1839 has been questioned (Eguchi 2003). Birket-Smith (1981) defined the cuspis as “a lobe produced from the apico-lateral corner of the volsella, lateral of the digitus.” In *Cardiocondyla* the lateral part of the volsella extends apicoventrally, but we could not reveal a process lateral to the digitus. We consequently assume that the cuspis is completely reduced in *Cardiocondyla* and that the parossiculus is completely formed by the basivolsella. It is possible, however, that in *Cardiocondyla*, the cuspis is derived and comprises the apical-most part of the parossiculus. Further studies are needed to determine the identity of the elements of the parossicular lobe of *Cardiocondyla*. We propose to use micro-CT analyses to examine the rather small volsellae of the genus.

So far, only few studies have focused on *Cardiocondyla* male external genitalia (summarized in Kugler 1983) and the information on their morphology is incomplete and has led to erroneous conclusions. The parameral hook has previously been described only in the ergatoid male of *Cardiocondyla papuana* (formerly *Prosopidris*, Reiskind 1965) and was not mentioned in other descriptions of male external genitalia of the genus (André 1881, Forel 1892, Minozzi 1918). Kugler (1983) apparently overlooked this morphological character in males of *C. emeryi*, *C. wroughtonii*, *C. stambuloffii*, and *C. elegans*. Based on this and other morphological characters, he supported the separation of the genus *Cardiocondyla* into the two subgenera *Cardiocondyla* and *Prosopidris* (Reiskind 1965). In the present study we show that the parameral hook is shared by all examined species of *Cardiocondyla* belonging to several species groups of the genus, including a partly damaged male of *C. emeryi* (data not shown). Similarly, in their study on *Nesomyrmex wilda* (see below), Francoeur & Loiselle (1988) en passant mention a ventrally protruding telomeral process in *Cardiocondyla* sp. and *C. wroughtonii*. This all supports Bolton (1982) who considered *Prosopidris* as junior synonym of *Cardiocondyla*.

At present we cannot determine if the parameral hook is an autapomorphy of *Cardiocondyla*, as the position of the genus within the Myrmicinae remains unclear and detailed information of males from many genera is still lacking. Based on genomic data, *Cardiocondyla* is nested within the Crematogastrini (sensu Ward *et al.* 2015), however, the clade is not well-resolved at the base and *Cardiocondyla* is a long-branched taxon with an unstable position across different analyses. Unfortunately, we were not able to examine males of potentially closely related taxa (*Ocymyrmex* Emery, 1886, *Cataulacus* Smith, 1853, *Atopomyrmex* André, 1889, *Carebara* Westwood, 1840, *Diplomorium* Mayr, 1901, *Xenomyrmex* Forel, 1885, and *Nesomyrmex* Wheeler, 1910, Ward *et al.* 2015). Bolton & Marsh (1989) examined males of the potential sister-group of *Cardiocondyla, Ocymyrmex* (Ward *et al.* 2015), and described the parameres to be “heavily sclerotized”. No information on a potential parameral hook is given, and the other valves remain undescribed, so we assume that genitalia were only roughly observed and the study does not give reliable information about the presence or absence of the hook. All three valves of male external genitalia of *Cataulacus* (Bolton 1974), several species of the genus *Carebara* (Wheeler 1922, Ettershank 1966, Fernandez 2004), and *Xenomyrmex* (Wheeler 1931) have been described without mention of the hook. No information on male external genitalia is available in the genera *Atopomyrmex* and *Diplomorium*. However, in several species of the genus *Nesomyrmex* (Francoeur & Loiselle 1988) a “dentiform process protruding angularly and ventrally from the inner wall of the valve” (valve = paramere) has been described and illustrated. We examined the genital apparatus of other Crematogastrini (*Formicoxenus nitidulus* Nylander, 1846, *Leptothorax gredleri* Mayr, 1855, *L. muscorum* Nylander, 1846, *Temnothorax unifasciatus* Latreille, 1798) and did not find a parameral hook in ventral view. However, detailed observations based on dissected genitalia are necessary to fully confirm the absence of a parameral process in these species. Absence of the hook in other potentially close relatives (see also Francoeur *et al.* 1985, Francoeur & Loiselle 1988) indicates that the parameral hook of *Cardiocondyla* and *Nesomyrmex* has either evolved convergently or that the parameral hook found in the two genera is homologous and the two taxa thus are sister-groups. A careful study of males from additional myrmicine genera might reveal whether the parameral hook constitutes a useful character to resolve the taxonomic position of *Cardiocondyla*.

An exclusively qualitative examination does not allow the identification of *Cardiocondyla* species, as males of closely related species have relatively similar external genitalia, as has also been reported for other genera (e.g. for *Crematogaster* Lund, 1831, Buren 1958). However, within the genus *Cardiocondyla*, several characters allow assigning species to their respective species group and corroborate previous genetic studies (Heinze *et al.* 2005, Oettler *et al.* 2010) (see table 3 above). Three characters (1, 2, and 5 in table 3) of male external genitalia are suitable to differentiate between the two major clades A and B of the genus (Heinze *et al.* 2005, Oettler *et al.* 2010). Within clade A, the basivolsellar projection is shared by males of the three examined species of the *C. wheeleri* and *C. "argentea"* groups and distinguishes this lineage from the *C. wroughtonii g*roup and also the other species of *Cardiocondyla*. The basivolsellar projection has also been described in *C. papuana* (Reiskind 1965), which together with the sickle-shaped mandibles of ergatoid males suggests that it is closely related to the former species groups.

In contrast to clade A, it is difficult to characterize discrete character states for the examined species groups of clade B. However, we reveal two trends concerning the development of morphological characteristics of the paramere and the volsella from earlier branching species groups (i.e. *C. minutior* group) towards more recently diverging groups (*C. batesii* group, *C. elegans* group). We found (1) an increasing sculpturation of the medial wall of the paramere, leading to the formation of deep depressions (*C. minutior* group), furrows (*C. nuda* group, *C. shuckardi* group, *C. batesii* group, *C. elegans* group) and large excavations (*C. nuda* group, *C. shuckardi* group, *C. batesii* group, *C. elegans* group), and (2) a more pronounced relief of the ventral surface of the parossiculus due to bulged margins (*C. minutior* group, *C. nuda* group) or protrusions (*C. shuckardi* group, *C. batesii* group, *C. elegans* group) accompanied by a more pronounced concave surface.

Although the function of the three paired valves of the external male genitalia during copulation has been studied (summarized in Boudinot 2013), to date nothing is known about the function of peculiar structures that deviate from the general pattern of hymenopteran genitalia. Projections of the parameres (e.g. Forbes 1967, *Myrmecia tarsata* Smith, 1858, *M. vindex* Smith, 1858: parameres with "dorsal, median projection"; Francoeur and Loiselle 1988, *Nesomyrmex wilda* Smith, 1943: paramere with "dentiform process protruding angularly and ventrally from the inner wall"; Yoshimura & Fisher 2011, *Aptinoma mangabe* Fisher, 2009, *Tapinoma* sp.: "basimere with apicoventral process"; Boudinot *et al.* 2013, *Megalomyrmex modestus* Emery, 1896: telomere with "dorso-median dentiform process"; present study: all examined species of *Cardiocondyla*;" a ventrolaterally oriented, curved hook originating at the medial wall of the telomere") and the parossiculus (Yoshimura & Fisher 2012, *Adetomyrma* sp., *Stigmatomma* sp., *Mystrium* sp., *Prionopelta descarpentriesi* Santschi, 1924, *Xymmer* sp.: "process on the basal portion of the cuspis on the volsella";

present study, *Cardiocondyla nigrocerea*, *C. "argyrotricha"*, *C. "microseta"*: "volsella with short, slender projection directed ventrally") have been revealed in several ant genera. The function of the potentially homologous parameral process found in *Cardiocondyla* and *Nesomyrmex* is so far unknown. We assume that it serves as a means to ensure close contact between the male and the female during mating, probably enhanced by the adjacent microsculpture present in several species. Francoeur & Loiselle (1988) speculated the hook to be associated with a telomeral gland in *Nesomyrmex wilda*. We could not find such a gland in *Cardiocondyla*; however, deep depressions or the furrow present in several of the examined species suggests that some kind of fluid is involved to enhance the firm connection between mating partners during copulation by wet adhesion. In the future, studies are needed to resolve the function of the parameral hook, the adjacent microsculpture and evaluate presence or absence of a potential fluid.

Interestingly, we observed a more pronounced sculpturation of the volsella and the paramere in species of clade B, in which wingless males do not obligatorily engage in lethal fighting but are territorial (*C. venustula*) or mutually tolerant (*C. elegans*, *C. batesii*; reviewed in Heinze 2017). At least in the latter two species, female sexuals are known to mate multiply (Schrempf *et al.* 2005, Lenoir *et al.* 2007), while wingless males typically do not have any rivals in *C. "argentea"* or *C. nigrocerea*, and genetic data suggest single mating for *C. obscurior* (Schmidt *et al.* 2016). More data on the function of the described structures are needed to clarify whether the observed variation is indeed a response to differences in queen mating frequency and male competition.

Although the length of the penisvalvae was different among winged and ergatoid males in diphenic species. This differs from the large size differences in male-diphenic *Technomyrmex albipes* Smith, 1861. However, in this species different male phenotypes mate assortatively with different female phenotypes (Ogata 1996), while in *Cardiocondyla* queens mate similarly with winged and ergatoid males (e.g. Kinomura & Yamauchi 1987, Cremer *et al.* 2002) We assume that the difference in variation in penisvalve length between ergatoid and winged male phenotypes is due to the different development time, which is faster in ergatoid males (Schrempf & Heinze 2006) and probably involves developmental instability.

Previous studies on sexual selection in *Cardiocondyla* focused on fighting and mating behavior. Our study adds data on the morphology of the male genital apparatus, which in the future might prove helpful both for infrageneric phylogeny and lead to a better understanding of the dynamics of sperm transfer and, if present, sperm competition. Furthermore, the parameral hook might be useful for resolving the phylogenetic relationships among the tribe Crematogastrini.

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Chapter 2

Mandible morphology reflects the type of male antagonism in the ant genus *Cardiocondyla*

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Key words: *Cardiocondyla*, ergatoid males, morphology, mandibles

Abstract

The ant genus *Cardiocondyla* is characterized by wingless, ergatoid males, which in some species have fully replaced the typical winged ant male. Depending on species, ergatoid males engage in lethal fighting for access to female sexuals, establish territories within their nests, or are mutually tolerant. Here we investigate, whether the morphology of ergatoid males and in particular the shape of their mandibles reflect phylogeny or male behaviour. In contrast to the worker mandible, which is relatively consistent in shape in the ten examined species, mandibles of ergatoid males show a great size and shape variation not only between the two clades of *Cardiocondyla* but also among species belonging to *Cardiocondyla* clade A. This diversity appears to reflect the diverse reproductive tactics of ergatoid males of different species, with extremely long, distally oriented mandibles associated with indirect killing of adult competitors, while particularly short mandibles possibly constitute an adaptation to a mating tactic with ergatoid males exclusively crushing the cuticle of young rivals.

Introduction

The genus *Cardiocondyla* is unusual among ants, as it exhibits two different male phenotypes, winged males and ergatoid males. Winged males are the typical males of most ant species but have been found only in less than 25% of the examined species of *Cardiocondyla* (Heinze, 2017). With fully developed wings, large eyes, ocelli, and a bulky thorax they are well adapted to dispersal, searching for female sexuals, and mating away from their natal nests. Ergatoid males are known from all examined species of *Cardiocondyla*. They stay inside the maternal nest and locally mate with the available female sexuals (Heinze, 2017). Their entirely non-dispersing life cycle is reflected in a superficially "worker-like" morphology: ergatoid males are wingless, have small eyes, and lack ocelli. They may be relatively long-lived (up to one year, Yamauchi *et al.* 2006), and, in contrast to all other males of social Hymenoptera, are characterized by life-long spermatogenesis (Heinze and Hölldobler 1993).

Winged *Cardiocondyla* males uniformly are docile and do not engage actively in fighting for access to female sexuals. In contrast, competition among ergatoid males ranges from complete mutual tolerance (Schrempf *et al.* 2005, Schrempf and Heinze 2007) and the defense of small territories in the nest (Frohschammer and Heinze 2009, Jacobs and Heinze 2016) to regular killing of immature rivals and fatal fighting among adults (e.g. Kinomura and Yamauchi 1987, Yamauchi and Kawase 1992). Different fighting techniques are clearly associated with different mandible morphology. In one branch of the phylogeny (clade A, Oettler *et al.* 2010), ergatoid males use their "sickle-shaped" mandibles to puncture the soft cuticula of young males ("callows"). In addition, they grasp older, sclerotized rivals and daub them with hindgut secretion, which elicits aggression by workers towards the besmeared individual (Kinomura and Yamauchi 1987, Stuart *et al.* 1987, Yamauchi and Kawase 1992). In the other branch, clade B, ergatoid males have "shear-shaped" mandibles superficially similar to those of workers. They eliminate unsclerotized callows by crushing or dismembering them (Heinze *et al.* 1993, 1998, Frohschammer and Heinze 2009) but are rarely capable of injuring competitors with their mandibles once the cuticula of the latter has hardened. Nests of species in clade B may therefore contain multiple adult males, which typically do not show agonistic behaviour (Heinze *et al.* 1993, 1998). In one particular lineage of clade B male fighting apparently has been completely lost (e.g., Schrempf *et al.* 2005; Lenoir *et al.* 2007), with male territoriality probably being an intermediate stage between fighting and tolerance (Frohschammer and Heinze 2009, Jacobs and Heinze 2016).

Here we compare males and workers of several species of *Cardiocondyla* and investigate whether it is possible to extrapolate male morphology from that of workers, which are known for many more species (Seifert 2003). More importantly, mandibles of most *Cardiocondyla* males so far have been described only superficially (e.g. Kugler 1983, Terayama 1999). We therefore describe the morphology of mandibles of ergatoid *Cardiocondyla* males with different reproductive life histories. By doing so, we aim to reveal possible correlations between male morphology and reproductive tactics.

Material and Methods

Examined species

For morphometric examination, specimens of eight different species (Table 1) belonging to different species groups (Seifert 2003) were examined. Morphometric measurements were carried out in both ergatoid males and workers, except in *C. elegans*, where only ergatoid males were available (see Table 1). In addition, mandible morphology was qualitatively assessed by the use of a WildM10 stereomicroscope and/or by scanning electron microscopy in the above mentioned species and two additional species (*C. wroughtonii*, *C. tjibodana*). Mandibles of winged males were qualitatively examined in four species (*C. obscurior*, *C. wroughtonii*, *C. minutior*, *C. tjibodana*).

Sample preparation and assessment of morphological characters

Ants were collected either in the field or from laboratory colonies and kept in small plastic flacons with 70% ethanol. Whole specimens were dehydrated in an ascending alcohol series (80%, 90%, 99.6% ethanol). Seven morphological characters were measured on mounted, dehydrated specimens using a WildM10 stereomicroscope (Leica Microsystems, Germany) with the aid of an ocular micrometer. Definition of numeric characters (mostly according to Seifert, 2003): CL: maximum cephalic length in median line, excavations of occiput and/or clypeus reduce CL; CW: cephalic width; across and including the eyes; ML: mesosoma length; measured in lateral view from the caudalmost portion of propodeum to the frontal-most point of the anterior pronotal slope; MW: Maximum mesosoma width; PEW: maximum width of petiole; PPW: maximum width of postpetiole. PEW and PPW could not be measured in ergatoid males of *C. venustula* because of their dense pubescence, which did not allow accurately identifying the actual borders of the body. Cephalic size CS was used as an indicator of body size of individuals (see Seifert 2003) and calculated by (CL+CW)/2. After measurement of the above mentioned characters, mandibles were removed and mounted in a ventral position to measure mandible length MDL: mandible length, straight-line distance between the ventral articulation point with the head capsule and the tip of the apical tooth (shear-shaped mandibles of ergatoid males and worker mandibles) and the tip of the mandible (sickle-shaped mandibles of ergatoid males). We assume that the landmarks chosen are homologous in the two mandible types. A mean measurement error of ± 1.4 µm was calculated based on three measurements for small structures (e.g. PEW, PPW) and of ± 2.5 µm for larger structures (e.g. ML). To allow for comparison within and between species, characters were divided by CS. In order to describe mandibles qualitatively, complete head capsules and detached mandibles of additional worker and male specimens were removed and mounted in different positions (dorsal, ventral, mesial, and lateral) on metal stubs with double-sided adhesive carbon pads and air-dried for at least 48 h. After this procedure, samples were coated with a mixture of gold-palladium with the use of a sputter-coater (*Fisons Instruments, UK*) before being examined in a DSM 950 Zeiss Scanning Electron Microscope. The terminology to describe mandibles largely follows Ettershank (1966) and Gotwald (1969), except for mandibles of ergatoid and winged males belonging to *Cardiocondyla* clade A (*C. obscurior, C. wroughtonii, C. nigrocerea,* and *C. "argyrotricha"*). Here, we use 'dorsal margin' because of the derived morphology of these mandibles, which makes it difficult to homologize this margin with the 'basal margin' of typical triangular mandibles. Furthermore, we avoid the term 'external margin' in the description of *Cardiocondyla* mandibles, as it is usually used in in-situ descriptions, and corresponds to the lateral margins seen in full-face view of ants. In our description of detached mandibles we use the term 'ventral margin' to describe the margin running ventrally from the ventral-most point of articulation with the head capsule to the frontal-most point of the mandible.

We used the software IBM SPSS Statistics 23 (IBM Corporation) and *the software R* 3.2.1 (*R* Development Core Team, 2015) to perform statistical analyses, including a Principal Component Analysis (PCA) to examine the variation in the morphometric data of six characters within and among species. We excluded mandible length from PCA. This character clearly distinguishes ergatoid males of clades A and B (Oettler *et al.* 2010) and might therefore have obscured other morphological trends. To take body size into account, we carried out a PCA of these six morphological characters divided by CS. PCA results were plotted in two dimensional PCA scatter plots. We used the first two principal components (PCs), which accounted for over 86% of the total variation.

Table 1: Examined species of different species groups, presence of male phenotypes and reproductive tactic.

species group	species	collection site	male phenotype	reproductive tactic
clade A: sickle-shaped mandibles				
C. wheeleri group	*C. nigrocerea**	Pulau Obi, Maluku Utara, Indonesien	ergatoid	besmearing callows and rarely adult males
C. "argentea" group[1]	*C. "argyrotricha"* *	Los Baños, Philippines	ergatoid	besmearing callows[2]
C. wroughtonii group	*C. wroughtonii*	Limay, Bataan, Philippines	ergatoid, winged	piercing callows and besmearing adults
	*C. obscurior**	Ilhéus, Bahia, Brazil	ergatoid, winged	piercing callows and besmearing adults
clade B: shear-shaped mandibles				
C. minutior group	*C. minutior**	Okinawa, Japan	ergatoid, winged	crushing callow cuticle
	C. tjibodana	Gua Londron, Indonesia	ergatoid, winged	crushing callow cuticle
C. nuda group	*C. kagutsuchi**	Okinawa, Japan	ergatoid	crushing callow cuticle
C. shuckardi group	*C. venustula* **	Kaua'i, Hawaii, USA	ergatoid	crushing callow cuticle, adult fighting and besmearing
C. batesii group	*C. batesii**	Padul, Spain	ergatoid	tolerant
C. elegans group	*C. elegans**	Chemilly, Allier, France	ergatoid	tolerant

* measurement of seven morphological characters

**measurement of five morphological characters

[1] *C. "argentea"* and *C. "argyrotricha"* are the preliminary names for taxa currently being described by B. Seifert

[2] authors' assumption based on occasional observations of lab-reared colonies

Results

Morphometric data

Principal component analysis using morphometric data of six morphological characters of six to 12 workers and ergatoid males of each of six species (*C. obscurior*, *C. nigrocerea*, *C. “argyrotricha”*, *C. minutior*, *C. kagutsuchi*, *C. batesii*) produced two axes, which together explained 91.42% of the total variance (Fig 1). The first axis accounted for 75.83% with nearly equal contribution of the measured characters (CW: 19.42%, CL: 13.54%, MW: 18.24%, PEW: 14.14%, PPW: 16.26%, ML: 18.40%). The second axis explained 15.59% and showed a relationship to CL, PEW and PPW (CL: 36.79%, PEW: 33.48%, PPW: 14.01%). Ergatoid males were mainly separated from conspecific workers by PC 2.

The diverse directions of the vectors between the centroids of relative worker and male measurements shows that from the limited number of species it is not possible to make more precise predictions about male morphology.

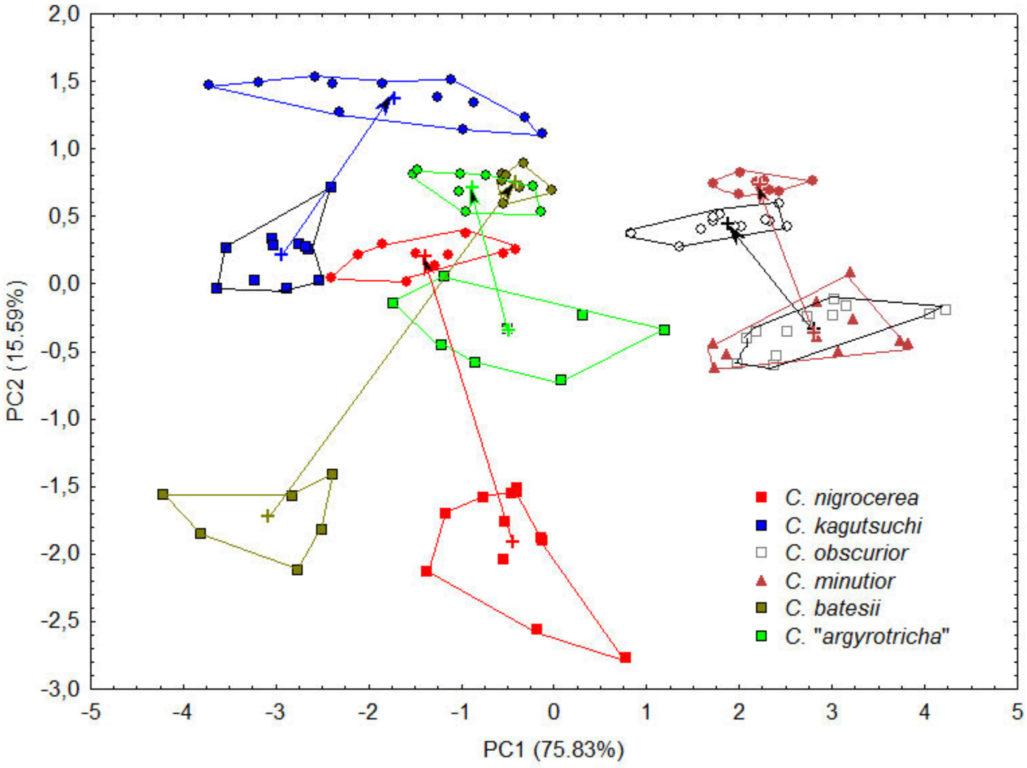


Fig. 1: Analysis of Principal Components of the morphometric data of ergatoid males (squares, triangle) and workers (circles) of six species of *Cardiocondyla*.

Several trends are present when comparing the data of seven characters (including mandible length) between workers and males in seven species of *Cardiocondyla*. CL/CW is generally smaller in ergatoid males (Fig. 2A), while PEW/CS (Fig. 2B), PPW/CS (Fig. 2C) and MW/CS (Fig. 2D) are larger in ergatoid males.

Concerning the morphology of mandibles, MDL/CS is generally larger in ergatoid males with sickle-shaped mandibles (clade A; > 0.5) than in workers (< 0.5). Contrary, in clade B MDL/CS is smaller in shear-shaped mandibles of ergatoid males (< 0.5) than in workers (> 0.5) (Fig. 2E), except for *C. venustula* (MDL/CS > 0.5 in both workers and ergatoid males, see Table 4). A similar pattern is seen when comparing total mandible length (Table 2-4) between ergatoid males and workers among the two clades. The length of the mandible is significantly positively correlated with body size (CS) both in workers and ergatoid males of the examined *Cardiocondyla* species except for the ergatoid males of *C. kagutsuchi* (Table 2-4).

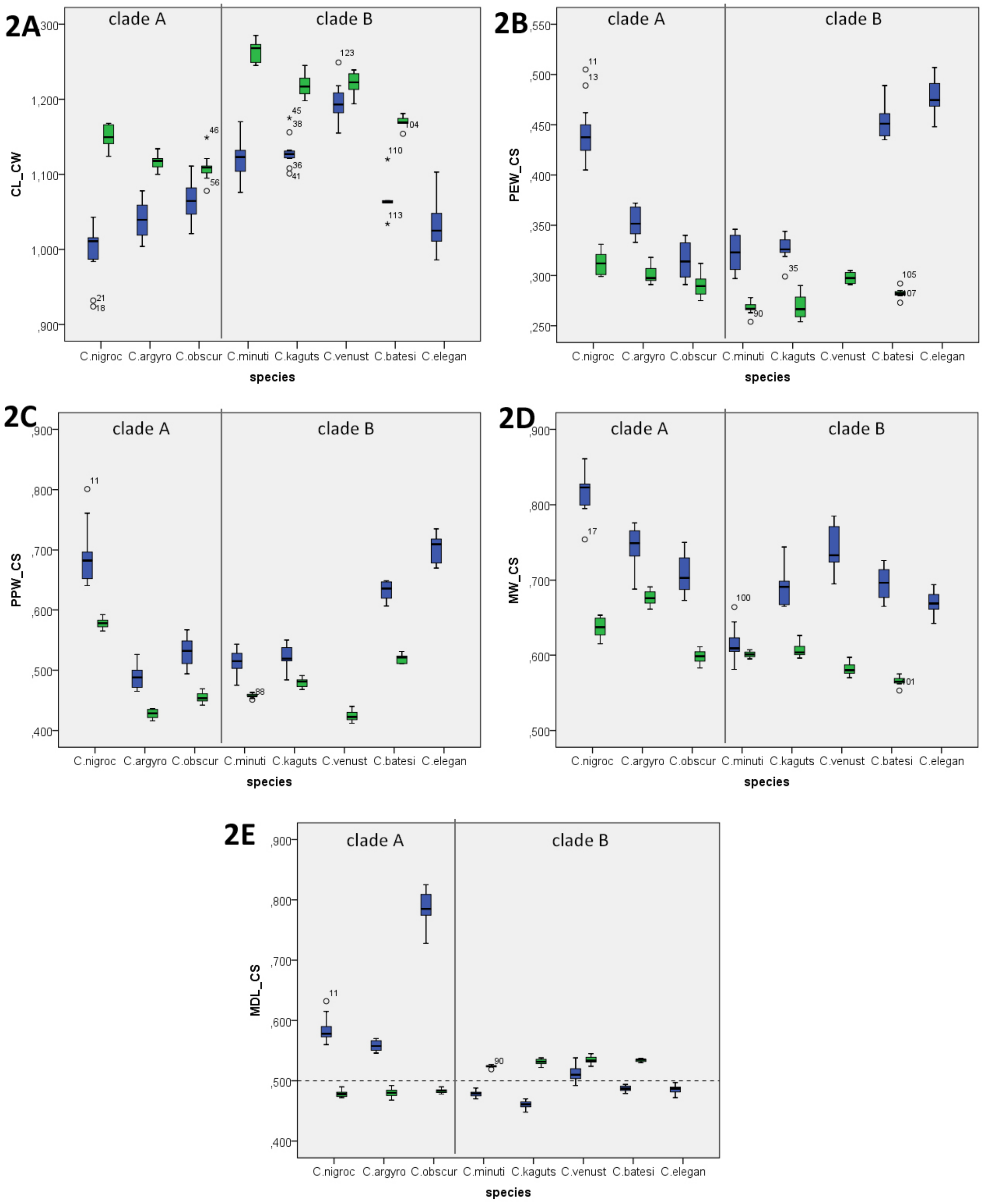


Fig. 2: Morphometric data of several characters of ergatoid males (blue plots) and workers (green plots) of eight species of *Cardiocondyla*. Medians, quartiles and ranges are given. A: CL/CW, B: PEW/CS, C: PPW/CS, D: MW/CS, E: MDL/CS. Characters of males and workers of all examined species are significantly different (two-sample t-test/ Welch's t-test, $p < 0.05$), except for MW/CS of *C. minutior*, $t = -1.792$, $p = 0.104$).

Table 2: Measurement data of workers and males of species of the *Cardiocondyla wroughtonii*, *C. "argyrotricha"*, and *C. nigrocerea* species-groups; arithmetic mean ± standard deviation [range].

species	***C. nigrocerea***	***C. nigrocerea***	***C. "argyrotricha"***	***C. "argyrotricha"***	***C. obscurior***	***C. obscurior***
caste	worker [N=10]	ergatoid male [N=12]	worker [N=8]	ergatoid male [N=8]	worker [N=12]	ergatoid male [N=12]
CS	500 ± 14 [475, 519]	417 ± 19 [369, 443]	515 ± 13 [495, 535]	472 ± 23 [441, 506]	441 ± 10 [427, 465]	379 ± 13 [351, 395]
CL/CW	1.150 ± 0.014 [1.124, 1.168]	0.996 ± 0.036 [0.924, 1.043]	1.117 ± 0.010 [1.100, 1.134]	1.040 ± 0.025 [1.004, 1.078]	1.108 ± 0.017 [1.078, 1.149]	1.064 ± 0.024 [1.021, 1.111]
MW/CS	0.637 ± 0.014 [0.615, 0.653]	0.816 ± 0.026 [0.754, 0.861]	0.677 ± 0.010 [0.661, 0.691]	0.744 ± 0.029 [0.688, 0.776]	0,599 ± 0.009 [0.590, 0.611]	0.708 ± 0.025 [0.673, 0.750]
ML/CS	1.325 ± 0.018 [1.292, 1.343]	1.413 ± 0.035 [1.356, 1.500]	1.172 ± 0.032 [1.119, 1.203]	1.237 ± 0.034 [1.165, 1.270]	1.201 ± 0.016 [1.177, 1.225]	1.384 ± 0.033 [1.318, 1.436]
PEW/CS	0.313 ± 0.011 [0.299, 0.331]	0.443 ± 0.029 [0.405, 0.505]	0.301 ± 0.009 [0.291, 0.318]	0.353 ± 0.015 [0.333, 0.372]	0.290 ± 0.011 [0.275, 0.312]	0.316 ± 0.017 [0.291, 0.340]
PPW/CS	0.578 ± 0.008 [0.565, 0.592]	0.690 ± 0.048 [0.641, 0.801]	0.428 ± 0.008 [0.416, 0.436]	0.489 ± 0.020 [0.465, 0.526]	0.455 ± 0.008 [0.442, 0.469]	0.531 ± 0.023 [0.494, 0.567]
MDL/CS	0.478 ± 0.005 [0.472, 0.490]	0.585 ± 0.021 [0.560, 0.632]	0.480 ± 0.007 [0.468, 0.492]	0.558 ± 0.009 [0.546, 0.570]	0.483 ± 0.003 [0.478, 0.490]	0.789 ± 0.027 [0.728, 0.825]
MDL [µm]	239 ± 7 [227, 249]	243 ± 5 [233, 251]	247 ± 8 [235, 263]	263 ± 11 [246, 276]	213 ± 5 [204, 223]	299 ± 19 [255, 320]
Pearson's correlation (MDL – CS)	0.924**	0.728**	0.905**	0.944**	0.952**	0.932**

** 0.01 significance level

Table 3: Measurement data of workers and males of species of the *C. minutior* and *C. nuda* species-groups; arithmetic mean ± standard deviation [range].

species	***C. minutior***	***C. minutior***	***C. kagutsuchi***	***C. kagutsuchi***
caste	worker [N=9]	ergatoid male [N=10]	worker [N=12]	ergatoid male [N=11]
CS	435 ± 6 [422, 448]	406 ± 16 [379, 425]	540 ± 29 [495, 586]	550 ± 7 [539, 564]
CL/CW	1.263 ± 0.014 [1.245, 1.285]	1.119 ± 0.026 [1.076, 1.170]	1.219 ± 0.014 [1.198, 1.245]	1,130 ± 0.021 [1.101, 1.175]
MW/CS	0.601 ± 0.004 [0.595, 0.607]	0.615 ± 0.024 [0.581, 0.664]	0.607 ± 0.010 [0.596, 0.626]	0.688 ± 0.024 [0.665, 0.744]
ML/CS	1.229 ± 0.006 [1.218, 1.238]	1.132 ± 0.029 [1.100, 1.198]	1.281 ± 0.014 [1.250, 1.319]	1.206 ± 0.022 [1.178, 1.248]
PEW/CS	0.268 ± 0.007 [0.254, 0.278]	0.324 ± 0.017 [0.297, 0.346]	0.269 ± 0.012 [0.254, 0.290]	0.328 ± 0.013 [0.299, 0.344]
PPW/CS	0.458 ± 0.004 [0.451, 0.463]	0.515 ± 0.020 [0.475, 0.543]	0.480 ± 0.007 [0.468, 0.486]	0.522 ± 0.019 [0.484, 0.550]
MDL/CS	0.524 ± 0.002 [0.519, 0.527]	0.478 ± 0.005 [0.470, 0.488]	0.531 ± 0.005 [0.522, 0.538]	0.460 ± 0.006 [0.448, 0.470]
MDL	228 ± 5 [219, 236]	194 ± 7 [182, 204]	287 ± 17 [259, 311]	253 ± 4 [247, 259]
Pearson's correlation (MDL – CS)	0.994**	0.959	0.990 **	0.525

** 0.01 significance level

Table 4: Measurement data of workers and males of species of the *Cardiocondyla shuckardi*, *C. batesii* and *C. elegans* species-groups; arithmetic mean ± standard deviation [range].

species	***C. venustula***	***C. venustula***	***C. batesii***	***C. batesii***	***C. elegans***
caste	worker [N=10]	ergatoid male [N=11]	worker [N=7]	ergatoid male [N=6]	ergatoid male [N=12]
CS	560 ± 14 [545, 582]	641 ± 26 [598, 671]	505 ± 4 [496, 509]	512 ± 19 [495, 545]	548 ± 18 [521, 577]
CL/CW	1.221 ± 0.015 [1.194, 1.239]	1.197 ± 0.025 [1.155, 1.249]	1.170 ± 0.008 [1.154, 1.181]	1.068 ± 0.028 [1.034, 1.120]	1.031 ± 0.031 [0.986, 1.103]
MW/CS	0.581 ± 0.008 [0.570, 0.597]	0.742 ± 0.030 [0.695, 0.785]	0.565 ± 0.007 [0.553, 0.575]	0.696 ± 0.023 [0.665, 0.726]	0.670 ± 0.016 [0.642, 0.694]
ML/CS	1.256 ± 0.021 [1.220, 1.284]	1.273 ± 0.068 [1.180, 1.380]	1.212 ± 0.011 [1.198, 1.229]	1.233 ± 0.024 [1.202, 1.267]	1.188 ± 0.021 [1.160, 1.232]
PEW/CS	0.298 ± 0.005 [0.291, 0.305]	n.a.	0.282 ± 0.006 [0.273, 0.292]	0.454 ± 0.020 [0.435, 0.489]	0.478 ± 0.016 [0.448, 0.507]
PPW/CS	0.424 ± 0.008 [0.412, 0.440]	n.a.	0.519 ± 0.008 [0.511, 0.531]	0.633 ± 0.017 [0.607, 0.649]	0.703 ± 0.023 [0.670, 0.735]
MDL/CS	0.534 ± 0.007 [0.524, 0.545]	0.512 ± 0.014 [0.492, 0.538]	0.534 ± 0.003 [0.530, 0.537]	0.487 ± 0.005 [0.479, 0.494]	0.486 ± 0.007 [0.472, 0.497]
MDL	299 ± 9 [290, 316]	328 ± 19 [296, 347]	270 ± 3 [264, 272]	249 ± 10 [237, 265]	266 ± 7 [255, 279]
Pearson's correlation (MDL – CS)	0.901**	0.892**	0.890**	0.962**	0.890**

** 0.01 significance level

n.a.: morphometric data not available

Morphology of mandibles of ten species of *Cardiocondyla*

Trulleum and mandalus present in workers, ergatoid males, and winged males of all examined species. Mandibles distally curved medially, generally opposable.

Worker mandibles

Worker mandibles show only very little morphological variation among the different species of *Cardiocondyla*. Worker mandibles possess a clear basal angle and thus well-defined basal and masticatory margins (Fig. 3 A). Masticatory margin bears five teeth, the apical two teeth are enlarged, the apical tooth is the largest one. In *C. nigrocerea* and *C.* "*argyrotricha*" masticatory teeth decrease in size from apical to basal (Fig. 3 D), while the prebasal tooth is the smallest one in workers of *C. obscurior*, *C. wroughtonii* (Fig. 3 E), *C. minutior*, *C. tjibodana* (Fig. 3 A, B), *C. kagutsuchi*, *C. venustula*, *C. batesii*, and *C. elegans*. Mandibles widening distally, with basal margin roughly parallel anterior clypeal margin in full-face view, ventral margin bent ventrad (Fig. 3 A, D). Canthellus distinct, almost reaching basal margin, trulleum open, heart-shaped in dorso-lateral view, in dorsal view disto-laterally distinctly bordered by basal margin, which is produced as a sharp blade widening towards the basal tooth (Fig. 3 E). Canthellus, trulleum und mandalus of similar shape among examined species. Mesial face distally with a short longitudinal ridge running towards the apical or preapical tooth (Fig. 3 G). Another ridge running beneath teeth along the masticatory margin, ranging from basal border of preapical tooth to apical border of basal tooth, producing a ledge with third and prebasal tooth out of line compared to remaining masticatory teeth (Fig 3 F, G). Long setae arranged below this ridge (Fig. 3 F) and very short setae present along the distal part of the basal margin. Mesial face distal to canthellus otherwise smooth (no microstructure or setae present).

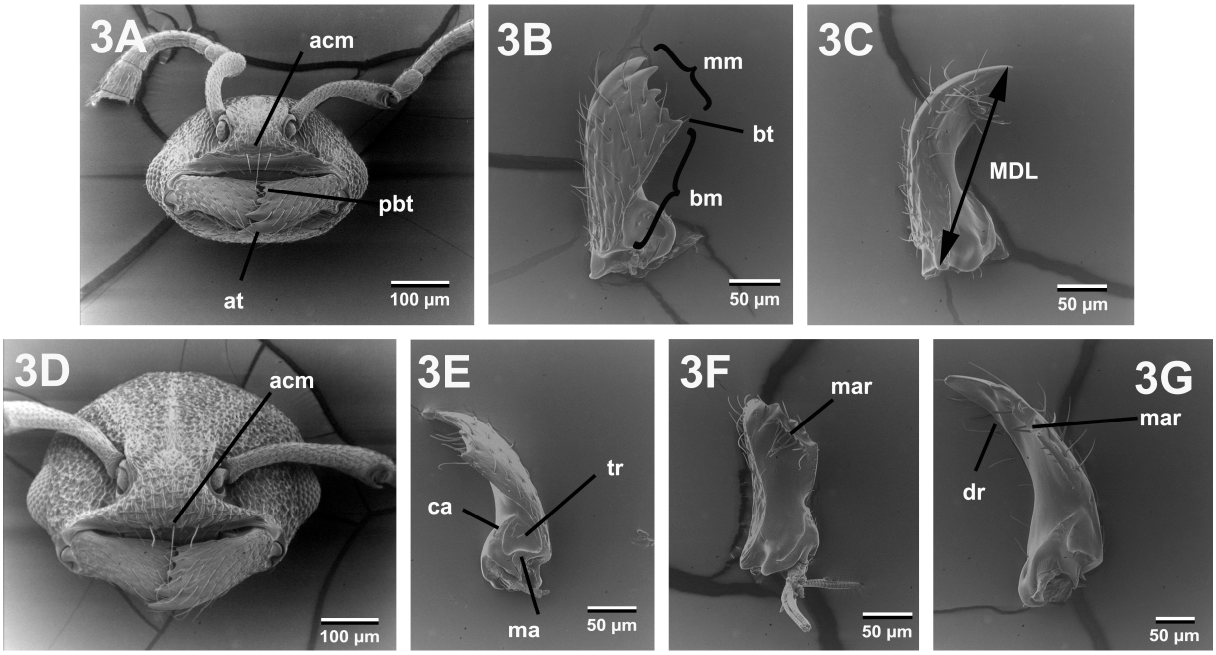


Fig. 3: worker mandibles; A: *C. tjibodana*, frontal view of head capsule, B: *C. tjibodana*, dorso-lateral view of left mandible, C: *C. tjibodana*, ventral view of right mandible (MDL: mandible length), D: *C. “argyrotricha”*, frontal view of head capsule, E: *C. wroughtonii*, dorsal view of right mandible, F: *C. wroughtonii*, mesial view of right mandible, G: *C. elegans*, dorso-mesial view of right mandible. acm: anterior clypeal margin, at: apical tooth, ba: basal angle, bm: basal margin, ca: canthellus, dr: distal ridge, ma: mandalus, mm: masticatory margin, mar: masticatory ridge, tr: trulleum, pbt: prebasal tooth. In 1A & D top-bottom corresponds to dorsal-ventral, in 1B, C, E & F top-bottom corresponds to distal-proximal.

Male mandibles

***Cardiocondyla* clade A, ergatoid males**:

Mandibles without distinct basal and masticatory margin, toothless, generally long, narrow in dorso-ventral dimension. Mandibles narrowing distally (in contrast to worker mandible), curved inwards. Mesial face longitudinally bulged and setose.

***C. wheeleri* group:** *C. nigrocerea* (Fig. 4), ergatoid male

Dorsal margin proximally bent ventrally with a distinct obtuse angle (arrow in Fig. 4 A, C, E), mandibles in full-face view oriented disto-ventrally. Mandibles narrow, tapering distal to the obtuse angle towards apex. Trulleum distinctly bordered latero-distally by dorsal margin, trulleum not fully visible in lateral view (Fig. 4 E). Mesial face with several setae, and with a longitudinal bulge directed towards the apex (Fig. 4 D).

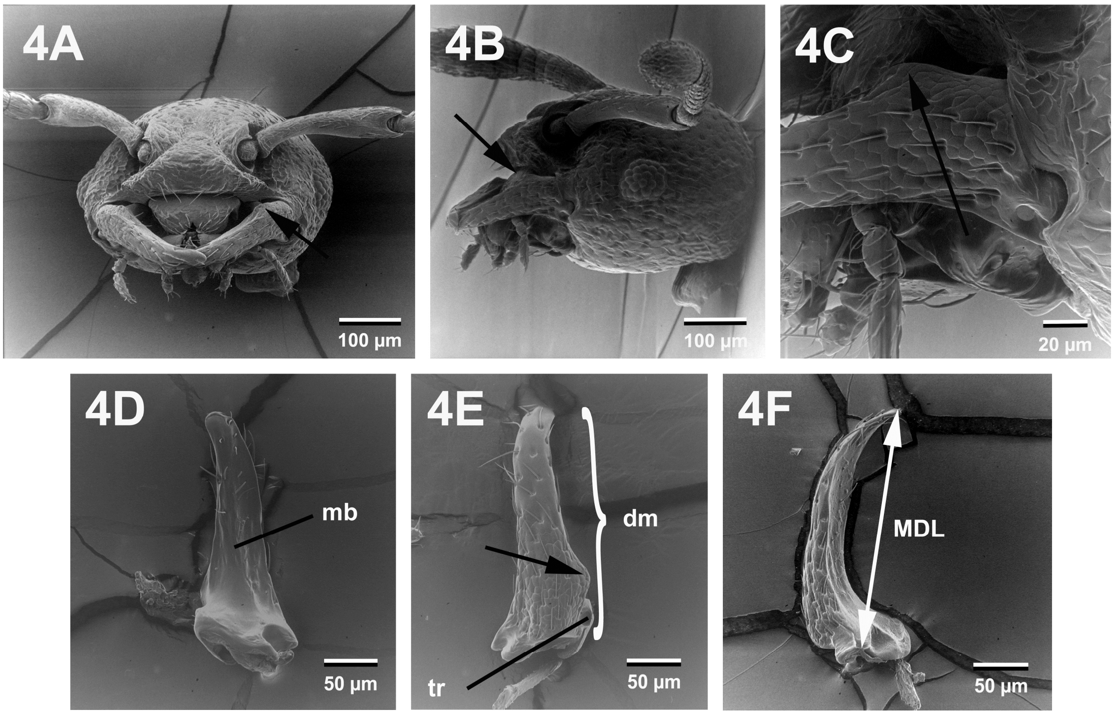


Fig. 4: *C. nigrocerea*, ergatoid male: A frontal view of head capsule, B: lateral view of head capsule, C: detail of left mandible in lateral view, D: dorso-mesial view of right mandible, E: lateral view of left mandible, F: ventral view of right mandible (MDL: mandible length). Arrows indicate angle on dorsal margin. ca: canthellus, dm: dorsal margin, mb: mesial bulge, tr: trulleum.

***C. “argentea”* group:** *C. “argyrotricha”* (Fig. 5), ergatoid male (preliminary names for taxa currently being described by B. Seifert)

Dorsal margin in the distal half bent ventrally, ventral margin bent ventrally (mandible in full-face view directed ventrally) (Fig. 5 A, B). Mandibles narrow, tapering in the distal half towards the apex. Dorsal margin proximally roundish instead of forming a distinct ridge, thus canthellus and trulleum fully visible in lateral view; trulleum in dorsal view not distinctly bordered lateral-distally. Mesial face with a median, longitudinal bulge, running towards the mandible apex, dividing the mesial face in two areas sloping towards the dorsal and ventral margin, respectively; dorsal area smooth, ventral area setose (Fig. 5 F). In lateral view a peculiar sclerotized structure is seen proximally (circle in Fig. 5 E).

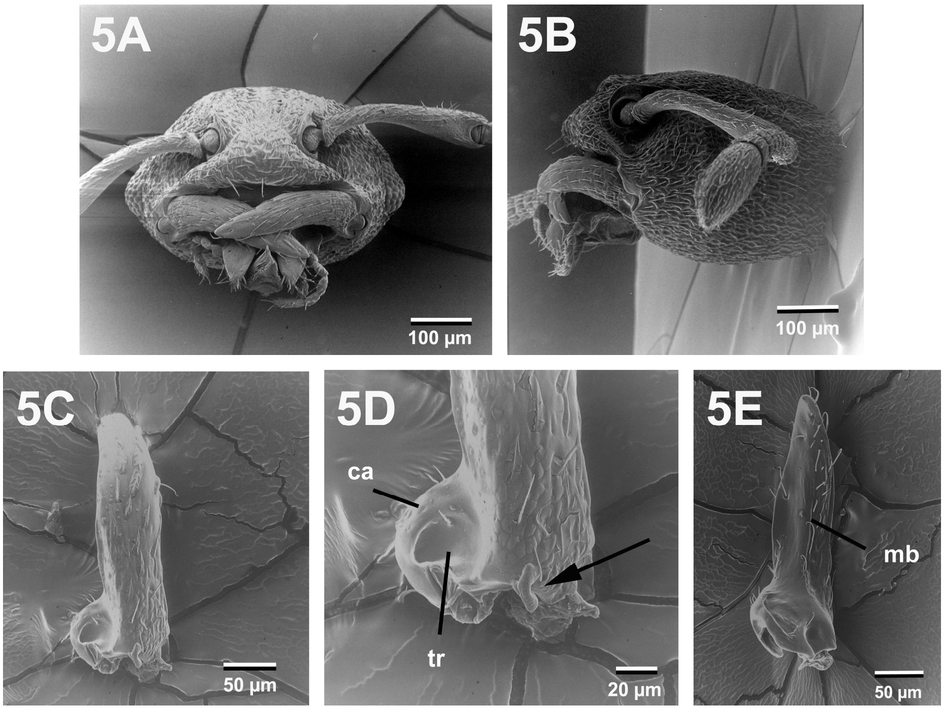


Fig. 5: *C. “argyrotricha”*, ergatoid male: A frontal view of head capsule, B: lateral view of head capsule, C: dorso-mesial view of left mandible, D: lateral view of right mandible, E: detail of right mandible in lateral view showing peculiar structure (arrow), F: mesial view of left mandible. Arrows indicate angle on dorsal margin. ca: canthellus, mb: mesial bulge, tr: trulleum.

***C. wroughtonii* group:** *C. wroughtonii*, ergatoid male (Fig. 6 A), *C. obscurior*, ergatoid male (Fig. 6B-E)

Dorsal margin slightly bent ventrad, mandibles in-face view oriented distally instead of disto-ventrally (Fig. 6 A, B), narrow, tapering towards the apex, pointed. Distal third notably narrow. Mandibles of *C. wroughtonii* even more tapered, as ventral margin is slightly bent dorsally in full-face view (Fig. 6 A). Mandibles longer than in the other studied species of clade A. In dorsal view, trulleum not distinctly bordered disto-laterally by dorsal margin, as margin is not produced as a sharp ridge but roundish. Mesial face bulged along the dorsal margin in the proximal half of the mandible (Fig. 6 E). The mesial face ventral to the bulge is concave and setose (Fig. 6 E).

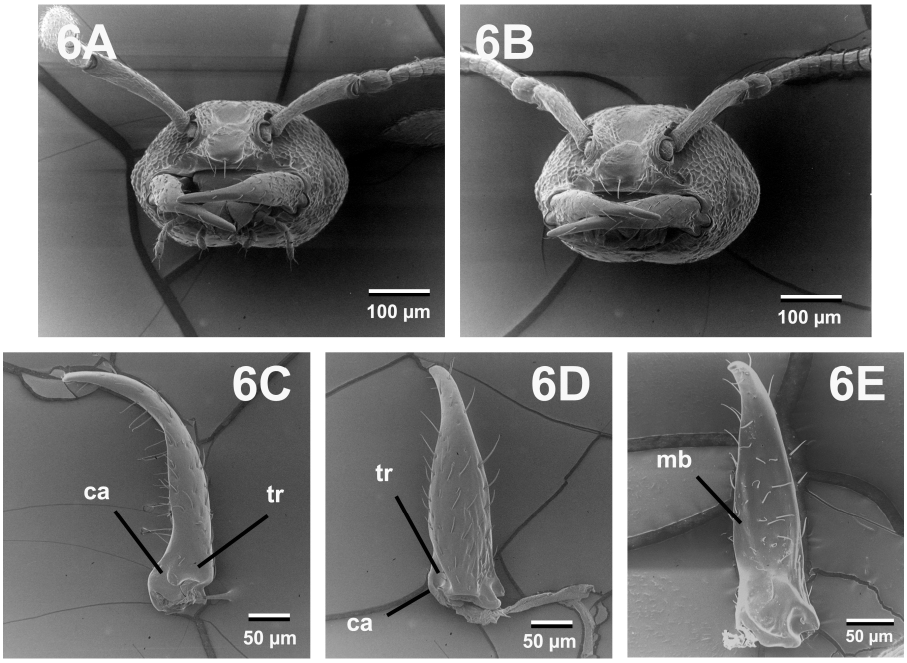


Fig. 6: ergatoid males of the *C. wroughtonii* species-group: A: *C. wroughtonii*, frontal view of head capsule, B: *C. obscurior*, frontal view of head capsule, C: *C. obscurior*, dorsal view of right mandible, D: *C. obscurior*, lateral view of right mandible, E: *C. obscurior*, mesial view of left mandible. ca: canthellus, mb: mesial bulge, tr: trulleum.

Cardiocondyla clade A, winged males:

C. wroughtonii* group*: *C. wroughtonii*, winged male (Fig. 7 A-C), *C. obscurior*, winged male (Fig. 7 E-F)

Mandibles without distinct basal and masticatory margin, toothless, narrowing towards the apex, pointed (Fig. 7 A-F). Shorter compared to ergatoid male mandibles, yet opposable, curved inwards, in full-face view oriented distally. Trulleum in dorsal view not distinctly bordered disto-laterally by dorsal margin (Fig. 7 A). Mesial face with a distinct ridge running from proximo-ventral towards the dorsal margin, reaching it at about half of the mandible length (Fig. 7 C, F). Mesial face covered with several setae distally to this ridge (Fig. 7 C, F).

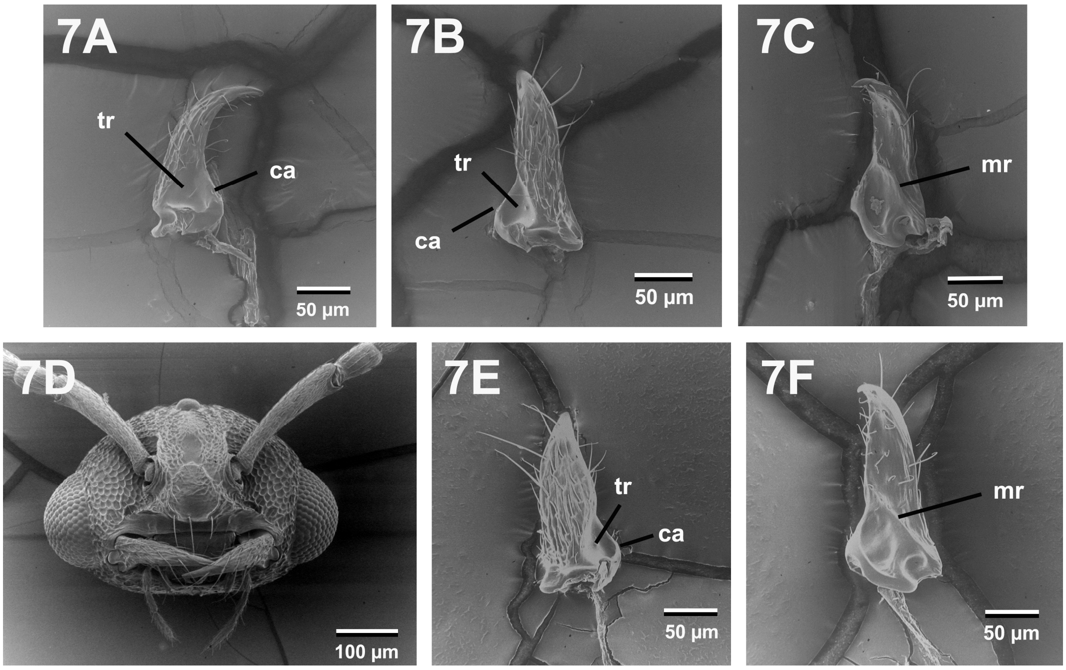


Fig. 7: Winged males of *Cardiocondyla* clade A. A: *C. wroughtonii*, dorsal view of left mandible, B: *C. wroughtonii,* lateral view of right mandible, C: *C. wroughtonii,* mesial view of left mandible. D: *C. obscurior*, frontal view of head capsule, E: *C. obscurior*, lateral view of left mandible, F: *C. obscurior,* mesial view of left mandible. ca: canthellus, mr: mesial ridge, tr: trulleum.

→

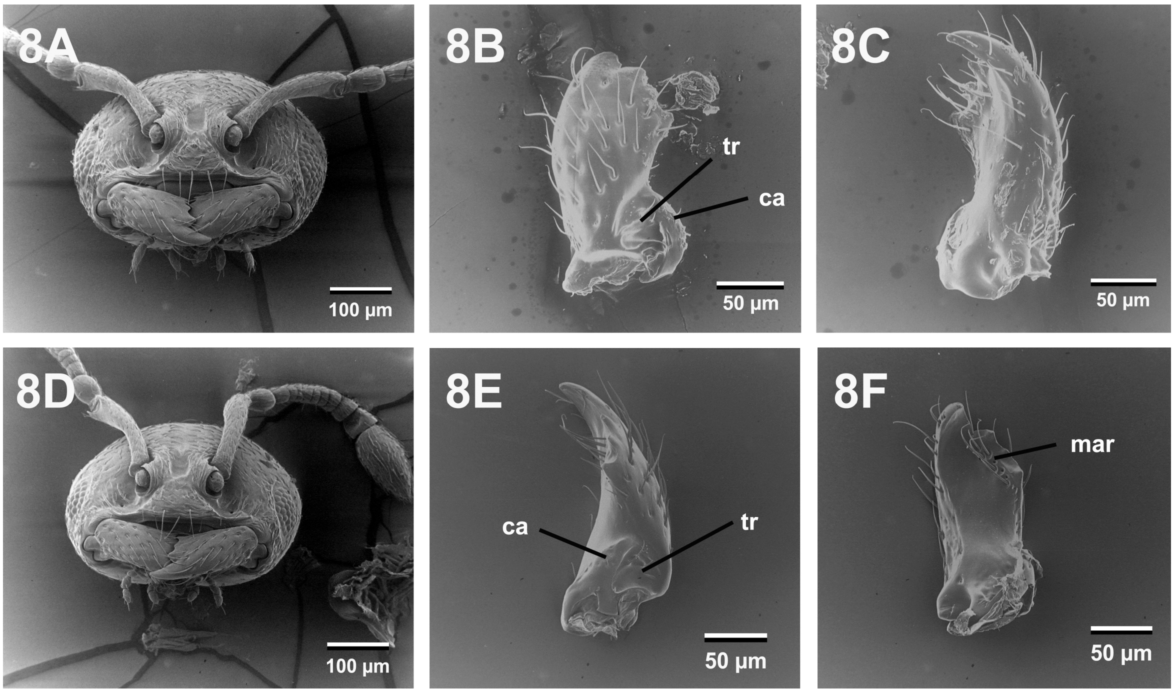
Fig. 8: ergatoid males of the *C. minutior* species group; A: *C. minutior*, frontal view of head capsule, B: *C. minutior*, dorso-lateral view of left mandible, C: *C. minutior*, ventral view of left mandible, D: *C. tjibodana*, frontal view of head capsule, E: *C. tjibodana*, dorsal view of right mandible, F: *C. tjibodana*, mesial view of left mandible. ca: canthellus, mar: masticatory ridge, tr: trulleum.

Cardiocondyla **clade B, ergatoid males:**

Mandibles with distinct basal and masticatory margin (Fig. 8 A-F, 9 A-G). Mandibles compact in appearance: distally widening, but to a lesser extent compared to worker mandibles, and shorter than worker mandibles. Masticatory margin with four teeth, except for some of the examined ergatoid males of *C. venustula*, which possess five masticatory teeth (Fig. 9 C, D). Apical tooth robust, about double size of preapical tooth, teeth decreasing in size from apical. Trulleum open, in dorsal view disto-laterally distinctly bordered by basal margin (Fig. 8 E). Presence of ridges on mesial face similar to worker mandibles: distally with a short longitudinal ridge extending to the apical or preapical tooth, and another ridge below teeth along the masticatory margin leaving one masticatory tooth (the prebasal one) out of line compared to the remaining masticatory teeth (Fig. 8 C). Mesial face distal to canthellus otherwise flat. Highly ordered arrangement of setae similar to worker mandibles: long setae arranged along the masticatory margin, short setae along the distal part of the basal margin (Fig. 8 F).

Contrary to ergatoid males of species assigned to clade A, we could not detect qualitative morphological differences among ergatoid male mandibles of species belonging to clade B.

C. minutior **group:** *C. minutior*, ergatoid male (Fig. 8 A-C), *C. tjibodana* (Fig. 8 D-F)



***C. nuda* group:** *C. kagutsuchi*, ergatoid male (Fig. 9 A, B)

***C. shuckardi* group**: *C. venustula*, ergatoid male (Fig. 9 C, D)

Individuals possess mandibles with either 4 or 5 (intercalary small, prebasal tooth) teeth, number of teeth is positively correlated to mandible size (Pearson's correlation: 0.899**, N=11, 0.01 significance level).

***C. batesii* group:** *C. batesii*, ergatoid male (Fig. 9E, F),

***C. elegans* group:** *C. elegans*, ergatoid male (Fig. 9 G)

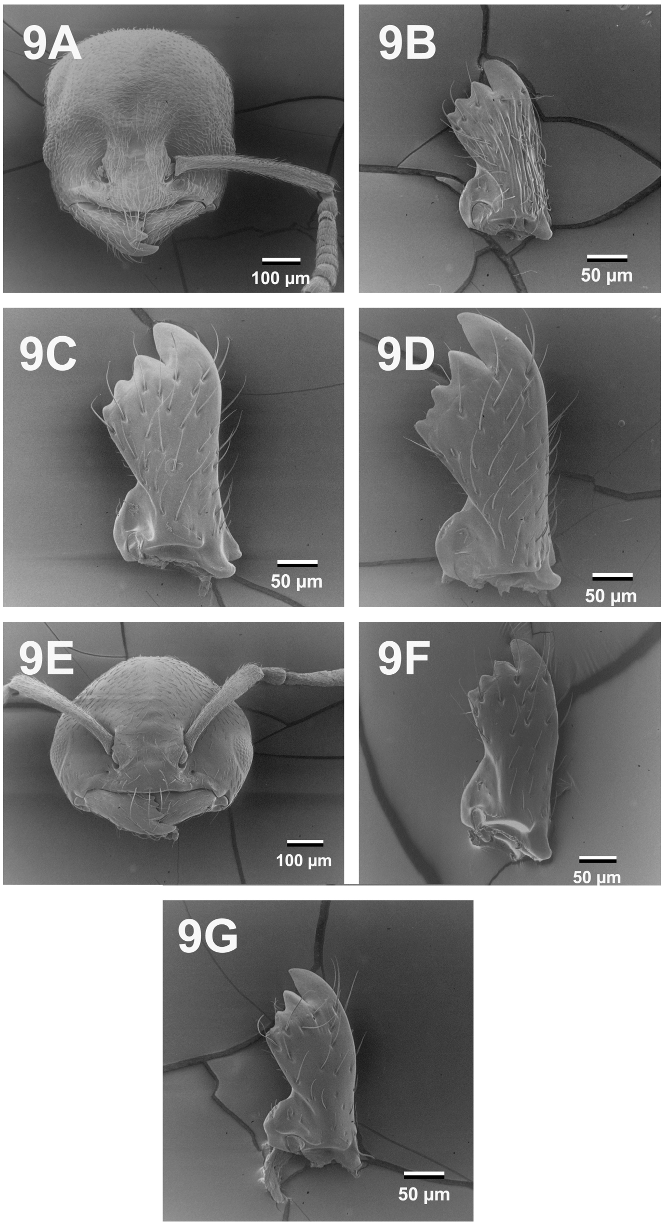


Fig. 9: ergatoid males of the *C. nuda*, *C. shuckardi*, *C. batesii*, and *C. elegans* species groups: A: *C. kagutsuchi*, frontal view of head capsule, B: *C. kagutsuchi*, dorso-lateral view of right mandible, C, D: *C. venustula*, dorso-lateral view of right mandible, E: *C. elegans*, frontal view of head capsule, F: *C. elegans*, dorso-lateral view of right mandible, G: *C. batesii*, dorso-lateral view of right mandible.

Cardiocondyla clade B, winged males:

***C. minutior* group:** *C. minutior*, winged male (Fig. 10 A-C), *C. tjibodana*, winged male (Fig. 10 D, E)

Contrary to clade A, where mandibles of winged males are more similar to ergatoid male mandibles, winged males of clade B possess mandibles, which resemble worker mandibles: mandibles with distinct basal and masticatory margins, five masticatory teeth with the subbasal one being the smallest (as in all workers of species of clade B). Trulleum open, distinctly bordered latero-distally by basal margin in dorsal view (Fig. 10 B, E). Arrangement of setae and ridges also similar to worker mandibles.

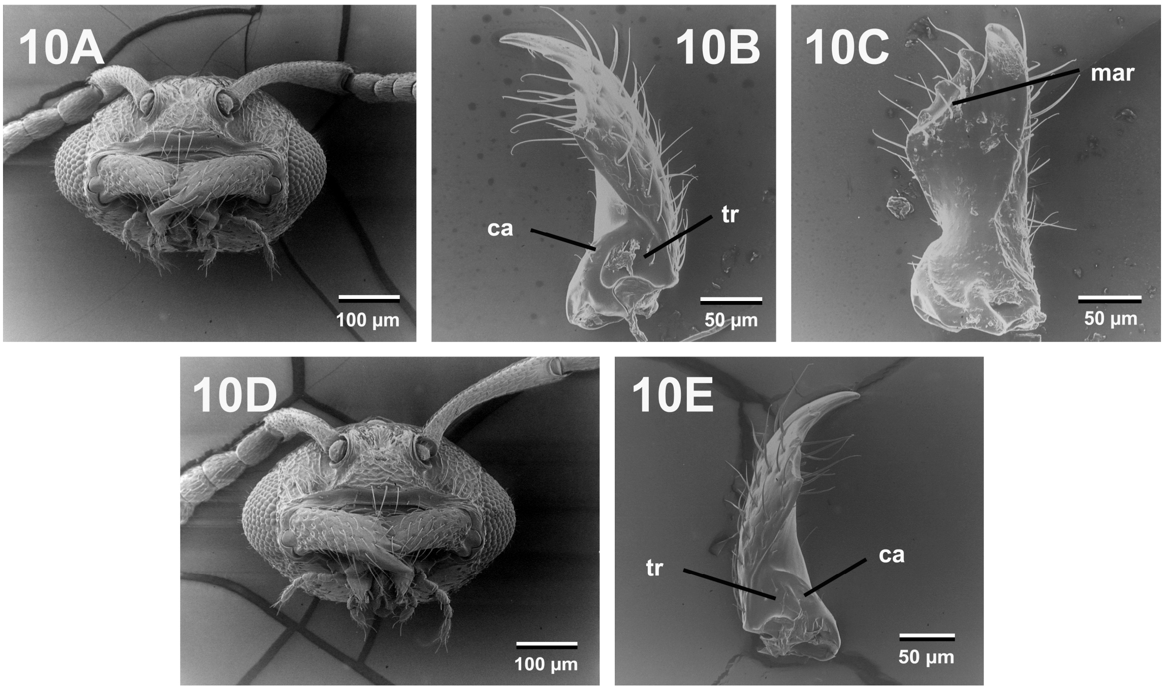


Fig. 10: Winged males of *Cardiocondyla* clade B. A: *C. minutior*, frontal view of head capsule, B: *C. minutior*, dorsal view of right mandible, C: *C. minutior*, mesial view of left mandible. D: *C. tjibodana*, frontal view of head capsule, E: *C. tjibodana*, dorsal view of left mandible. ca: canthellus, mar: masticatory ridge, tr: trulleum.

Table 5: Summary of qualitative and quantitative morphological data of the mandibles in the examined species.

***Cardiocondyla* clade A:** **(*C. wheeleri, C. "argentea", C. wroughtonii* species-groups)**			***Cardiocondyla* clade B:** **(*C. minutior, C. nuda, C. shuckardi, C. batesii, C. elegans* species-groups)**		
worker	**ergatoid male**	**winged male**	**worker**	**ergatoid male**	**winged male**
widening distally	narrowing distally	narrowing distally	widening distally	widening distally	widening distally
5 teeth	toothless	toothless	5 teeth	4 teeth (5 in some individuals of *C. venustula*)	5 teeth
mesial face distal to canthellus flat	mesial face distal to canthellus bulged	mesial face distal to canthellus with distinct ridge	mesial face distal to canthellus flat	mesial face distal to canthellus flat	mesial face distal to canthellus flat
setae specifically arranged	setae without specific distribution	setae without specific distribution	setae specifically arranged	setae specifically arranged	setae specifically arranged
MDL/CS < 0.5	MDL/CS > 0.5	n. a.	MDL/CS > 0.5	MDL/CS < 0.5 (except *C. venustula*)	n. a.
worker MDL/CS < ergatoid male MDL/CS >		n. a.	worker MDL/CS > ergatoid male MDL/CS		n. a.
worker MDL < ergatoid male MDL		n. a.	worker MDL > ergatoid male MDL (except *C. venustula*)		n. a.

n. a.: morphometric data not available

Discussion

This comparative morphological survey of several species of the genus *Cardiocondyla* provides morphometric data that reveal several trends in how ergatoid male morphology differs from worker morphology in the different species. Seifert (2003) described several of these trends, namely the increase in petiolus, postpetiolus, and anterior mesosomal width. The present study for the first time provides detailed morphometric data confirming these previous findings and additionally reveals CL/CW to be generally larger in workers than ergatoid males. This directional deviation is found in all examined species, irrespective of the mating strategy and the clade they are assigned to.

The increase in the width of various body parts is probably correlated to male fighting: ergatoid males use their mandibles to injure or dismember their competitors, with the narrowest body parts being most vulnerable to such attacks. Several species have been reported to possess morphological structures adapted to protect these sensitive regions, such as extensions of the pronotum (Seifert 2003, Heinze *et al.* 2010). Interestingly, in the two examined species with non-fighting, but tolerant ergatoid males (*C. batesii*, *C. elegans*), the waist segments (petiolus and postpetiolus) are large compared to those of the respective workers. We assume that these morphological characteristics, although they may have lost their functional significance in non-fighting males, are conserved across species. This indicates that fighting is an ancestral trait as was previously concluded from molecular data (Oettler *et al.* 2010).

Male ant mandibles are typically described as ‘reduced’ and ‘falcate’ (short, curved, without distinct basal and masticatory margins), ‘often without function’ (Ettershank 1966, Gotwald 1969, Boudinot 2015), although possible adaptations of male mandibles (as of male morphology in general) have only rarely been examined (for exceptions see, e.g., Abell *et al.* 1999). This is presumably due to the erroneous view that ant males are elusive “sperm delivery vessels” (but see Boomsma *et al.* 2005, Shik *et al.* 2012, 2013), only adapted to locate virgin queens and subsequently mate with them. Typical falcate mandibles are found in the winged males of *C. obscurior* and *C. wroughtonii*, while the mandibles of winged males of *C. minutior* and *C. tjibodana* are similar to those of workers. In contrast, ergatoid males of *Cardiocondyla* either develop compact four-toothed mandibles with an increased apical tooth (“shear-shaped”), or long and narrow (“sickle-shaped”) mandibles lacking distinct basal and masticatory margins. The difference in the shape of ergatoid male mandibles of species of *Cardiocondyla* has proven to be useful assigning species to one of the two clades of the genus

(Oettler *et al.* 2010), and mandibular shape (long and narrow vs. four-toothed worker-like) is clearly correlated to the different ways of killing male competitors in several species of the two clades. Ergatoid males of several species of clade B use their robust mandibles equipped with a large apical tooth to crush the weakly sclerotized cuticle of young males (Heinze *et al.* 1993, 1998, Frohschammer and Heinze 2009). In contrast, ergatoid males of clade A use their mandibles both to pierce the cuticle of emerging male rivals (Kinomura and Yamauchi 1987; Stuart *et al.* 1987) and to grasp the competitor’s body and hold it for up to several hours (Kinomura and Yamauchi 1987; Yamauchi and Kawase 1992; Yamauchi *et al.* 2006). Mandibles of such ergatoid males are presumably selected to become both longer, to better clasp around wide body parts, and narrower, as this allows for better grasping in the narrow gap between different body parts, such as between the head and the mesosoma.

Mandible shape and length are more variable in clade A than in clade B. In clade A, ergatoid males of *C. obscurior* and *C. wroughtonii* are smaller but have longer mandibles than males of *C. nigrocerea* and *C. “argyrotricha”*. Furthermore, the mandible apex is oriented distally in *C. obscurior* and *C. wroughtonii* but disto-ventrally in *C. nigrocerea* and *C. “argyrotricha”*. This might reflect different fighting tactics. *C. nigrocerea* primarily besmears freshly eclosing males and only rarely adult ones (Heinze *et al.* 2010). Furthermore, casual observations of lab colonies indicate that adult ergatoid males of *C. “argyrotricha”* quickly detect and besmear young rivals (pers. observation). Freshly eclosing males are less mobile than adult males. They may be easily approached from behind and above and seized by an adult aggressor. In contrast, ergatoid males of *C. obscurior* and *C. wroughtonii* have been observed to also fight with fully-sclerotized adult rivals (e.g. Kinomura and Yamauchi 1987, Yamauchi and Kawase 1992). Their extremely long, distally oriented mandibles of the ergatoid males of these two species may facilitate seizing mobile adult rivals and staying attached to them.

According to Fisher and Bolton (2016) long and narrow mandibles without distinct basal and masticatory margins can evolve in that 1) “the base of the mandible narrows and the basal angle is obliterated” (basal and masticatory margins forming a single margin), (2) “the masticatory margin becomes elongated and the basal margin contracted”, and 3) “the basal margin becomes elongated and the masticatory margin contracted”. Ergatoid males of *Cardiocondyla* clade A have mandibles, which bear only few features, and no intermediate condition, such as mandibles with fewer than five teeth, are known. This makes it difficult to retrace the evolution of this derived mandible type. Based on the data obtained, we cannot determine if the angle of the dorsal margin corresponds to the basal angle present in worker-like mandibles, or if these structures are not homologous. However, two characteristics appear to be correlated with the formation of narrow mandibles in *Cardiocondyla*: (1) a bulge or ridge on their mesial surface and (2) an unordered distribution of setae (probably derived from the highly ordered setae in triangular mandibles).

Short mandibles have been suggested to apply greater forces than longer mandibles because of the smaller moment arm (Abell *et al.* 1999). Mandibles of ergatoid males of clade B are not only relatively shorter than those of males of clade A, but, relative values are significantly smaller compared to those of conspecific workers (two-sample t-test/ Welch’s t-test, $p < 0.05$). The mandibles of ergatoid males of clade B retain the overall structure found in workers and therefore are not degenerated as the short mandibles of many other, short-lived ant males. In contrast, selection has maintained strong mandibles in those ergatoid males that use them to crush the cuticle of rivals (e.g. Heinze *et al.* 1993, 1998).). Mandibles of ergatoid males of *C. minutior* and *C. kagutsuchi*, which exclusively kill young males by crushing their still soft cuticle, have the shortest mandibles relative to head size. We hypothesize that the loss of one masticatory tooth is associated with the reduction of mandible length, as mandibles are distally widening to a lesser extent compared to the worker mandible. This assumption is corroborated by the sporadic occurrence of five masticatory teeth in large mandibles of ergatoid males of *C. venustula*. It appears that the prebasal tooth of the worker mandible has been reduced in the evolution of the ergatoid male mandible, as this is usually the smallest in workers of species of *Cardiocondyla* clade B, and also the smallest in five-toothed mandibles of ergatoid males of *C. venustula*.

Surprisingly our study does not reveal differences in the shape of mandibles between species of clade B with actively fighting males (*C. minutior*, *C. tjibodana*, *C. kagutsuchi*, *C. venustula*) and mutually tolerant, peaceful males (*C. batesii*, *C. elegans*). However, it appears that mandible length correlates with the reproductive strategy in the worker-like mandibles of ergatoid males of clade B species. Interestingly, the mandibles of ergatoid males of *C. venustula* are the longest mandibles (total values) in clade B and, in contrast to other species, also absolutely longer than those of conspecific workers. Ergatoid males of *C. venustula* kill young rivals by crushing the soft cuticle and also defend small territories by biting and holding intruders with their mandibles and besmearing it (Frohschammer and Heinze 2009, Jacobs and Heinze 2016). As *C. venustula* is the only species known species in clade B in which males regularly show this behaviour it cannot be determined whether here mandible elongation might be similarly associated with besmearing as in clade A.

To conclude, this study adds another piece to the puzzle of the evolution of ant male biology. Detailed observation of the reproductive behaviour of ergatoid males and examination of mandible size and shape of additional species of *Cardiocondyla* may help to evaluate possible adaptations of ergatoid male mandibles and shed light on the evolution of the diverse male traits and reproductive life histories in this fascinating ant genus.

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Chapter 3

Microsatellite markers for the tramp ant, *Cardiocondyla obscurior* (Formicidae: Myrmicinae)

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Introduction

Cardiocondyla obscurior is a very successful, but unobtrusive tramp ant, which is widely distributed throughout the tropics and subtropics. To understand its population structure and biology, we established six polymorphic microsatellite markers. We determined the general variability of the six loci by analyzing individuals from four different populations. In addition we analyzed samples from a Japanese population and revealed first insights into the population and colony structure of this tramp species, including high nestmate relatedness in multi-queen colonies and a relatively high inbreeding coefficient.

Numerous plants and animals have become established in areas far away from their native ranges due to human activities (Hulme 2009). Among these are several dozen tramp species of ants. Some of them, such as the red imported fire ant, the Argentine ant, and the Yellow crazy ant, quickly become dominant in new ecosystems and are among the worst invasive species worldwide, on a par with tiger mosquitoes, black rats, or water hyacinths (reviewed by Holway *et al.* 2002; Tsutsui and Suarez 2003). The ecological impact, behavioural ecology, and sociobiology of these eye-catching invaders have been intensively studied, but much less is known about tramps that less dramatically integrate in novel environments. Among the most widespread of these inconspicuous tramp ants are the “sneaking ants”, *Cardiocondyla*. At least seven of its approximately 100 species are tramps, and a subset of two or three of these can be found reliably on most tropical beaches and in plantations and parks around the tropics and subtropics (Seifert 2003; Heinze *et al.* 2006; Wetterer 2012ab, 2014).

Cardiocondyla exhibits a bizarre male diphenism with so-called “ergatoid” wingless males in addition to the typical winged male of ants. While winged males are peaceful and disperse, wingless males stay inside their maternal nests throughout their lives and mate with young nestmate queens (Kinomura and Yamauchi 1987; Stuart *et al.* 1987). This results in considerable inbreeding: from fixation coefficients in single-queen colonies of non-invasive *Cardiocondyla* from Mediterranean Europe and Madagascar it has been estimated that up to 90% of the matings are between brothers and sisters (Schrempf *et al.* 2005; Lenoir *et al.* 2007; Schrempf 2014; Heinze *et al.* 2014).

Due to the lack of variable genetic markers, nothing is known about the genetic structure of the tramp species. Their colonies typically contain multiple queens and, like in other invasive species, individual nests might not be closed against alien individuals (Heinze and Delabie 2005). Our study species *Cardiocondyla obscurior* was originally described from Taiwan

(Wheeler 1929) and has spread from its presumed origin in Southeast Asia as far as to the Ryukyus, the Caribbean, the Canary Islands, Hawaii, and other areas (Seifert 2003). In this study, we report the sequences of primers for microsatellites developed for *C. obscurior* and data on nestmate relatedness and inbreeding in colonies of this ant from Okinawa, Japan. Our results show that the microsatellite primers are suited well to conduct more detailed analyses, which will give further insights into the population structure of this ant.

Material and Methods

For the development of microsatellite primers, we followed a method based on selective hybridization (Tenzer *et al.* 1999, Gautschi *et al.* 2000). In detail, 50 individuals from a colony from Okinawa (collected in 2007) and 200 individuals from three Brazilian colonies (collected at CEPLAC, Ilhéus, Brazil in 2004 and 2009) were pooled and genomic DNA was subsequently extracted using a modified CTAB (cetyltrimethyl ammonium bromide) protocol (Sambrook and Russell 2001). According to the protocol of Kautz *et al.* (2009) we ligated the adapters onto the DNA after restriction with Tsp 509I. Microsatellite sequences were fished with the help of magnetic beads. PCR fragments were cloned using the TOPO TA Cloning kit (Invitrogen) following the manufacturer’s protocol. Successful recombinants were easily identified by the white color of colonies. 286 of them were dot-blotted on nylon membranes (Hybond-N Amersham) and probed with $(GA)_{13}$ oligonucleotide labeled with fluorescein (MWG Biotech). For detection, we used the Gene Images CDP-Star detection module (Amersham Life Science). In total, 104 clones yielded a positive signal, and for the strongest 92 of them, plasmids were purified and sequenced by GATC Biotech. The obtained sequences were edited in Sequencing Analysis 3.4.1 (PE Biosystems) and visually checked for microsatellites. Primers flanking the core microsatellite repeats were manually designed and tested for 38 loci.

Standard PCR was carried out in a final 20 µl reaction volume containing 2.5 ng DNA, 1x buffer, 2.5mM $MgCl_2$, 200µM dNTP (MBI Fermentas), 0.5 µM (Cobs8), 1µM (Cobs9, Cobs13) and 1.25 µM (Cobs P3, Cobs13.2, Cobs 42) unlabeled reverse primer, the same amounts of FAM-, HEX-, or TET-labelled forward primer, and 0.5U *Taq* DNA polymerase (Fermentas). Cycling conditions were 94°C for 70s, T_A for 45s, 72°C for 25s for 34 cycles with an initial denaturation step at 94°C for 4min and a final extension step at 72°C for 1min. PCR products were visualized on an ABI PRISM genetic analyzer (PE Biosystems). Allele size was analyzed using a GeneScan-500 size standard (TAMRA) and GENESCAN 3.1 software (PE Biosystems).

To determine the general variability of the six microsatellite loci for which primers were developed, we analyzed individuals from four different populations: from Ilhéus, Brazil (collected in 2004 and 2009), Lake Alfred, Florida (2004), Okinawa, Japan (2007 and 2011) and Ishigaki, Japan (2007; for number of investigated individuals see Table 1). Finally, we analyzed the genetic structure of eight colonies of *C. obscurior* from four different sites in and around Naha, Okinawa, by genotyping 2 to 9 queens and 7 to 15 workers per colony (Table 2). Six colonies, originate from two nearby trees in Onoyama Park, Naha (tree 1: OypC51, OypC32, OypC48, 26°12.255' N, 127°40.656' E; tree 2: OypE3, OypE5, OypE4, 26°12.334' N, 127°40.592' E). Two additional colonies came from Urasoe Sports Park, Naha (7km distance to Onoyama, 26°15.134' N, 127°43.262' E) and the Campus of the University of the Ryukyus (11km distance to Onoyama, 26°14.782' N, 127°45.864' E). Relatedness within the colonies and within the whole population was estimated using the software Relatedness 4.2 (Goodnight and Queller 1994) based on the algorithm by Queller and Goodnight (1989). Groups were weighted equally and standard errors were estimated by jackknifing over loci. The polymorphic information content (PIC, Botstein *et al.* 1980) was calculated using the software Cervus 3.0 (Kalinowski *et al.* 2007). To obtain information about the amount of inbreeding, we calculated Weir’s F (inbreeding coefficient) with the software GDA (Lewis and Zaykin 2001) for the six colonies of the two nearby trees. Even though sample size is low, for these six colonies we also give the Θ_P-value, which gives information on population structure. Confidence intervals were calculated by bootstrapping 5.000 times over loci.

Results and Discussion

From the 38 loci, which yielded a good amplification product, six were polymorphic, with three to nine alleles per locus (see table 1). Interestingly, there is no overlap in allele length between the Brazilian and the Japanese and Florida population at three primers, respectively, which suggests a long separation of the Brazilian population, and / or alternatively, different source populations during colonization. A recent study could show that populations differ strongly in several aspects (Schrader *et al.* 2014). Our preliminary analysis confirms that there is sufficient genetic variation for detailed studies, and hence, a future analysis will allow gaining further insights into possible differences concerning the colony and population structure between the two populations.

Our analysis of a small number of colonies from Okinawa yielded some important insights into the breeding structure of *C. obscurior*. Generally, relatedness varied greatly among colonies and in some cases was surprisingly low. Over all, worker relatedness and queen relatedness are not different from each other (worker relatedness: 0.580 ± SE 0.127 (102 individuals) , queen relatedness: 0.612 ± SE 0.366 (61 individuals); t- test for paired samples: $t = -0.22$, $p=0.83$; see table 2). The relatively low relatedness among queens in some of the colonies (colonies OypC48, OypE3, OypE5, and OypE4, $r < 0.35$) might indicate that alien queens can be easily adopted in the colonies, as has been already suggested by Kinomura and Yamauchi (1987). Queen adoption appears to be the rule in other tramp species, such as *Solenopsis invicta* and *Linepithema humile*, resulting in low relatedness values close to zero (e.g. Goodisman and Ross 2003, Krieger and Keller 2001). The occasionally higher worker relatedness in comparison to queen relatedness in some colonies might be either the result of reproductive skew, i.e., some queens producing more worker offspring than others, or due to a common father (unrelated queens having mated with the same male). Half-sisters in insect societies usually have the same mother but different fathers (as in honeybees or other species with multiply- mated queens). In contrast, the special mating biology of *Cardiocondyla* with non-dispersing, wingless males that monopolize mating raises the possibility of paternal half-sibs. Even though we found significant inbreeding in the population (six colonies: $F = 0.293$, 95% CI 0.145 – 0395; Θ_P: 0.260), sib-mating appears less common than in monogynous *Cardiocondyla* species. This indicates that the likely adoption of alien queens allows the opportunity for outbreeding despite mating in the nest in *C. obscurior*. The same holds for the tramp ant *L. humile*. Although mating takes place in the nest, inbreeding coefficients have also been found to be very low as foreign queens are adopted in the colonies (Krieger and Keller 2001).

Table 1. Primer sequences of microsatellite loci in *Cardiocondyla obscurior*. The name, accession number, annealing temperature, repeat sequence, primer sequence, allele size and number of investigated individuals are given, as well as the PIC (polymorphic information content) resulting from the genetic analysis of the population of Okinawa, Japan.

Locus Name	Accession no. (EMBL)	TA [°C]	Repeat sequence	Primer sequences (5' to 3')	Alleles (bp) Japan, Okinawa	Alleles (bp) Japan, Ishigaki	Alleles (bp) Brazil	Alleles (bp) Florida
Cobs8	HE608811	55	$(AG)_{10}$	F: TTATCGTGAGGATTTTGAGGC R: TTTCGACAATGACAAACCGAGC	160,180 (N=8) *148*152*156*160 (N=130) PIC: 0.46	156 (N=4)	156,160 (N=10)	156 (N=6)
Cobs9	HE608812	55	(TC)11TT(TC)11 TT$(TC)_{6}$	F: ACTCAGTGCCAATTCGAATAAACAGC R: TGAACCGGGTAGAATCAATTA	163, 203 (N=7) *163*203 (N=148) PIC: 0.09	161 (N=6)	163,203 (N=10)	161 (N=4)
Cobs13	HE608813	60	$(CT)_{35}$	F: TATCTTTTCAACCCTCTCGC R: TATTCCGCGATAGCTTAAAT	189 (N=8) *185*187*189 (N=137) PIC: 0.12	189 (N=6)	159,161,183,185 (N=10)	185 (N=4)
Cobs P3	AJ784426	50	$(AG)_{31}$	F: ACTCTCACAATCGCTACGC R: GACGTACGGCCAGATGTCA	*110*130 (N=149) PIC: 0.04	110 (N=6)	152,154,156,158 (N=30)	110 (N=6)
Cobs 13.2	AJ784427	45	$(AG)_{29}$	F: AATCGCGCCTGCGACGGCG R: AGTTTCTCACTTTTGCTCG	*200*204*206*208 (N=133) PIC: 0.55	206 (N=2)	212,216,218,222,226 (N=30)	206 (N=5)
Cobs 42	AJ784429	45	$(AG)_{34}$	F: TCAGAGAAGTAAATATCAG	*137*139*145 (N=140) PIC: 0.40	145 (N=5)	157,161,163,165,171 (N=30)	139 (N=6)

*alleles resulting from the genetic population analysis

$T_{A,}$ annealing temperature

Table 2. Relatedness (mean ± SE) over all individuals and for queens and workers separately, in *Cardiocondyla obscurior* colonies from Okinawa.

colony	**Number of queens**	**Number of workers**	**r (queen) (number of analyzed individuals)**	**r (worker) (number of analyzed individuals)**
OypC32	21	76	0.838 ± 0.202 (N=9)	0.619 ± 0.176 (N=15)
OypC48	15	61	0.331 ± 0.197 (N=9)	0.582 ± 0.216 (N=15)
OypC51	26	34	0.817 ± 0.155 (N=9)	0.784 ± 0.133 (N=15)
OypE3	14	17	0.227 ± 0.170 (N=9)	0.636 ± 0.201 (N=15)
OypE5	7	11	-0.014 ± 0.229 (N=7)	0.448 ± 0.208 (N=11)
OypE4	9	25	0.297 ± 0.276 (N=9)	0.357 ± 0.149 (N=15)
Urasoe Sports Park	2	9	1.000 (N=2)	0.778 ± 0.119 (N=9)
Univ. of the Ryukyus, Campus	10	8	0.913 ± 0.109 (N=7)	0.411 ± 0.206 (N=7)

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Chapter 4

Virgin ant queens mate with their own sons to avoid failure at colony foundation

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Keywords *Cardiocondyla* · ants · mother-son mating · inbreeding · colony foundation

Abstract

Mother-son mating (oedipal mating) is practically non-existent in social Hymenoptera, as queens typically avoid inbreeding, mate only early in life and do not mate again after having begun to lay eggs. In the ant genus *Cardiocondyla* mating occurs among sib in the natal nests. Sex ratios are extremely female-biased and young queens face the risk of remaining without mating partners. Here, we show that virgin queens of *C. "argyrotricha"* produce sons from their own unfertilized eggs and later mate with them to produce female offspring from fertilized eggs. Oedipal mating may allow *C. "argyrotricha"* queens to found new colonies when no mating partners are available and thus maintains their unusual life history combining monogyny, mating in the nest, and low male production. Our result indicates that a trait that sporadically occurs in solitary haplodiploid animals may evolve also in social Hymenoptera under appropriate ecological and social conditions.

Introduction

Queens of most social Hymenoptera (ants, bees, and wasps) mate usually only once at the beginning of their life away from their natal nests. Thereafter, they produce workers and later also new queens from fertilized eggs and haploid males from unfertilized eggs without ever mating again (Wilson 1971). Because of the standard mechanism of single-locus complementary sex determination (sl-CSD), inbreeding is particularly detrimental in social Hymenoptera, and mating with close relatives is usually avoided (van Wilgenburg *et al.* 2006). In contrast, mating in the ant genus *Cardiocondyla* occurs in the natal nest and regularly involves close sibs. Wingless *Cardiocondyla* males engage in fatal fighting with their rivals and the only survivor monopolizes mating with all virgin queens reared in the nest (Yamauchi *et al.* 2006). Especially in single-queened (monogynous) species, mother queens are selected to adjust their offspring sex ratio to an extreme female bias, as brothers locally compete for mating chances ("local mate competition", Hamilton 1967). Indeed, queens of monogynous *Cardiocondyla* lay only relatively few male-destined, haploid eggs (Schrempf *et al.* 2005), and data from the field suggest that colonies may lack male offspring despite containing several young virgin queens. Virgin queens thus face the risk of failing to acquire a mating partner, in particular when they eclose after the death of their mother. Here, we show that virgin queens of *C. "argyrotricha*" (provisional name of a recognized morphospecies currently described by B. Seifert) emerging in nests without a male may establish a new colony by raising males from their unfertilized eggs and later mating with their own sons.

Materials and Methods

All workers and brood used in the experiment came from nests that originally were set up by splitting a single stock colony of *Cardiocondyla "argyrotricha"* from the botanical garden of UPLB, Los Baños, Philippines. Colonies of *C. "argyrotricha*" consist of 20 to 60 completely sterile workers (*Cardiocondyla* workers generally lack ovaries, Heinze *et al.* 2006) and a single reproductive queen. Though males of this and related species are extremely long-lived compared to other ant males (up to one year, Yamauchi *et al.* 2006), about one third of colonies that contained winged virgin queens when collected in the field in various parts of Southeast Asia were without a male (median sex ratio 0.92, n = 10 colonies). In colonies with a male, matings typically occur between brothers and sisters.

To examine the behaviour of virgin queens in the absence of males, we set up a total of 31 experimentally queenless colonies. Each colony consisted of ten workers, one or two queen pupae (of which one was removed after the first queen had emerged), and several second and third instar larvae (in order to be able to differentiate between initially provided larvae and larvae later produced by the emerged virgin queen). Experimental colonies were kept in climate chambers under near-natural conditions with 28 °C / 23 °C temperature and 12 h / 12 h day-night cycles and fed *ad libitum* with honey and pieces of cockroaches twice a week.

We checked experimental colonies for the presence of adults and brood three times a week. When necessary, new workers were added to keep initial worker numbers constant. The dates of emergence of the queen, the appearance of the first eggs, the emergence of the first male and worker pupae, and the hatching of the first male and first worker were noted. In addition, we counted the number of eggs, larvae and pupae produced by the queen until the first worker pupae appeared. Scans were continued for about four weeks after the emergence of the first worker pupae to control for male offspring (i.e., to check whether a large number of diploid males were produced). Queens mated with their own sons and thereafter produced diploid worker offspring in 13 of the 31 experimental colonies. Three of these colonies were not included in the statistical analysis because the numbers of brood items were censused only at more irregular intervals. In the other 18 colonies, queens died either before rearing males or before mating with their sons. Statistical tests were conducted with the software Statistica 6.0 and the slopes of regression lines were compared using the online tool Comp2Regs_Pgm at www.statstodo.com.

In a supplemental experiment we set up nine additional colonies as above. In four of these colonies we regularly removed all male pupae to determine whether virgin queens might ever produce female offspring. In the other five colonies we dissected the ovaries of queens 7 to 28 days after the eclosion of the first male pupae and presumed mating to determine whether their spermathecae contained sperm (Buschinger and Alloway 1978). In order to directly observe mother-son mating, we in one colony separated the male pupae shortly before eclosion from the queen and put them together only after the male had hatched. We videotaped the 55 day old queen mating with her 1 day old son 10 minutes after the latter had been returned into the nest.

Results

After eclosion, the virgin queens readily shed their wings and began to lay eggs within one or two weeks after emergence (median 11 days, quartiles 9.25-12, n = 10 queens). The number of eggs increased with time only slightly (Fig. 1, slope of regression line before eclosion of first offspring β_1 = 0.058, SE = 0.026, sign. different from zero: t = 2.192, P = 0.043), and only 1 to 5 eggs (median 2.5, quartiles 1.75-4) hatched into male pupae several weeks later (first male pupa after queen emergence, median 46 days, quartiles 51-56; total production 44 males, median 4, quartiles 3-5.75). All other eggs were fed to the larvae, and not a single egg developed into a female pupa. The first adult male eclosed on average 63 days after queen emergence (median 56 days, quartiles 46.25-62.75), and mated thereafter with its mother. Dissection verified the presence of sperm in the spermathecae of the five additional queens, which in a supplemental experiment with the same design had mated with their own sons. In one case, we could directly observe and videotape a 55 day old queen mating with its 1 day old son.

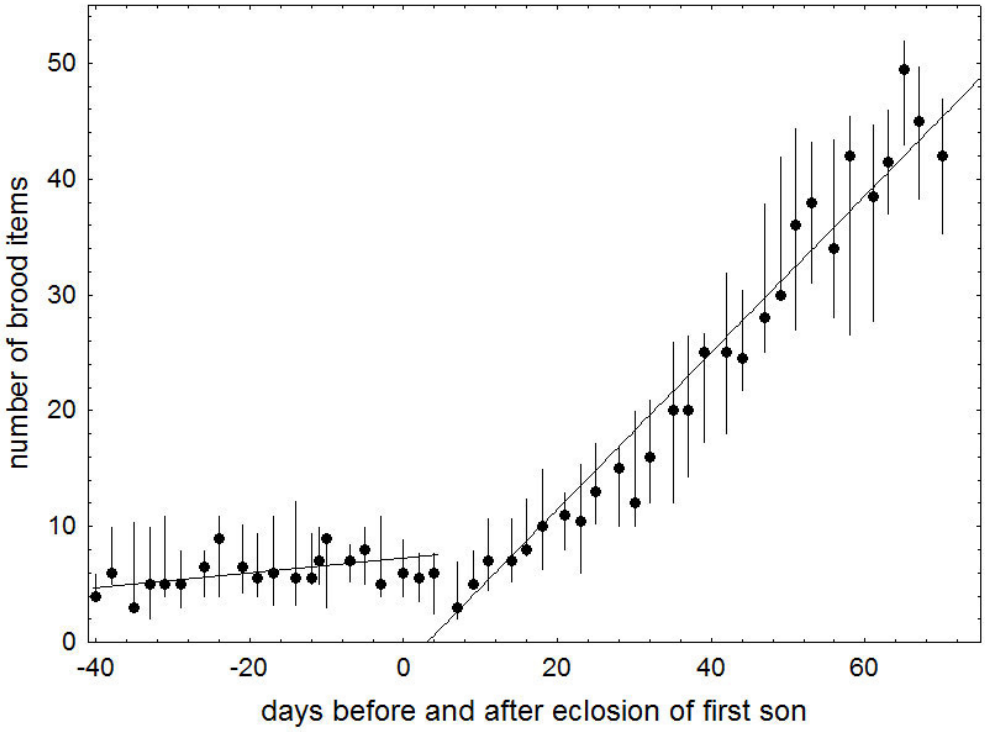

Fig. 1 Number of brood items (eggs, larvae, pupae) produced by initially unmated queens of the ant Cardiocondyla “argyrotricha” before and after the eclosion of their first sons at day 0 (n = 10 queens). Given are medians, upper (75%) and lower (25%) quartiles, and separate linear regression lines for the relationship between the number of brood items and time in the two periods before and after day 0.

After mating, egg numbers increased significantly more rapidly than before (Fig. 1, β_2 = 0.677, SE = 0.027, sign. different from zero: t = 23.024, P < 0.00001; difference in slopes $\beta_1 - \beta_2$ = 0.618, SE = 0.057, t = 10.871, P < 0.0001). First adult workers eclosed on average 40 days after male emergence (median 38 days, quartiles 33-48.5, range 26-57). During the four weeks following the emergence of the first worker pupae, 9 of 10 colonies produced exclusively workers, suggesting that most eggs laid after the emergence of males have been fertilized. One colony produced two males in addition to workers, but throughout our experiment no new virgin queens were reared. Four additional queens that were prevented to mate with their sons by regular removal of all male pupae produced only male brood throughout the whole experiment (median 101.5 days after queen emergence, range 93.25-106.25) and their colonies failed, documenting that the production of female offspring requires the fertilization of eggs and is not due to parthenogenesis.

Discussion

Mother-son mating (oedipal mating) has previously been reported from a number of haplodiploid species, such as mites, beetles, and parasitoid wasps (see, e.g., Adamson and Ludwig 1993), and also from termites (Kobayashi *et al.* 2013). It was suggested to be beneficial in habitats in which dispersing females face a particularly high risk of remaining without mating partners (Adamson and Ludwig 1993).

In social Hymenoptera, however, mother-son mating is a rare phenomenon. A thorough literature search revealed only three reports of oedipal mating in social Hymenoptera, one of them turning out to be an erroneous citation, and the other two not clearly showing that sperm was actually transferred during mating. Garófalo (1973) reports that one laboratory-mated queen of the bumble bee *Bombus atratus* laid eggs, which, when the experimenter provided them with pollen from a honeybee colony, developed into male and female offspring. Two males were later observed to mate with their mother, but as the queen had mated before it is not completely clear whether sperm was transferred during these copulations or whether most probably males just tried to copulate with available individuals. Liebig *et al.* (1998) provides anecdotal evidence suggesting mother-son mating in the ant *Harpegnathos saltator*, which regularly has mated workers in addition to queens. Workers were observed to first produce males but later switch to the production of female offspring. Finally, Wilson (1971) cites Peacock *et al.* (1954) for mother-son mating in the pharaoh ant, *Monomorium pharaonis*, but a careful reading of the original paper does not support this claim. Rather, Peacock *et al.* (1954) describe male production by virgin queens.

Oedipal mating appears to be very rare in social Hymenoptera because of two fundamental traits of their life history. First, queens are generally receptive only during a short time window following adult emergence and before laying eggs (Wilson 1971; Thornhill and Alcock 1983; Kronauer and Boomsma 2007). Second, as mentioned above, the typical sex determination mechanism of social Hymenoptera, sl-CSD, makes sib-mating especially harmful because under inbreeding half of all fertilized eggs develop into usually sterile or inviable diploid males instead of workers (van Wilgenburg *et al.* 2006). The initial production of only workers by *C.* "*argyrotricha*" queens that mated with their sons shows that, in contrast to the majority of social Hymenoptera, in *C.* "*argyrotricha*", sex is not determined by sl-CSD but another, as yet unknown mechanism. The absence of sl-CSD has also been revealed in other species of *Cardiocondyla*, where sister-brother mating is the rule (Schrempf *et al.* 2006). This pre-disposes *Cardiocondyla* ants to evolve the capability of mother-son mating under appropriate ecological and social conditions. Hymenopteran species more prone to diploid male load might have responded to the risks of female sexuals remaining without mating partners by evolving thelytokous parthenogenesis (Rabeling and Kronauer 2013).

Oedipal mating might underlie the evolutionary stability of the life history of *C.* "*argyrotricha*" combining monogyny, fatal fighting among males, and local mating. It still remains to be seen how widely distributed mother-son mating is within this genus and in other ants with sib-mating in the nest, but its occurrence in *C.* "*argyrotricha*" again highlights the striking plasticity of social insect life histories.

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Chapter 5

First come, first served: The first emerging queen monopolizes reproduction in the ant *Cardiocondyla "argyrotricha"*

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Abstract

While colonies of most tropical species of the ant genus *Cardiocondyla* regularly contain multiple egg laying queens (polygyny), single-queening (monogyny) evolved convergently in a Palearctic clade of *Cardiocondyla* and in Southeast Asian *C. "argyrotricha"*. In the latter species, monogyny is probably an adaptation to patchily distributed, but highly stable nest sites. In experimentally orphaned colonies of *C. "argyrotricha"* the first emerging queen shed its wings, began to lay eggs, and stayed mostly on the brood pile. Later emerging queens remained in the peripheral areas of the nest without dispersing but retained their wings even after mating in the nest. Aggressive interactions among queens and between workers and queens were occasionally observed, but it appears that the order of emergence of queens determines which of them will become reproductive and inherit the nest. We conclude that young queens commonly compete for nest inheritance in some species of *Cardiocondyla* and that queen-queen antagonism does not necessarily involve lethal fighting.

Introduction

Rich food sources, territories, and breeding partners are valuable resources. Individuals may therefore greatly benefit from inheriting such capital from their ancestors (e.g., Woolfenden and Fitzpatrick 1978; Pusey and Packer 1987a; Myles 1988), and kin conflict over resource inheritance is a common phenomenon throughout the animal kingdom. A stable nest, with an established worker force and provisions, may be particularly valuable in social Hymenoptera (ants, bees, wasps). During dispersal and colony founding, young queens have to find an appropriate nest site and to escape predators and pathogens, which both is associated with a high risk of failure (Cronin *et al.* 2013). In contrast, young queens that avoid solitary founding by mating and laying eggs in their natal nest have far better fitness perspectives (Myles 1988).

Philopatric reproduction is easily possible when colonies may contain multiple reproductives (polygyny), but in species with single queens it may be associated with fights over nest inheritance. In paper wasps and other social insects with limited caste differentiation, females establish rank orders in which a high-ranking individual may replace an old reproductive and take over its resources (Monnin and Peeters 1998; André *et al.* 2001; Field and Cant 2009). Similarly, virgin honeybee queens fatally fight over the breeding position once their mother queen has left the nest with a part of the workers to found a new society (Gilley 2001). In monogynous ants with pronounced caste diphenism, fighting over inheritance of an established nest appears to be uncommon (e.g., Heinze 1993). The lifespan of the colony is typically bound to the lifespan of the single reproductive queen, and the colony quickly declines after the death of the reproductive.

Here we describe nest inheritance in colonies of the ant *Cardiocondyla “argyrotricha”* (provisional name of a recognized morphospecies to be described by B. Seifert). Species of the genus *Cardiocondyla* are characterized by the occurrence of wingless fighter males, which permanently stay and mate in their natal nests (e.g., Kinomura and Yamauchi 1987). Polygyny is ancestral in this genus, but several Palearctic species have secondarily evolved single-queening (Heinze *et al.* 2005; Schrempf and Heinze 2007; Oettler *et al.* 2010). *C. “argyrotricha”* belongs to a clade of Southeast Asian species of *Cardiocondyla,* in which queens in some species are mutually tolerant and form facultatively polygynous colonies (e.g., Heinze *et al.* in press), while queens of other species build reproductive hierarchies (Yamauchi *et al.* 2007) and fight for nest inheritance (Heinze and Weber 2011). Our study aimed to determine the number of reproductive queens in *C. “argyrotricha”*, to investigate whether emerging queens in queenright and experimentally orphaned colonies fight for nest inheritance, and to examine if age positively affects the likelihood of a young queen to be dominant over later eclosing queens.

Methods

Ant collection and rearing

Colonies of *C. "argyrotricha"* were originally collected by S. Frohschammer and A. Schulz in 2007 and 2008 in various places in Malaysia and the Philippines (details will be given with the species description). In 11 of 14 collected colonies, only a single wingless queen was present (Table 1). Additional winged female sexuals (mean 6.3 ± SD 6.9) were present in 7 of 13 colonies.

Lab observations (see below) showed that female sexuals may shed their wings and lay eggs without mating but may as well retain their wings for several weeks after mating. As we did not dissect all studied female reproductives we here use a morphological definition of the term "queen" and in the following refer to all potential female reproductives as queens.

Colonies were reared in the lab in three-chambered plastic nests with a plaster floor (Buschinger 1974) and fed twice a week with cockroaches and honey. The nesting site was an artificial cavity in the plaster, which was covered with a red foil. Colonies were kept in incubators at near-natural temperature cycles (14 hours 28°C, 10 hours 23°C). Queens could leave the nest cavity, but could not escape from the three-chambered nest box.

Behavioural observations

In summer 2010, we set up 13 experimental colonies (ten from the Philippines, three from Malaysia) each with 30 workers and brood (eggs and larvae) to examine the behaviour of colonies of *C. "argyrotricha*". As the number of adult males or male pupae was limited, only nine colonies contained a wingless male and only in these colonies queens had a chance to mate. However, male presence did not affect queen behaviour (see below).

Two different behavioural experiments were performed:

(a) Observation of the behaviour in colonies with an egg-laying queen and several emerging virgin queens. In addition to the initial composition of experimental colonies, we added one queen pupa into each nest. Two weeks after hatching of the queen, i.e., when the queen had shed its wings and started egg laying, we placed five queen pupae of different developmental stages into the colonies (experiment A, $n = 6$).

(b) Observation of the behaviour in orphaned colonies with subsequently emerging virgin queens. Five queen pupae of different developmental stages (in order to have approximately one hatching queen per day) were placed into the colony (experiment B, $n = 7$).

We expected that queens in experiment A would already have obtained a stable top rank before their sisters had eclosed and that the rate of aggression among queens would therefore be lower than in experiment B.

Emerging queens were marked by tying thin wires of different colour around different parts of their bodies (Mirenda and Vinson 1979). Colour and position were chosen randomly. Queen behaviour was not influenced by the wire.

Table 1: Colonies collected on the Philippines and in Malaysia, including the numbers of winged and wingless queens, males, and workers.

colony	**winged queens**	**wingless queens**	**males**	**worker**
P08-27	-	-	-	18
P08-28	-	1	-	1
P08-30	-	1	-	20
P08-31	1	1	-	30
P08-37	-	1	1	60
P08-42	5	1	1	60
P08-44	3	1	-	40
Mal-11	-	1	1	50
Mal-12	-	1	-	27
Mal-16	11	2	1	70
Mal-18	20	4	1	130
Mal-19	-	1	-	20
Mal-26	2	1	1	60
Mal-123	2	1	-	50

For both experiments, we recorded queen number (winged, wingless), the day of queen emergence, aggressive interactions both among queens and among workers and queens (biting, threatening, boxing, stinging, mounting, see Heinze and Weber 2011), and the location of the queens in the nest (core area of brood pile, periphery of brood pile, away from brood pile but in the nest, outside of nest). We applied the scan sampling method (Altmann 1974, Hepworth and Hamilton 2001) to assess the behaviour and location of the individuals studied. During each observation period (experiment A; 15min, once per day, experiment B: 10 min twice per day) each queen was observed in the same predefined order. In total, experiment A was observed over 900 minutes during 10 days, experiment B over 1050 minutes during 14 days after the emergence of the first queen. The total observation time of each colony amounted to 150 minutes. Each colony was observed at different times of the day to avoid possible effects of daytime on behaviour.

All statistical analyses were performed and graphs were generated with the statistical software R 3.2.1 software (R Development Core Team, 2015) and the glmmPQL function from the MASS library of R. We used generalized linear mixed models (GLMM) to analyse the frequency of scans spent in different locations in the nest, and aggressive interactions. As we had proportional data, we used binomial error distributions. Because of overdispersion, the standard errors were corrected using a quasi-GLM model (Zuur *et al.* 2009). We ran GLMMs with location of the queens in the nest (core area of the brood pile, periphery of the brood pile or away from the brood pile) as response variables (we did not include the location “outside of the nest” in statistical analyses and figures as queens stayed there only very rarely, see below) and female status (winged, wingless) or behavioural experiments (A and B) as explanatory variables. The models to test aggression (attacks received from both queens and workers, received from queens, and received from workers) included female status or behavioural experiments as explanatory variables. The variable “male presence” was also included in all the models to control for a possible effect of the mating status of the queens. Because several colonies from two populations and several queens from the same colonies were observed and to account for pseudoreplication, the variable “colony” was nested within the variable “population” and entered as a random variable in all models.

Determination of reproductive status

To determine the mating status of queens present in colonies of *C. "argyrotricha"*, we dissected all queens of two additional laboratory colonies following Buschinger and Alloway (1978). We determined if their spermathecas contained sperm and categorized three states of ovarian development: (a) elongated ovaries containing several large eggs, (b) short ovaries containing only few small eggs, and (c) short ovaries without any eggs.

Results

In each colony of both experiments, only a single queen shed its wings and started to lay eggs (n = 13). This was always the first emerging queen except in one colony of experiment B, in which two queens emerged on the same day. Wing shedding took place four days after eclosure regardless of the presence of a male, i.e., several queens shed their wings queens without having the chance to mate. Surprisingly, male presence and thus mating status did not affect queen location and behaviour (p > 0.05 in all GLMM of the study).

First and later eclosing queens did not only differ in wing shedding but also in where they stayed during most of the observation time (Fig. 1). In both experiments, the queen eclosing first in a colony was more frequently observed in the core area of the brood pile compared to later eclosing queens (experiment A: t = 7.980, p < 0.001; experiment B: t = 9.163, p < 0.001). Queens were only occasionally observed outside of the nest (in only 2.4% of the total number of scans).

The location of winged and wingless queens differed significantly between the two experiments (Fig. 1). In experiment A, the wingless queen was found significantly more often in the core area of the brood pile (t = -2.319, p = 0.046) and significantly less frequently in its periphery (t = 3.104, p = 0.013) than in experiment B. Winged queens of experiment A were observed significantly less frequently in the periphery of the brood pile (t = 3.386, p = 0.007) and significantly more often away from it (t = -3.104, p = 0.011) than in experiment B.

Aggressive interactions among queens and between workers and queens were only rarely observed (experiment A, observation time 900 minutes: total attacks by queens: 26, total attacks received by queens: 93; experiment B, observation time 1050 minutes: total attacks by queens: 26, total attacks received by queens: 46). In experiment A, both winged and wingless queens showed aggression in four of six colonies. In two of seven colonies of experiment B, only the wingless queen attacked winged queens. No lethal fighting was observed in any of the colonies. Analysis of aggressive behaviour of workers and queens directed to queens (aggression received) showed that while mounting was the most frequent aggressive behaviour in experiment A (62 of a total of 93 aggressive interactions) it was completely absent in experiment B (0 of a total of 46 aggressive interactions).

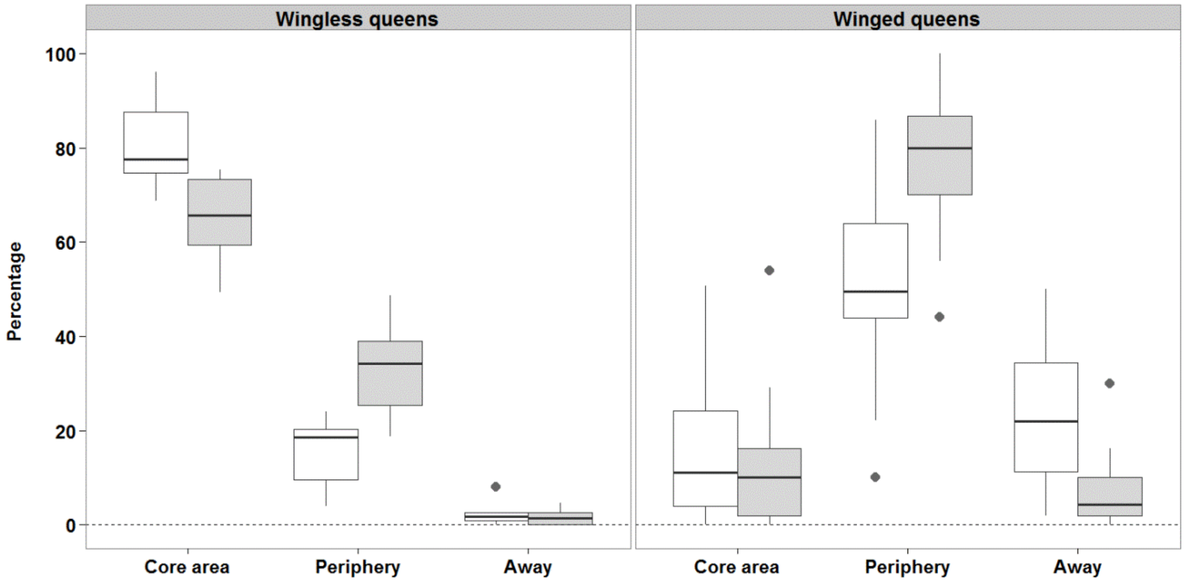

Fig. 1: Comparison of the percentages of scans during which winged and wingless queens of the ant *C. “argyrotricha”* were observed in different locations of the nest (core area of the brood pile, periphery of the brood pile, away from the brood pile) between the two experiments (white plot: experiment A, grey plot: experiment B) (medians, quartiles, range, and outliers).

Pooling all types of aggressive behavioural interactions showed that in experiment A wingless queens were significantly less frequently attacked than winged queens (*t* = - 2.423, *p* = 0.026) (Fig. 2A), but not so in experiment B (*t* = 0.432, *p* = 0.231) (Fig 2B). In experiment A, worker aggression was directed more often to winged than to wingless queens at a marginal significance (*t* = -1.978, *p* = 0.063). In experiment B, the amount of received aggression did not differ significantly between winged and wingless queens (*t* = - 0.412, *p* = 0.685) (Fig. 2B). Winged queens tended to receive more aggression in older, established colonies (experiment A) (*t* = -1.950, *p* = 0.083). Aggression directed to wingless queens did not differ between the two experiments (*t* = -1.112, *p* = 0.295). The frequency of aggressive interactions was too low to determine dominance relationships among individual queens and to construct hierarchies.

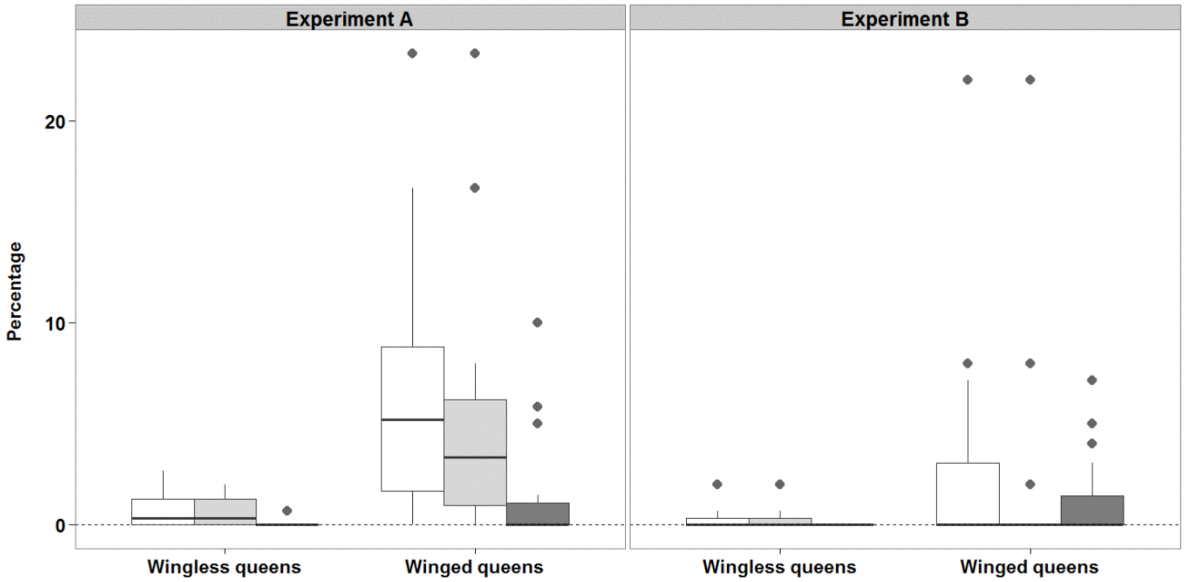

Fig. 2: Aggressive behaviour received by winged and wingless queens from other queens and workers (white plot), solely from workers (light grey plots), or solely from queens (dark grey plots) in the two experiments (medians, quartiles, range, and outliers).

The dissection of all wingless (*n* = 2) and winged queens (*n* = 21, 20 of them having a fully sclerotized black cuticle, and thus not being freshly emerged) of two additional laboratory colonies showed that all but one queen, which had just emerged (grayish cuticle), were inseminated by the single wingless male present in the colony. However, only the single wingless queen of each colony was reproducing and had elongated ovarioles with several large eggs. In one colony with one wingless queen and 14 inseminated winged queens, four small eggs were visible in one winged queen. However, neither yellow bodies nor mature eggs were present.

Winged queens were rarely observed outside of the nest in both our experimental colonies also additional laboratory colonies during the first three or four weeks of their adult life. In contrast, in very large laboratory colonies with more than 100 workers, winged queens were often away from the maternal nest, e.g., in another chamber of the three-chambered plastic boxes. Such large colonies occasionally contained two wingless queens in different parts of the nest (see also field data).

Discussion

Our study reveals a reversal from polygyny with mutually tolerant, reproductive queens to the monopolization of reproduction by a single queen in the ant *Cardiocondyla "argyrotricha"*. While colonies of related species are facultatively polygynous (Oettler *et al.* 2010), in *C. "argyrotricha"* a) the majority of natural colonies (12 out of 14) contained only a single wingless queen, b) in all experimental colonies with multiple female sexuals only a single queen shed its wings, and c) the dissection of all queens from two additional laboratory colonies with multiple inseminated female reproductives revealed that only a single queen was laying eggs. Our observations show that the first young queen to emerge after the removal of the fertile queen will inherit the colony, mate in the nest, shed its wings, and begin to reproduce. Subsequently emerging queens may similarly mate but retain their wings and at least temporarily stay in the nest without laying eggs. The single wingless queen is found significantly more often in the core area of the brood pile than the winged queens. This matches previous observations in the related species *C. "latifrons" and C. "microseta"* (provisional names of recognized morphospecies to be described by B. Seifert) that the social and reproductive status of queens is not only reflected in their behaviour but also in their location in the nest (Yamauchi *et al.* 2007).

Our study suggests that colonies of *C. "argyrotricha"* are at least temporarily functionally monogynous, i.e., of several inseminated queens found in the nest only a single queen reproduces (Buschinger 1968). Such high reproductive skew is typically associated with patchily distributed habitats with a limitation of suitable nest sites (Bourke and Heinze 1994). Like its congener *C. "latifrons"* (Heinze and Weber 2011), colonies of *C. "argyrotricha"* inhabit crevices in large boulders, which are relatively stable but not very common (S. Frohschammer, pers. comm.). Hence, the nest and also its workforce are valuable resources to the queens. In addition, dispersal and solitary founding might be particularly risky.

Functional monogyny, high reproductive skew and competition over an established nest are typically based on queen antagonism, such as biting, stinging and violent antennation, which leads to the formation of a dominance hierarchy with a single dominant, reproducing queen. Worker aggression (policing) may additionally stabilize these rank orders (Heinze and Smith 1990; Gill and Hammond 2011; Trettin *et al.* 2011). In contrast to the high-skew species *C. "latifrons"*, where in a similar experiment half of the winged queens were killed by others within a few weeks after emergence (Heinze and Weber 2011), aggression between *C. "argyrotricha"* queens occurred only rarely and we did not observe any lethal fighting. When emerging in orphaned colonies, queens received only very few attacks from workers and other queens but nevertheless in each colony only a single queen shed its wings and began to lay eggs. From the finding that in all but one colony the first emerging queen became wingless, we conclude that it is mainly the order of emergence of queens in an orphaned colony that determines which queen will inherit the nest. A head start of even one day appeared to be sufficient to consolidate its reproductive role. This is again strikingly different from the situation in *C.* "*latifrons*", where the oldest queen did not consistently have a reproductive advantage over queens that emerged one or a few days later (Heinze and Weber 2011).

In colonies in which one queen had eclosed two weeks before the other queens (experiment A), aggression against the later emerging queens tended to be more frequent than in experimental colonies in which all female sexuals eclosed within a few days (experiment B). Aggression in colonies with an established queen appeared to be more ritualized and consisted of one queen or one worker mounting another queen and standing on top of it. Aggression among queens in experiment B usually consisted of antennal boxing, biting, pulling on the antennae or legs, or threatening with opened mandibles. Like in other high-skew species (Heinze and Smith 1990; Gill and Hammond 2011; Trettin *et al.* 2011), worker aggression presumably served to maintain the reproductive monopoly of the wingless queen and to drive away winged queens from the brood pile. Winged queens were crowded out from the core area of the brood pile without completely leaving the nest at least during the 24 days of our study.

Typically, young ant queens shed their wings rapidly after insemination. In *C.* "*argyrotricha,*" all queens but the single reproductive remained winged though being inseminated. This might suggest that winged queens wait for favourable environmental conditions to disperse on the wing (e.g., Kinomura and Yamauchi 1987). In casual observations of stock colonies we indeed noticed winged queens trying to disperse from the storage box. An alternative option would be to bet on the possibility of inheriting the nest after the death of the wingless queen. As *Cardiocondyla* queens are short-lived compared to the queens of other ants (in most species of *Cardiocondyla* queens live for approximately 1 year or less, e.g., Yamauchi *et al.* 2006), such "hopeful reproductives" might have a considerable change of obtaining fitness while avoiding the risks of dispersal and independent founding. Furthermore, in the largest colonies in the field and the laboratory, mutually intolerant queens might space out in different parts of the nest and begin to reproduce ("oligogyny", as in *Camponotus ligniperdus* or *Iridomyrmex purpureus*, Hölldobler and

Carlin 1985; Gadau *et al.* 1998). Casual observations suggested that these wingless queens engage in lethal fighting when they meet (S. Frohschammer, pers. comm.).

The reversal from ancestral polygyny to obligatory or functional monogyny in *C. "argyrotricha*" and related taxa evolved convergently to that in the Palearctic monophylum comprising *C. elegans*, *C. batesii*, and others (Schrempf and Heinze 2007; Oettler *et al.* 2010). Interestingly, the two monogynous or functionally monogynous clades differ in queen mating frequency: in the Palearctic clade, monogyny is associated with multiple mating of the queens (e.g., Schrempf *et al.* 2005; Lenoir *et al.* 2007). Lethal male fighting has been observed in *C. "argyrotricha*" and *C. "latifrons*" (Yamauchi *et al.* 2006) and multiple mating is therefore unlikely. The taxa have therefore been considered to be monandrous though genetic proof is lacking (Hughes *et al.* 2008). The genus *Cardiocondyla* therefore provides an interesting model system for investigations on the evolution of queen number, reproductive skew, and mating frequency and their ecological correlates in social insects.

Acknowledgements

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General Discussion

The results of the studies carried out in the course of this thesis are discussed in the individual chapters (1-5). The following paragraphs highlight and discuss the main findings, and add some further considerations potentially useful for future investigations.

Male Traits in *Cardiocondyla*

In the course of this thesis, both qualitative and quantitative data of several morphological structures of ergatoid, and if present, winged males of several species of *Cardiocondyla* were obtained in order to provide information suitable for retracing the evolution of male traits, such as alternative reproductive tactics and exaggerated morphologies.

The studies presented in chapter 1 and 2 provide data which are valuable in the context of sexual selection and phylogenetic relationships. Chapter 1 reveals are more pronounced structuration of male genitalia in species with tolerant and territorial males, in which strength of sperm competition is assumed to be higher because multiple males compete for mating with available virgin queens, which are known to mate multiply at least in the two tolerant species (Schrempf *et al.* 2005, Lenoir *et al.* 2007). Future studies may focus on the function of these structures to clarify whether the observed variation results from differences in queen mating frequency and male competition.

As another consequence of sexual selection, males may evolve weapons used in fights over access to females (Emlen 2008). Ergatoid males of several species of *Cardiocondyla* kill ergatoid rivals in order to monopolize mating with available virgin queens, and they use their mandibles to do so (Anderson *et al.* 2003). Ergatoid males of species belonging to different clades exhibit different ways of eliminating their competitors, reflected in different mandible shapes (Oettler *et al.* 2010). The study presented in chapter 2 reveals that ergatoid male mandible morphology is correlated to the type of male antagonism. It demonstrates that males, which are fighting against mobile adult competitors, possess sickle-shaped mandibles that are the longest mandibles relative to body size of all examined male mandibles. In addition, these mandibles are directed distally in contrast to the mandibles of males and workers of all other examined species, which are directed distoventrally. These mandibles are not used to kill the competitor directly, but allow getting a firm grip around the rival's body and subsequently besmear him with a hindgut secretion, which elicits worker aggression. As a consequence, the rival will be eliminated in an indirect way (e.g. Kinomura and Yamauchi 1987; Stuart *et al.* 1987). The shortest mandibles relative to body size are found in ergatoid males with worker-like mandibles, which exclusively kill young males by crushing their not yet fully sclerotized cuticle. Future studies might compare the mandibles' content of zinc between tolerant ergatoid males and ergatoid males that crush the cuticle of young males, because tooth hardness has been shown to be positively correlated with zinc content in leaf-cutting ants (Schofield *et al.* 2002).

Most often, phylogenetic trees are based on genomic data only. Yet, for instance in the case of myrmicine ants, phylogenetic relationships remain obscure (Ward *et al.* 2015). The study of the morphology of males of the social Hymenoptera, which have been largely ignored to date, may provide valuable information useful e.g. for resolving phylogenetic relationships. In *Cardiocondyla*, mandible morphology of ergatoid males allows for the discrimination between the two clades of the genus (Oettler *et al.* 2010). Chapter 1 presents morphological characters of males valuable for both taxonomic and phylogenetic purposes, and thus demonstrates the importance of detailed comparative morphological studies not only of workers and queens, but also of males.

With regard to the Myrmicinae, in particular the tribe Crematogastrini, the study in chapter 2 proposes one character of the male external genitalia of *Cardiocondyla* to be potentially useful for resolving phylogenetic relationships. Future researchers are encouraged to examine the genitalia of potentially closely related species in order to determine presence or absence of a minute structure (the "parameral hook") developed by the outer valves, the parameres, as it may reveal the as yet unknown sister taxon of the genus.

Unfortunately, many morphological studies have been carried out before the advent of modern techniques such as scanning electron microscopy and micro-CT scanning. As a consequence, potentially important information may not have been assessed and wrong conclusions may have been drawn. For instance, with regard to *Cardiocondyla*, a separation of the genus in two subgenera has been advocated because a minute structure of the male external genitalia has been overlooked (Kugler 1983). It is therefore recommended to only carefully include previously published data obtained using devices with low magnification and resolution power.

Genetic structure of *C. obscurior*

As typical for members of the genus *Cardiocondyla*, members of the polygynous species *Cardiocondyla obscurior* regularly mate within the nest with close relatives. As sex is not determined by single locus complimentary sex determination (sl-CSD) in *C. obscurior* (Schrempf *et al.* 2006), mating between close relatives thus does not lead to the production of diploid males, which are typically sterile or not viable (Stouthamer *et al.* 1992, Cook and Crozier 1995). Yet, continuous inbreeding over several generations has been shown to cause negative effects after several generations (Schrempf *et al.* 2006). The study presented in chapter 3 provides first insights into the population and colony genetic structure and allows making some assumptions about the mechanisms to avoid inbreeding depression in natural populations.

Microsatellite primers were developed using a method based on selective hybridization (Tenzer *et al.* 1999, Gautschi *et al.* 2000), which is usually very efficient. Yet, although 48 microsatellite loci were detected, only six loci were variable. This is presumably due to the high level of inbreeding in the species, resulting from intranidal mating usually involving close sib, which in consequence leads to low genetic variability. Yet, using the six established polymorphic microsatellite markers allowed analyzing several colonies of a Japanese population of *C. obscurior* (chapter 3). Interestingly, relatedness varied greatly among colonies, with some of the colonies having relatively high relatedness values and some having surprisingly low values. High nestmate relatedness is a consequence of intranidal mating and regular mating between close sib. Previous analyses of relatedness in monogynous species of *Cardiocondyla* also revealed such high relatedness values (Schrempf *et al.* 2005). Analysis of a small colony of *C. obscurior* consisting of only few individuals showed that the two queens of the nest were highly related. This colony was most probably only recently founded by budding by two sister queens accompanied by few workers. Yet, in some of the larger and thus probably older colonies, relatedness of colony members was surprisingly low, indicating that alien queens are adopted, as has been suggested before (Kinomura and Yamauchi 1987). These outbreeding events counteract negative effects due to regular inbreeding in *C. obscurior*. Colonies of other tramp species, such as e.g. *Linepithema humile* appear to regularly adopt foreign individuals and thus have only low relatedness values despite mating in the nest (Krieger and Keller 2000). This presumably is an adaptation to avoid negative effects of low genetic variability probably arising through founding effects.

Alternative Reproductive Strategies of Virgin Queens

Chapter 4 reveals an extraordinary reproductive strategy of virgin queens not described in members of the social Hymenoptera before. To avoid failure at colony foundation, young uninseminated queens of *C. "argyrotricha"* may first produce male offspring resulting from haploid, unfertilized eggs, later mate with their own sons and thereby switch to the production of diploid offspring, i.e. workers and female sexuals. This is extraordinary, as queens of social Hymenoptera typically mate only during a brief period early in life and appear to be unattractive to males after the start of reproduction, which in consequence avoids remating (Wilson 1971). What is more, due to the standard mechanism of sex determination of the social Hymenoptera (sl-CSD), mother-son inbreeding will result in diploid male production at the expense of workers (van Wilgenburg *et al.* 2006). Yet, sex has been shown to be determined by another mechanism in species of *Cardiocondyla* (Schrempf *et al.* 2006), making mother-son mating a possible strategy founding colonies with the help of only a few workers in the absence of a mating partner. Usually, unmated virgin ant queens initiate haploid male egg production when they remain uninseminated for several weeks. Such queens may remain in a polygynous colony and contribute to male offspring, but will not be able to establish a colony on their own. The production of diploid worker offspring of queens, which have mated with their sons, as well as the lack of diploid males, reveals sl-CSD to be absent in *C. "argyrotricha"*, too.

Mating in *C. "argyrotricha"* typically occurs between brothers and sisters, i.e. virgin queens mate with their brother. The described case of mother-son mating appears to be restricted to cases where no male is available for mating. Mother-son mating probably evolved due to the unusual life-history of this species, which combines monogyny (chapter 5), intranidal mating, and extremely female biased sex ratios, which in consequence involves the risk of queen emergence in colonies in which no male is present.

Yet, the study shows that mother-son mating can evolve under certain ecological conditions. Based on the findings presented in chapter 4, other species of *Cardiocondyla* have been studied, and indeed mother-son mating has been found to occur in at least one further species of the genus (Moske *et al.* unpubl.) Though it has only been rarely described before from other species, mother-son mating might not be restricted to *Cardiocondyla* queens but might occur more widely in species with intranidal mating.

Nest Inheritance

Chapter 5 describes a reversal to single-queening in *C. "argyrotricha"*, as, despite the presence of several inseminated queens, only one queen shed wings and reproduced in experimental colonies. As mating typically occurs inside the nest in all species of the genus, several potential reproductive queens are temporarily present even in monogynous species (Schrempf and Heinze 2007). Yet, due to the limited observation time and the experimental conditions, which did not allow queen dispersal from experimental colonies, it is not possible to state whether colonies of *C. "argyrotricha"* are monogynous or rather functionally monogynous. Functional monogyny is a rare state in ants, in which several inseminated queens are present in the nest, however, only a single queen is reproducing (Buschinger 1968). Young inseminated queens stay in the maternal nest during the 14 days of the observation time. Yet, winged queens of large lab-reared colonies of *C. "argyrotricha"*, which are housed in three chambered plastic boxes, were observed staying in one of the chambers not used as the nesting chamber and leaving the plastic box as soon as the lid was opened. Because of this, functional monogyny, as observed in the experiment, may only be a temporary state in colonies of *C. "argyrotricha"*. Furthermore, functionally monogynous species are typically characterized by queen dominance behaviour (Heinze and Lipski 1990, Heinze and Smith 1990), such as biting, stinging and antennating. In *C. "argyrotricha"*, however, aggressive interactions occurred on a low level only, and it appeared that mainly the order of emergence determined which queen will become reproductive. This might indicate, that functional monogyny is only a temporary state in the species, probably linked to the habitat, which is characterized by only limited availability of suitable nest sites and high risks of failure for queens seeking to found colonies solitarily. These young inseminated queens in a queenright monogynous colony may avoid the risks of dispersal and independent colony foundation and stay as "hopeful reproductives" at least for some weeks in the natal colony in order to wait for the opportunity of future reproduction after the death of the mother queen, and thus to inherit the nest. The nest is an especially valuable resource in such habitats, which are associated with high costs for solitarily founding queens (Myles 1988). By retaining their wings, however, they may later nevertheless disperse and try to found a new colony independently.

The lifespan of monogynous ant colonies is typically believed to be bound to the lifespan of the single reproductive queen. Yet, it has been indicated that colony inheritance by a related individual may be much more common than previously thought (Peeters and Molet 2010).

Queen turnover is difficult to examine, as ant queens are typically long-lived. As workers are much shorter lived, genetic analyses are only suitable to detect queen turnover shortly after the death of the mother queen, before the emergence of worker offspring of the secondary reproductive queen. Colonies of *Cardiocondyla* are rather small, and may be easily reared in the lab. In addition, queens of monogynous species of *Cardiocondyla* are relatively short-lived if compared to the queen lifespan of other monogynous ant species (Schrempf and Heinze 2007). As several species of *Cardiocondyla* have not yet been described and studied in the lab, further single-queened species may be detected in the future, and queen turnover and its consequences may be easily observed in such species in the lab.

Conclusions

This thesis examines a variety of different aspects of reproduction in the ant genus *Cardiocondyla* and provides data, which are not only important to retrace the evolution of alternative reproductive tactics in both male and female sexuals of the genus *Cardiocondyla*, but may also be relevant to the study of social hymenopteran biology in general.

The extraordinary ant genus *Cardiocondyla* has been extensively studied in the past years. Yet, many species remain undescribed, and the position of the genus among the Crematogastrini is still unclear. Future research surely will reveal more fascinating aspects of its biology and thereby add more facets to the study of the evolution of the social Hymenoptera.

Summary

This thesis focusses on a variety of different aspects of the reproductive life cycle in the myrmicine genus *Cardiocondyla*, deviating from the typical pattern of reproduction in the social Hymenoptera:

Cardiocondyla is characterized by intranidal mating involving close sib, and, as a consequence, high inbreeding coefficients and extremely female-biased sex ratios. Linked to mating in the nest are alternative reproductive tactics: two different phenotypes are produced in several species of the genus, differing in morphology, physiology and behaviour. Winged males are adapted to disperse, search for female reproductives, and subsequently mate with them. In contrast, wingless, ergatoid (= worker-like) males stay in the maternal nest and mate with available virgin queens. In several species, ergatoid males monopolize mating with female reproductives by eliminating their competitors. Different strategies of rival killing are correlated to the shape of ergatoid male mandibles. Not only male, but also female reproductive strategies are variable in the genus, as, despite polygyny (multiple reproductive queens per colony) representing the ancestral condition in *Cardiocondyla*, several Palearctic species show a reversal to single-queening. Regular inbreeding without negative effects indicates that sex is not determined by single-locus complementary sex determination in *Cardiocondyla*.

The thesis wants to shed light on the evolution of the diverse male traits in *Cardiocondyla*. To do so, **Chapter 1** provides the first detailed comparative morphological study of the male genitalia of *Cardiocondyla*. Several morphological characters of external genitalia of ergatoid males and, if present, winged males, of most major species groups are described. Some characters of male genitalic morphology are useful for infrageneric taxonomy, as they allow distinguishing between the two clades of the genus, as well as between several species groups. Furthermore, one of the examined structures, the parameral hook, potentially has value for resolving relationships of the myrmicine tribe Crematogastrini. Interestingly, male external genitalia of species in which males are tolerant of each other’s presence or territorial exhibit a more pronounced structuration, probably linked to higher levels of sperm competition.

Chapter 2 investigates whether different male reproductive tactics are reflected in ergatoid male morphology, especially in mandible size and shape. Mandibles of ergatoid males turned

out to be highly variable in both size and shape not only between the two clades of *Cardiocondyla*, but also among species belonging to *Cardiocondyla* clade A. Extremely long, distally oriented mandibles appear to be associated with indirect killing of mobile adult competitors. Particularly short mandibles are presumably adapted to a mating tactic in which ergatoid males exclusively kill their rivals by crushing their cuticle.

Chapter 3 provides first insights into the breeding structure of the tramp ant *Cardiocondyla obscurior*, which is distributed throughout the tropics and subtropics of all continents. Six polymorphic microsatellite markers were established and used to analyse the genetic structure of several colonies of a Japanese population. Nestmate relatedness turned out to be very variable in the different colonies. Despite regular mating in the nest and between close sib, which is characteristic for species of the genus *Cardiocondyla*, several colonies exhibited surprisingly low values of nestmate relatedness. This indicates that colonies regularly adopt alien queens in order to avoid negative effects of inbreeding by increasing genetic variability.

Chapter 4 reveals an outstanding reproductive tactic of virgin queens to avoid failure at colony foundation. Virgin queens of *C. "argyrotricha"*, which face the risk of emerging in the absence of a mating partner due to highly female-biased sex ratios, may first produce male offspring resulting from unfertilized, haploid eggs. Later, they may mate with their own sons and subsequently switch to the production of diploid offspring (workers and female sexuals). In consequence, queens are able to start their own colony. This is extraordinary, as queens of the social Hymenoptera typically avoid inbreeding, mate only during a short period early in life and do not remate thereafter. The study shows that the standard mechanism of sex determination of the social Hymenoptera (single locus complementary sex determination) is absent in *C. "argyrotricha"*, as otherwise mother-son inbreeding would result in diploid male offspring. In addition, it demonstrates that mother-son mating, which is known to occur in solitary haplodiploid animals, may also evolve under certain conditions in the social Hymenoptera.

Chapter 5 reveals a reversal from polygyny (multiple egg laying queens per nest), which is the ancestral condition in the genus *Cardiocondyla*, to single-queening in *C. "argyrotricha"*. In experimentally orphaned colonies it was mainly the order of queen emergence which determined which queen became reproductive and thus inherited the nest. The first emerging queen shed wings, began to reproduce and stayed mostly on the brood pile. Although several queens were inseminated, all but the reproductive queen retained their wings and only rarely

behaved aggressively towards the reproductive queen. Presumably these queens stay at least temporarily in the nest as “hopeful reproductives”, in order to bet on the opportunity of future reproduction and thus nest inheritance, instead of facing the risks associated with dispersal and independent colony foundation.

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