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Genetic Diversity in Pioneer Ants: The *Cardiocondyla shuckardi* Group

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Abstract

The myrmicine ant genus *Cardiocondyla* consists of around 80 species of small, inconspicuous ants, which live mainly in subtropical and tropical Africa, Eurasia, and Australia. Several species have been accidentally introduced to America and have also invaded numerous originally ant-free islands around the world. The diversity of life histories in this genus, with lethally fighting wingless males, dominance hierarchies among queens, and considerable variation in the sociogenetic organization of colonies across species, has made it an interesting model to investigate the evolution and behavioral ecology of reproductive strategies. Taxa of the African *Cardiocondyla shuckardi* group are of particular interest, as in a phylogeny, they lie between facultatively polygynous species with fatal male competition and monogynous species with mutually tolerant males. Studies on male behavior and sociogenetics in *C. "venustula,"* a widespread member of the *C. shuckardi* group, showed that males defend small territories inside their subterranean nests. The aim of our study is to document the surprisingly large variability in mtDNA haplotypes both within and between populations in South Africa, Angola, and Côte d'Ivoire. As the different lineages are very similar in morphology and nuclear markers, more research is needed to clarify the very confused taxonomic situation in this fascinating group of ants.

Introduction

Cardiocondyla is a genus of approximately 80 species of small to minute, inconspicuous ants, which are widely distributed throughout Africa, Australia, and Eurasia. Over the last decades, the genus has received increased attention because of its peculiar male polyphenism, with "normal", docile, winged disperser males and wingless males, which engage in lethal fighting with rival males and attempt to monopolize mating with all young queens that emerge in their natal nests over several weeks or even months (Kugler 1983; Stuart et al., 1987; Kinomura & Yamauchi, 1987; Heinze & Hölldobler, 1993; Cremer et al., 2002). This reproductive life history is presumably an adaptation to life in small, defendable societies with a year-round availability of female

sexuals (Heinze, 2017). With the colonization of xeric and temperate habitats, alternative strategies have evolved. For example, in the Palearctic species, winged males have been lost completely, and wingless males have become mutually tolerant. Together with variation in queen number and queen mating frequencies, this makes the genus a suitable model for studies on the evolution of life histories and reproductive strategies (Oettler et al., 2010; Heinze, 2017; Jaimes-Nino et al., 2022).

Several *Cardiocondyla* species are cosmopolitan tramps, which live in parks, plantations, along beaches, and roadsides around the world (Seifert, 2003; Heinze et al., 2006; Wetterer, 2014, 2015). Among these is *Cardiocondyla venustula* Wheeler, 1908, a species originally described from the Caribbean islands of Culebra and Puerto Rico. *C. venustula* belongs to the



C. shuckardi group, a complex of ants that are widespread throughout the Afrotropical realm (Seifert, 2003, 2023). Colonies of these taxa consist of only a few dozen workers and may live in high densities of two or more nests per m² in the ground in natural grassland but also in parks and plantations (Jacobs & Heinze, 2019). Colonies have often been found close to water, e.g., in flood plains, near irrigation or roadside ditches, and in regularly watered lawns, but also abound in dry, rocky places and rehabilitated mining areas and ash dams (Majer & De Kock, 1992; van Hamburg et al., 2004).

Species delimitation by morphometry can be notoriously difficult in *Cardiocondyla* as cryptic species abound (Seifert, 2009, 2016; Okita et al., 2015). This appears to be particularly the case in the *C. shuckardi* group. Seifert (2003) recognized six valid species (in addition to *C. shuckardi* Forel, 1891 and *C. venustula*, *C. fajumensis* Forel, 1913, *C. unicalis* Seifert, 2003, *C. melana* Seifert, 2003, *C. longiceps* Seifert, 2003) and recently (Seifert, 2023) added *C. zoserka* Bolton, 1982, and *C. sekhemka* Bolton, 1982 to this group. Seifert's (2023) inclusion of *C. globinodis* Stitz, 1923 in this list must be disregarded as it is currently still considered a synonym of *C. venustula*, even though this synonymy is almost certainly erroneous (B. Seifert, pers. comm.).

The taxonomy of the *C. shuckardi* group is rather confused after an incomplete treatment by Seifert (2003), who transferred *C. globinodis* and *C. badonei* from synonymy with *C. shuckardi* to synonymy with *C. venustula*, but did not consider the positions of the other taxa (*C. brevispinosa* Weber, 1952; *C. fusca* Weber, 1952; *C. shuckardoides* Forel 1895; *C. wasmanni* Santschi, 1926 and *C. wasmanni sculptor*, Santschi, 1926) previously synonymized with *C. shuckardi* by Bolton (1982). These taxa thus remain synonyms of *C. shuckardi*, although they were omitted from Seifert's (2003) synonymic list of this species. This leaves a biogeographically anomalous picture, with the forms from mainland Africa that remain synonyms of *C. shuckardi* being those that have type localities (in the Democratic Republic of the Congo, Uganda, Cameroon, and Gabon respectively) geographically most removed from its type locality (in Madagascar), while *C. badonei*, Arnold, 1926 (described from Mozambique) and *C. globinodis* (described from Namibia) are classified as synonyms of *C. venustula*. Specimens of *C. zoserka*, originally described as female sexuals of a workerless social parasite of *C. shuckardi*, have now been identified as the regular winged males of a non-parasitic species (Heinze, 2020).

Investigations on how local mate competition among wingless males of *C. "venustula"* affects the structure of its colonies and populations (Jacobs & Heinze, 2017, 2019) suggested a large variation in sequences of the mitochondrial genes *cytochrome oxidase I* and *II* not only among but also within sampling sites. This indicated the co-occurrence of several distinct genetic lineages and prompted additional investigations in several parts of Africa. As full coverage of the entire range of the *C. shuckardi* group is beyond the

scope of an individual project, the aim of the present study is to document this diversity and to sensitize ecologists and entomologists about the possible occurrence of cryptic species in this interesting taxon.

Material and Methods

Partial and complete colonies of taxa belonging to the *C. shuckardi* group were collected in various sites in South Africa (Rietvlei Nature Reserve, Gauteng; Hlalanathi Drakensberg resort and other sites in uThukela valley, KwaZulu Natal), Angola (Luanda; Lubango, Huíla; Cussequê, Bié; Candelega, Cuando Cubango), Côte d'Ivoire (Comoé National Park), Madagascar (for details see Heinze et al., 2014), Hawai'i (Nualolo Trail, Kaua'i), and Puerto Rico (Coco Beach, Rio Grande). Nests were found by following foragers back to the needle-prick like nest entrance and excavated for studies on colony composition and male behavior. Individual workers from other areas were provided by colleagues.

Samples for sequencing were stored in 100% EtOH. DNA was extracted using a modified CTAB protocol (Sambrook & Russell, 2001), and the mitochondrial genes *CO I/CO II*, including intergenic tRNA^{Leu}, were amplified by PCR. As previously reported (e.g., Jacobs & Heinze, 2019), amplification using the primer combination C1-J 2183/C2-N-3661 (Simon et al., 1984; yielding 1450bp) did not work in all samples of the *C. shuckardi* group, and instead we had to amplify several individual fragments by combinations of C1-J-2813/CW-3031 (Brandt et al., 2007, 810bp) and C516ven-for (5' - ATT TTT TTC CAT ATT TAT YGG - 3', S. Jacobs, unpubl.)/A8-N-3914 (Simon et al., 1994, yielding 1200bp), or C1-J-2813/CW-3031 (810bp), C516ven-for/C2-N-3661 (930bp) and COICv-f/COICv-r (Jacobs & Heinze, 2019, 1100bp). The fragments were then combined to obtain the 1450bp sequence. PCR products were purified with the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Düren, Germany).

DNA was sequenced by LGC Genomics (Berlin, Germany), aligned using the Clustal W algorithm (Thompson et al., 1994) in BioEdit v 7.09 (Hall, 1999), and thereafter manually corrected for variation in the tRNA^{Leu} intergenic area, using the complete mtDNA sequence of *C. obscurior* for comparison (GenBank KX951753, Liu et al., 2019). For several specimens, only shorter sequences of around 460 bp of *CO I* could be retrieved, probably due to inadequate DNA quality or variation in the primer target sequences. Translation of the sequences into amino acids did not suggest any unexpected stop codons. It resulted in amino acid sequences broadly identical to that obtained by Liu et al. (2019) for *C. obscurior*. A fasta-file with all sequences is available upon request from the first author.

To compare variation in the *C. shuckardi* group with intra – and interspecific variation in previously obtained *CO I/CO II* sequences (1350 – 1445bp) of other species of *Cardiocondyla* (e.g., Oettler et al., 2010; Heinze, 2017) the

number of base substitutions per site was calculated using MEGA11 (Tamura et al., 2021). Genetic diversity among 154 specimens of the *C. shuckardi* group was illustrated by constructing a minimum spanning network (Bandelt et al., 1999) for 460bp using PopArt (<http://popart.otago.ac.nz/>). The evolutionary history was inferred from 91 longer sequences (up to 1445bp) using the Neighbor-Joining method (Saitou & Nei, 1987), with missing data coded as question marks. Branching patterns found in less than half of all bootstrap replicates were collapsed.

Results

Ants of the *C. shuckardi* group were abundant in grassland, parks, lawns, and particularly on river benches in all visited sites. Many nests were found in extremely wet, gravelly soil, e.g., in the flood plains of Comoé River, Côte d'Ivoire, the occasionally flooded sea shore at Coco Beach, Puerto Rico, and even in muddy ground at a tributary of Cussequ,

Angola. Nests were often found only a few centimeters below the surface, but in drier places, also down to a depth of 30cm and more (Fig 1). In many cases, solitary, dealate queens were found outside of the nest, probably dispersing on foot after mating in their natal nests. Males eclosing in colonies from South Africa, Madagascar, Côte d'Ivoire, Angola, Puerto Rico, and Kaua'i were almost invariably the wingless males typical for *Cardiocondyla*, occasionally with wing buds and traces of ocelli (Heinze et al., 2013, 2014; Jacobs & Heinze, 2017; Fig 2), but colonies in saturated soil, between the low and high river stages along the Orange River in the Richtersveld National Park, had both wingless and brachypterous males. The latter had a well-developed medial ocellus, weakly-developed lateral ocelli, and wings extending to about the mid-length of the first gastral tergite (PGH, unpublished observations). Winged males with spoon-shaped antennal tips were only found in two colonies of *C. zoserka* from Comoé, Côte d'Ivoire (Heinze, 2020).

CO I/CO II haplotypes exhibited surprisingly large



Fig 1. Unpaved parking lot at the entrance to Rietvlei Nature Reserve in Southern Tshwane, Gauteng, South Africa, with the entrances of nests of ants of the *Cardiocondyla shuckardi* group marked by little green flags. The inset shows how colonies were excavated and collected.

variation among individual sequences across and also within sampling sites, regardless of whether the full sequences (mean genetic distance $0.029 \pm \text{SE } 0.005$; nucleotide diversity π 0.0175), or only a short 460bp fragment of *CO I* was compared (mean genetic distance $0.037 \pm \text{SE } 0.008$; nucleotide diversity π 0.0345). The mean genetic distance in the full sequence estimated for colonies from lawns and sparsely vegetated patches at Hlalanathi Drakensberg Resort was 0.0245 (ranging from 0 to 0.0715, 24 colonies), from an unpaved parking lot at the entrance of Rietvlei Nature Reserve

0.0157 (0 – 0.0482, $n = 11$), the flood plain of Comoé River 0.0111 (0 – 0.0318, $n = 20$), and a moist meadow near Cussequ River 0.0132 (0 – 0.0271, $n = 5$). These values were much higher than in other species of this genus, in which mean pairwise genetic distances ranged from 0.0014 in *C. elegans* (18 colonies from several sites in France and Italy) to 0.009 in *C. nuda* (nine colonies from two sites in Queensland, Australia) (J. Heinze, unpublished; average over six species 0.0042).

The largest differences among samples of the *C. shuckardi* group from Hlalanathi or Cussequ were in the

range of genetic distances between closely related species of *Cardiocondyla* (*C. nuda* – *C. atalanta* 0.0662; *C. batesii* – *C. nigra* 0.0305; *C. obscurior* – *C. wroughtonii* 0.00478; *C. tjibodana* – *C. minor* – 0.0273; *C. latifrons* – *C. micropila* 0.0044; see also Okita et al., 2015, Seifert et al., 2017). In contrast, almost no variation was found in the introduced population at Puerto Rico (0.00125, 0 – 0.00472, 8 colonies). The two identical sequences from Kaua’i were also extremely close to the ones from Puerto Rico (0.0014, 0 – 0.00235). The 460bp fragment of *CO I*, which could be reliably obtained in all materials, corroborated the results obtained from the smaller set of samples with the full sequence. The mean pairwise differences between the haplotype of *C. zoserka*, which Bolton (1982) considered morphologically indistinguishable from *C. shuckardi*, and the other sequences of the *C. shuckardi* group was 0.1010 (0.0899 – 0.1134).

A minimum spanning network based on the 460bp fragment (Fig 3) revealed several divergent clusters of haplotypes, most of which correspond to well-supported branches in the NJ tree based on the complete sequence of



Fig 2. Laboratory colony of a taxon of the *Cardiocondyla shuckardi* group with three winged female sexuals, several black workers, a large, brown wingless male, and several pupae. Photo by S. Jacobs.

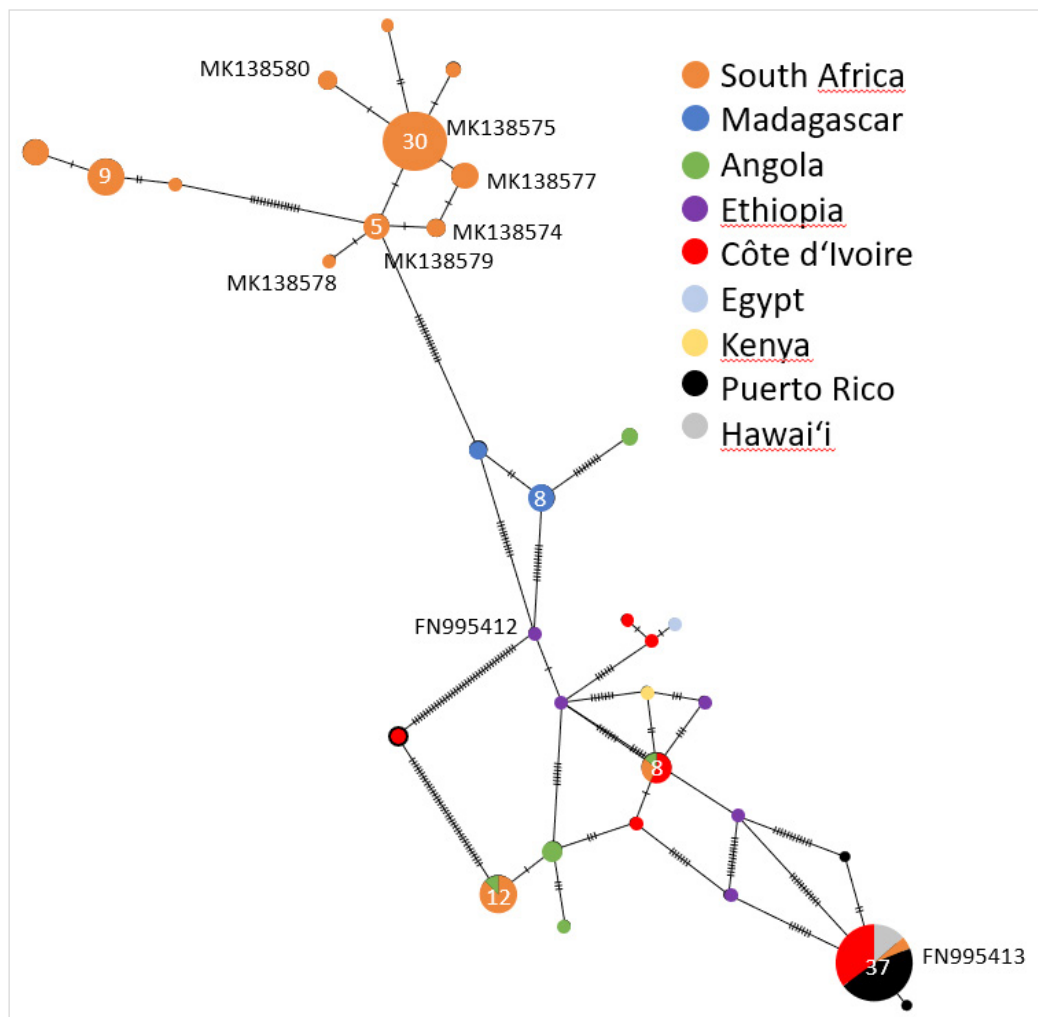


Fig 3. Minimum Spanning network based on 460bp of the mitochondrial *CO I* gene from 154 ants of the *Cardiocondyla shuckardi* group from various parts of Africa, Puerto Rico, and Hawai'i. The size of circles indicates the number of samples representing a particular haplotype (exemplarily indicated in white). The colors indicate the origin of the ants. For previously published sequences, Genbank accession numbers are given next to the pie.

a subset of the specimens (Fig 4). The two most divergent clusters in the network consisted of 11 relatively similar sequences found in ants exclusively from South Africa (“cluster I”, $n = 68$ samples) and of two almost identical haplotypes found in ants from Puerto Rico ($n = 17$), Kaua’i ($n = 5$), Côte d’Ivoire ($n = 13$), and one sample each from Hlalanathi (HGC23) and Rietvlei (C9), South Africa (“cluster II”). The two major clusters are connected by sequences from various places in Africa, including haplotypes of ants from Madagascar (previously referred to as *C. shuckardi*, Heinze et

al., 2014) and of ants from Côte d’Ivoire (some of which had been mentioned as *C. melana*, Heinze et al., 2021).

Translation of the sequences into amino acids showed that most substitutions were neutral and did not affect protein composition. For example, in the 460bp fragment, the 16 or 17 bp differing between the most divergent samples from Angola translated into only two changes in amino acids, the 23 bp difference between samples from South Africa in “cluster I” and specimens from Puerto Rico and Kaua’i in “cluster II” into three changed amino acids.

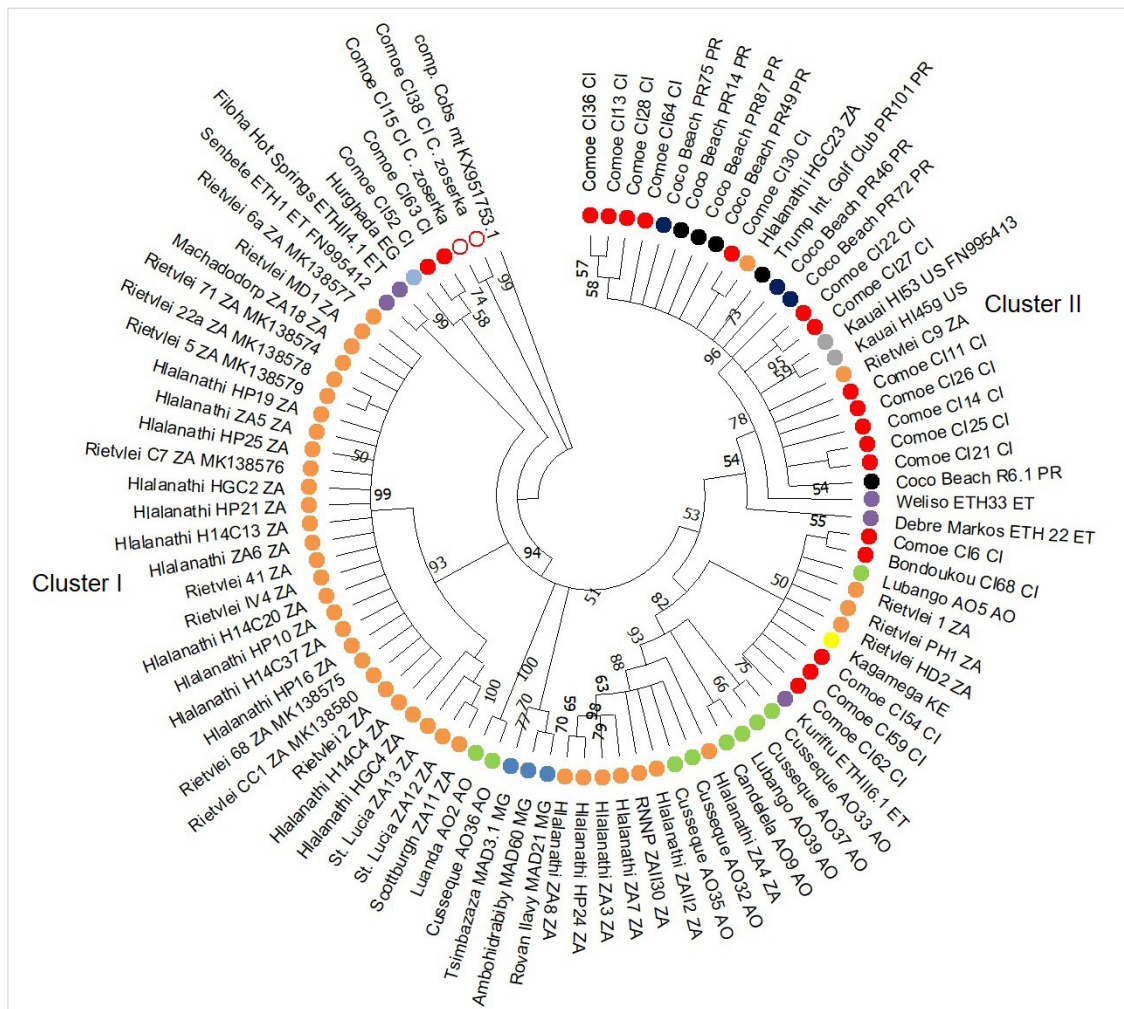


Fig 4. Bootstrap consensus tree of *CO I/CO II* sequences of 91 ants belonging to the *C. shuckardi* group inferred from 500 replicates using the Neighbor-Joining method. Numbers indicate the percentage of trees in which the respective node was supported. Branches with bootstrap values below 50% are collapsed. The respective part of the full mtDNA sequence of *C. obscurior* (Genbank KX951743) was defined as outgroup. The colors indicate the origin of the specimens. Cluster I comprises samples from South Africa (Rietvlei 6a to Scottburgh ZA11), Cluster II includes the samples Comoé CI36 to Coco Beach R6.1.

Discussion

Ants of the *C. shuckardi* group are widespread throughout Sub-Saharan Africa and the Middle East and often abound in anthropogenically disturbed habitats, such as gardens, plantations, and unpaved parking lots, but also along rivers, irrigation ditches, in ephemeral pans and other areas with at least temporarily high humidity and possibly subject to natural disturbance by flooding. Despite considerable effort

to distinguish among the various taxa in this group, species delimitation is difficult, and it remains unclear how many separate species it includes. In recent revisions of the genus, Seifert (2023) employed his “gene and gene expression” species concept (Seifert, 2020) to characterize the Oriental, Australasian, and European representatives of *Cardiocondyla*. The *C. shuckardi* group awaits such an analysis and currently consists of numerous ill-defined taxa, many of which are considered synonyms of *C. shuckardi* or *C. venustula*.

The present study compiles information on genetic variation in samples of these ants from various parts of Africa. It is not the aim to revise the whole group but rather to document the syntopic presence of multiple divergent mtDNA lineages, which asks for additional work concerning large-scale genomic comparisons. In phylogenetic trees and networks based on sequences of mitochondrial *CO I/CO II*, the sequences cluster in several distinct lineages, of which the two most diverging contain most of the available material: “Cluster I” consists of 11 slightly differing haplotypes from various places in South Africa, “cluster II” comprises two almost identical sequences from Kaua’i, Puerto Rico, Côte d’Ivoire, and two sites in South Africa. Between these two main clusters lie haplotypes of ants from Madagascar, which previously have been determined as *C. shuckardi* (Heinze et al., 2014), Côte d’Ivoire, which in part have been assigned to *C. melana* (Heinze et al., 2021), and undetermined material from other places in Africa.

Ants from “cluster I” and “cluster II” do not show clear differences in morphology and behavior, and both have therefore been considered as *C. “venustula”* in previous studies (e.g., Heinze et al., 2013; Jacobs & Heinze, 2017, 2019). However, the strong divergence of haplotypes might suggest that these samples alone actually constitute two or probably even more species. Interestingly, barcodes retrieved from GenBank (<https://www.ncbi.nlm.nih.gov/genbank>) and BOLD (Ratnasingham & Hebert, 2007) vary to a similar degree (mean 0.0720, 0.0000 – 0.1492), with specimens labeled *C. “venustula”* from two introduced populations (Kaua’i, workers from the same colony were also used in the present study) and Honduras differing considerably from workers of *C. “venustula”* from South Africa. The barcode sequences represent a 620 – 658bp stretch of the *CO I* gene, which does not overlap with the sequences analyzed here.

Given that the type material of *C. venustula* was originally described from Puerto Rico, “cluster II” likely represents this species, whereas samples in “cluster I” might constitute at least one separate taxon restricted mostly to Southern Africa, probably *C. globinodis*, which was described from Namibia (Stitz, 1923). The large genetic variability also reflects the broad range of habitats in which colonies were collected, from tropical savannas in Côte d’Ivoire to temperate areas in Drakensberg, South Africa, where temperatures may occasionally fall below 0 °C. Furthermore, it is suggestive that all available material from outside of Africa showed only the “cluster II” haplotype.

Despite the widespread application of mtDNA for species identification by barcoding, several studies in ants have previously highlighted the occurrence of discordance between morphometry, mtDNA, and nuclear markers, e.g., due to hybridization and introgression (e.g., Wild, 2009; Wagner et al., 2010; Hakala et al., 2018). Nuclear markers are, therefore, an indispensable tool for the separation of cryptic species. Unfortunately, sequences of previously studied nuclear

genes in *Cardiocondyla* vary little and appear unsuitable for robust species delimitation. The two samples considered as *C. venustula* in Oettler et al. (2010), one from Kaua’i (cluster II), the other from a sample from Ethiopia (in Figs 3 and 4 with CO I/CO II accession number FN995412), differ in only one of 500 base pairs in *LwRhod* and are completely identical in *wingless*, *EF aF1*, and *EF aF2* but diverge in 33 of 1433bp of *CO I/CO II*. Similarly, workers from the only two studied South African colonies with a “cluster I” haplotype showed the same alleles in all seven microsatellite loci as workers from numerous syntopic colonies from “cluster II” (Jacobs & Heinze, 2019; Jacobs, 2020). This might indicate ongoing gene flow between the different genetic lineages. Furthermore, individual colonies from South Africa occasionally contained workers with strongly diverging haplotypes. For example, sequences of workers from the same colony from Hlalanathi showed a haplotype of “cluster I” and a haplotype similar to “cluster II” (ZAI-7 in Heinze et al., 2013). This not only suggests the occasional adoption of alien queens into colonies but might also indicate conspecificity or at least hybridization. The two Southeast Asian species *C. micropila* and *C. longiseta* readily hybridize and produce fertile offspring in the laboratory (Yamauchi et al., 2007; Seifert, 2023), and even though sexuals of *Cardiocondyla* in nature usually mate within their natal nests, the occasional adoption of alien queens or the active transfer of sexuals by workers (Vidal et al., 2021) may eventually promote gene flow. Furthermore, specimens were only available from a few sites, and collecting these ants throughout Africa might provide missing haplotypes linking the two most divergent clusters (e.g., Pante et al., 2015). Clarification of the taxonomy of the *C. shuckardi* group might assist in the interpretation of evolutionary patterns of reproductive behavior but seems unattainable at present based on the material available. Geographically broad sampling might, therefore, help clarify this group’s taxonomy. We encourage researchers, wherever possible, to collect samples from multiple nests per locality across the Afrotropical region in the hope that this might facilitate the resolution of the taxonomic uncertainties within the *C. shuckardi* group.

One final result from the mtDNA study is that *C. zoserka* appears to be a valid species. Though its workers are morphologically extremely similar to those of other taxa in the *C. shuckardi* group (Bolton, 1982), its separate position is supported by the presence of winged males – the only fully winged males known from the *C. shuckardi* group – and the unique morphology of the antennae of these winged males (Heinze, 2020; Heinze et al., 2021).

Authors’ Contribution

JH: conducted fieldwork and genetic analyses and wrote the manuscript.

PGH: conducted fieldwork, provided data on ecology, and took part in writing the manuscript.

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