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Morphometry and colony structure of ants of the genus *Cardiocondyla* (Hymenoptera: Formicidae) from Georgia

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The ant genus *Cardiocondyla* is characterised by a remarkable variability of reproductive life histories and colony structures. Little is known about the *C. stambuloffii* group, whose distribution extends from southeast Europe to central and eastern Asia. We here present data on the structure of *Cardiocondyla* colonies from southern Georgia. Morphometry and sequencing of the mitochondrial cytochrome c oxidase subunits I and II indicates that it is distinct from the other species of this group (*C. gibbosa*, *C. koshewnikovi*, *C. stambuloffii*, and *C. tibetana*). Microsatellite analysis revealed a high nestmate relatedness and frequent inbreeding, matching the observation of exclusively wingless males and the phylogenetic position of the group in the "Palaearctic clade," in which colonies are typically single-queened.

Keywords: Ergatoid ants; monogyny; *Cardiocondyla stambuloffii*; *Cardiocondyla koshewnikovi*

Introduction

The ant genus *Cardiocondyla* shows a remarkable variability of reproductive strategies and sociogenetic colony structures (Heinze, 2017). The cosmotropical tramp species *C. emeryi*, Forel, 1881, *C. minutior* Forel, 1899, and *C. obscurior* Wheeler, 1929, for example, are characterised by a male diphenism with winged disperser males and wingless fighter males (ergatoid males) (Stuart, Francoeur, & Loiselle, 1987; Heinze, Hölldobler, & Yamauchi, 1998). Their colonies may contain multiple fertile queens (polygyny) (Kinomura & Yamauchi 1987; Stuart et al., 1987), which at least in *C. obscurior* mate only with a single male each (Schmidt, Trindl, Schrempf, & Heinze, 2016). A phylogeny suggests polygyny and male diphenism to be ancestral (Oettler, Suefuji, & Heinze, 2010; Heinze, 2017), but both male phenotype and queen number have evolved in various ways. In the West African *C. zoserka* Bolton, 1982, winged males with bizarrely shaped antennae are known (Heinze, 2020), while numerous other taxa have completely lost winged males: in a number of species from Southeast Asia, long-lived wingless males monopolize mating with all young queens, which later fight for inheritance of the natal nest (Schmidt, Heimbucher, Bernadou, & Heinze, 2017). In *C. venustula* Wheeler, 1908 from South Africa, wingless males defend small territories in the multi-queen nests (Jacobs & Heinze, 2017), and colonies of the members of the Palaearctic *C. elegans* Emery 1869 and *C. batesii* Forel, 1894 groups (sensu Seifert, 2002) have single, multiply mated queens (monogyny, polyandry) and mutually tolerant wingless males (Schrempf, Reber, Tinaut, & Heinze, 2005; Lenoir, Schrempf, Lenoir, Heinze, & Mer-

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cier, 2007; Schrempf, 2014). This large variation justifies studying further taxa of this genus, as each might reveal novel facets of life history evolution in *Cardiocondyla*.

Little is known about the colony structure and male behaviour of the members of the *C. stambuloffii* Forel, 1892 group, which is distributed from southeast Europe eastwards to Tibet and Mongolia (Seifert, 2002). Forel (1892) described yellowish wingless males from nests of *C. stambuloffii* found close to the shore of the Black Sea in Bulgaria, and Arnoldi (1926) reported on the presence of multiple wingless males in colonies excavated from sandy beaches and salt marshes on the northeastern shores of the Black Sea. Multiple wingless males have also been observed in colonies of *C. koshewnikovi* Ruzsky, 1902 from Kazakhstan (Seifert, 2002), suggesting that species of the *C. stambuloffii* group have a reproductive life history similar to the members of the *C. elegans* or *C. batesii* groups. Aim of our study was to test this hypothesis by investigating the colony structure of a taxon of the *C. stambuloffii* group from southern Georgia by microsatellite genotyping. Many species of *Cardiocondyla* are morphologically very similar and can only be determined by detailed morphometry and comparison with material of other species (Seifert, 2002, 2009). Furthermore, workers of the *C. stambuloffii* group may vary considerably in their morphology (Arnoldi, 1926). We therefore compared specimens from Georgia by morphometry and mtDNA sequencing with other species of the *C. stambuloffii* group in order to assess species identity.

Material and Methods

Collecting site. Fragments of colonies of a species belonging to the *Cardiocondyla stambuloffii* group (sensu Seifert 2002) with workers and occasionally alate and dealate female sexuals and wingless males were collected in September 2013 in Parekha, Akhaltsikhe, Samtskhe–Javakheti, Georgia from their nests near the banks of Potskhovi river (41'39' N, 42°52' E, 1023m, Figure 1) and stored in 100% EtOH. Five additional samples were collected at the same site and at Grakali, Shida Kartli region of Georgia (41° 57'N, 44°17'E) in summer 2019.

Morphometry. Morphometric analyses were done following Seifert (2002) and Seifert, Okita, and Heinze (2017) with seven workers collected in June 2019 in Parekha, Akhaltsikhe, three workers collected in June 2019 in Grakali, six workers of *C. koshewnikovi* from Kazakhstan, Zayan region (47° 42'N, 85°18'E, 496 m), three workers of *C. stambuloffii* from Stepnoje, Stavropolij Kraj, Russia, and two workers of *C. stambuloffii* from Slantshev Brjag, Bulgaria (the specimens from Russia and Bulgaria provided by B. Seifert).

The dried specimens were mounted using a pin-holding stage, which allowed rotation in all dimensions and measured under a UNITRON Z650HR stereo-microscope equipped with an apochromatic objective at a magnification of 200×. For each specimen, we measured 13 morphological characters and two indices as defined by Seifert (2002) and Seifert et al. (2017). The parameters measured are given in Table 1. The mean value, standard deviation (SD) for each morphological character and ratios were calculated. Patterns of morphometric variation were analysed by Principal Component Analysis (PCA) using the software packages Past v. 2.16 (Hammer, Harper, & Ryan, 2001).

Genetic analysis. mtDNA was extracted from workers stored in EtOH using a CTAB protocol (Sambrook & Russell, 2001). We amplified partial sequences of mitochondrial cytochrome c oxidase subunits I and II including tRNA-Leu by PCR using the primer combinations C1-J 2183/C2-N-3661 and C1-J 2183/A8-N- 3914 (Simon, Frati, Beckenback, Crespi, Liu, & Flook, 1984) as described before (Heinze, Trindl, Seifert, & Yamauchi, 2005), and sequenced by LGC Genomics (Berlin, Germany). The sequences obtained and the sequences of three species belonging to the *C. stambuloffii* group (*C. koshewnikovi*, *C. stambuloffii*, *C. tibetana* Seifert, 2002) and several other species from GenBank were aligned using the CLUSTAL W Algorithm (Thompson, Higgins, & Gibson, 1994) in the software BioEdit v 7.09 (Hall, 1999). After manual correction of the alignment, we used she software RAxML (Stamatakis, 2014) and the graphical interface raxmlGUI2 (Edler, Klein, Antonelli, & Silvestro, 2019) to infer the evolutionary history of the

Figure 1. Collecting site of the ant *Cardiocondyla* sp. on the banks of Potshkovi River near Akhaltsiche, Georgia. Nest entrances were found on the pasture on the right, in particular close to the low scarp. The insert shows partly excavated, superficial nest chambers in the sandy soil with two ~3mm long workers. More chambers were found deeper down and they presumably reached down to the water table.

samples using the maximum likelihood method and a general time reversible model with gamma-distributed rate variation among sites and allowing for invariable sites (GTR+G+I, Nei & Kumar, 2000). Support for the tree was assessed by bootstrapping (500 replicates, Felsenstein, 1985). In addition, we performed a Bayesian analysis using MrBayes 3.2.2 (Ronquist et al., 2012) with three heated and one cold Markov chains and a MCMC length of 3,000,000 generations.

Microsatellite analysis. A first attempt to detect variability in a sample of ten colonies using primers for various microsatellite loci (Schrempf et al., 2005: Card 8, 9, 16 & 21; Lenoir et al., 2007: Ce2-3a, 2-4a, 2-5d; Schmidt et al. 2016: Cobs 13-2 & 13), failed due to the low variability of *Cardiocondyla* ants. However, we found variability at three loci (Cobs 13 & P3, Schmidt et al., 2016; Ce2-12D, Lenoir et al., 2005). To determine colony structure, we genotyped 238 workers from 25 colonies at three microsatellite loci.

DNA was extracted from individual ants following a modified CTAB (cetyltrimethylammonium bromide) protocol (Sambrook & Russell, 2001). PCR was performed in 20 µl reaction volumes with labelled forward primers (TET, HEX, FAM) using the following program: initial denaturation step at 94°C (4 min), 33 cycles of 94°C (75 s), 55°C/60°C (45 s), 72°C (25 s) and a final extension step at 72°C (60 s). The amplified fragments were visualized on an ABI Prism 310 Genetic Analyzer.

Population genetic analyses were done using the software Genetic Data Analysis (Lewis & Zaykin, 2001) and GenAlEx 6.5 (Peakall & Smouse, 2006, 2012). The confidence interval of the fixation coefficient was obtained in GDA by bootstrapping (999 replicates). The confidence interval of mean relatedness among nestmates per colony was obtained in GenAlEx 6.5 by bootstrap sampling, the mean relatedness across all colonies and its standard error by bootstrapping over the obtained colony means (999 replicates) using the respective module (Wessa, 2020).

Table 1. Proportion of contribution and variable coefficients of the first two eigenvectors (principal components) for PCA in workers of *Cardiocondyla koshewnikovi*, *C.* sp. and *C. stambuloffii*.

Variables	PC1	PC2
CL: maximum cephalic length in median line	0.330	0.079
PoOc: postocular distance	0.334	0.007
CW: maximum cephalic width	0.334	0.008
SL: maximum straight line scape length	0.128	-0.460
MW: maximum mesosoma width	0.313	-0.174
SPTI: distance of tips of epinotal spines in dorsal view	0.172	0.427
PEW: maximum petiole width	0.334	0.003
PPW: maximum width of postpetiole	0.209	0.389
PEH: maximum height of petiole	0.325	0.100
EYE L: large diameter of the elliptical compound eye	-0.033	0.495
EYE W: small diameter of the elliptical compound eye	-0.332	0.056
ML: mesosoma length	0.334	0.027
MGr: depth of metanotal groove	0.200	-0.399
Percentage of total variation	68%	31%

Table 2. Morphometric measurements and ratios of workers of *Cardiocondyla koshewnikovi*, *C. stambuloffi*, and *C.* sp. from Georgia (mean±S.D., [range]). EYE=(EL+EW) / (CL+CW), for the other measurements see Table 1.

|	*C. koshewnikovi*	*C. stambuloffii*	*C.* sp.
N	6	5	10
CS	561±12 [547; 583]	523±7 [517; 531]	546±13 [519; 562]
CL	591±8 [583; 602]	549±9 [541; 559]	577±15 [552; 597]
CW	530±27 [511; 582]	497±5 [494; 503]	515±15 [486; 537]
PoOc	271±7 [263; 280]	248±7 [239; 257]	261±8 [242; 269]
SL	439±20 [420; 470]	427±16 [409; 451]	410±18 [433; 374]
EYE L	121±9 [108; 134]	123±6 [119; 134]	133±6 [125; 147]
EYE W	103±9 [89; 112]	108±7 [97; 116]	106±5 [97; 116]
MW	330±6 [324; 336]	299±7 [292; 306]	305±13 [283; 327]
SPTI	127±6 [116; 134]	121±13 [104; 142]	134±10 [112; 145]
PEW	169±8 [160; 184]	151±8 [143; 161]	160±9 [149; 175]
PPW	319±9 [307; 330]	312±5 [307; 318]	323±16 [292; 341]
PEH	202±8 [190; 210]	189±6 [179; 197]	198±7 [190; 209]
ML	644±34 [586; 686]	602±3 [598; 606]	626±20 [597; 649]
MGr	29±4 [22; 36]	25±5 [21; 31]	23±3 [19; 30]
CL/CW	1.115±0.058 [1.002; 1.161]	1.104±0.008 [1.095; 1.114]	1.120±0.026 [1.071; 1.161]
PoOc/CL	0.459±0.015 [0.440; 0.480]	0.452±0.013 [0.439; 0.473]	0.452±0.010 [0.433; 0.467]
SL/CS	0.783±0.024 [0.749; 0.814]	0.816±0.033 [0.788; 0.869]	0.750±0.029 [0.692; 0.779]
EYE	0.200±0.014 [0.183; 0.218]	0.221±0.010 [0.209; 0.232]	0.219±0.005 [0.214; 0.232]
MW/CS	0.590±0.017 [0.563; 0.610]	0.571±0.181 [0,551; 0,591]	0.559±0.021 [0.531; 0.587]
SPTI/CS	0.227±0.011 [0.211; 0.240]	0.230±0.024 [0.201; 0,267]	0.246±0.021 [0.199; 0.270]
PEW/CS	0.301±0.169 [0.282; 0.329]	0.289±0.015 [0.276; 0.311]	0.294±0.016 [0.274; 0.319]
PPW/CS	0.568±0.014 [0.550; 0.590]	0.595±0.010 [0.582; 0.608]	0.592±0.020 [0.555; 0.624]
PEW/PPW	0.529±0.021 [0.502; 0.557]	0.484±0.020 [0.463; 0.512]	0.497±0.025 [0.464; 0.547]
PEH/CS	0.360±0.013 [0.344; 0.374]	0.361±0.016 [0.337; 0.381]	0.363±0.010 [0.349; 0.378]
ML/CS	1.149±0.053 [1.064; 1.218]	1.150±0.020 [1.127; 1.172]	1.146±0.026 [1.096; 1.189]
MGr/CS	0.052±0.008 [0.040; 0.065]	0.048±0.009 [0.040; 0.059]	0.042±0.005 [0.034; 0.053]
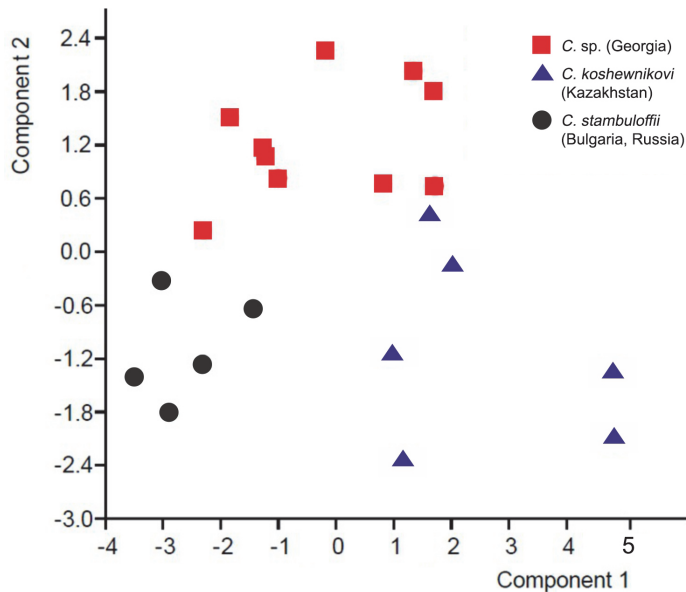
Figure 2. Principal component ordination of 21 workers of *Cardiocondyla* based on the analysis of 13 morphological characters onto the first and second principal axes.

Results

Both our morphometric analysis and mtDNA sequencing revealed considerable differences between the samples collected in Georgia and other taxa of the *C. stambuloffii* group and did not yet allow to assign it to a recognised species.

Morphometry. Although the PCA analysis is based on only a small number of samples and measures, the specimens from Georgia do not clearly cluster neither with *C. koshewnikovi* nor with *C. stambuloffii* (Figure 2). The main contributions to PC 1 (68% of total variation) were CW, PEW, PoOc, contrasting with EYE W, those to PC 2 (31% of total variation) were EYE L, contrasting with SL (Table 1). Plotting PC 1 against PC 2 reveals three different clusters, with PC1 separating *C. koshewnikovi* and *C. stambuloffii*, and PC2 separating the samples from Georgia from the two other taxa. Dimensions of diagnostic morphological characters in workers and their ratios are given in the Table 2.

mtDNA analysis. COI/COII sequences obtained from *Cardiocondyla* samples from Georgia differed from those of three previously studied species of the *C. stambuloffii* group, *C. koshewnikovi* (GenBank accession numbers FN995399, Yingbazar, Xinjang, China, and FN995400, Khovd, Mongolia), *C. stambuloffii* (DQ023107, Slantshev Brjag, Bulgaria, and a 100% identical sequence from Karapınar Meke, Konya, Turkey, which became available after completing the anbalysis), and *C. tibetana* (FN995410, Yingbazar, Xinjiang, China). Both in the maximum likelihood and Bayesian analyses, the samples from Georgia formed a branch that was clearly separate from *C. koshewnikovi*, *C. stambuloffii*, and *C. tibetana*. *Cardiocondyla koshewnikovi* formed the sister group to a clade consisting of Georgian *Cardiocondyla* and *C. stambuloffii* with mean genetic distances of 0.084 and 0.11 (Figure 3).
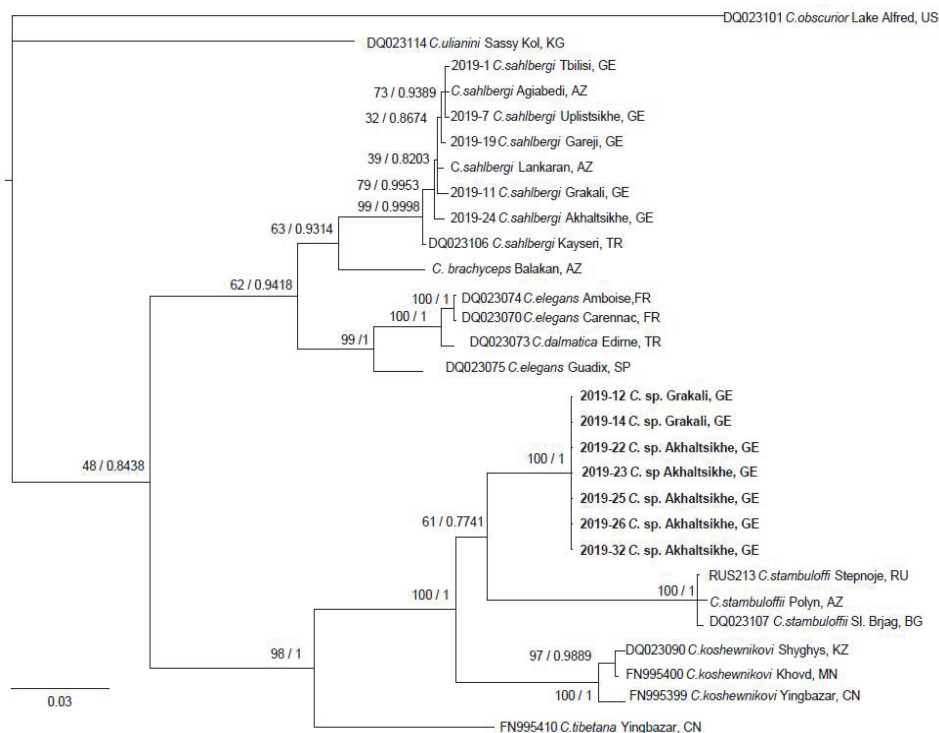
Figure 3. Relationships among *Cardiocondyla* species as inferred by Bayesian and Maximum Likelihood analysis from 1435bp sequences of the mitochondrial genes CO I and CO II including tRNA-Leu. The percentage of trees in which the associated taxa clustered together (500 bootstrap replicates, and Bayesian posterior probabilities are shown next to the nodes. The bar indicates expected changes per site. The final standard deviation of split frequencies for the Bayesian analysis was 0.00648.

Microsatellite analysis. Allelic richness determined in 238 workers from 25 colonies was extremely low, with two alleles at loci Ce2-12D and Cobs 13 and seven alleles at Cobs P3 (Table 3). Three of the seven alleles at Cobs P3 were also present in samples from five colonies collected in 2019. The estimate of mean relatedness over all colonies collected in 2013 (following Queller & Goodnight, 1989; 0.714±0.052, 95% CI 0.626–0.795) was not significantly different from the expected value for full sisters, 0.75 (one sample t-test, t=0.689, p=0.497) suggesting that many colonies in this population are headed by single, singly-mated queen. A visual inspection of genotypes supports this hypothesis: in eight colonies, the segregation of genotypes of workers indicated that all inspected workers were full sisters; in two additional colonies the queen may have mated with two males. In four colonies, all individuals shared the same multilocus genotype, and each of the remaining colonies contained one or two workers with genotypes that did not match the assumption of single queening. Colony mean relatedness varied between 0.076 and 1.000. The inbreeding coefficient was 0.430 (95 % CI 0.141–0.489), which indicates a proportion of sib-mating of 75%.

Table 3. Nestmate relatedness in colonies of the ant *Cardiocondyla* sp. from Georgia. n is the number of individuals examined in each colony sample, r the mean relatedness and CI95 the confidence interval of the mean relatedness as determined by bootstrap sampling.

Sample no.	n	r	95% CI	
01	8	0.587	0.352	0.823
02	12	1.000	1.000	1.000
03	3	0.622	0.433	1.000
04	8	0.703	0.532	0.846
05	8	0.858	0.757	0.939
06	12	0.076	-0.089	0.237
07	9	0.743	0.640	0.843
27	11	0.712	0.639	0.784
28	12	0.163	-0.010	0.303
29	12	1.000	1.000	1.000
32	12	0.840	0.799	0.881
33	10	0.943	0.911	0.975
34	12	0.561	0.456	0.664
36	12	0.906	0.854	0.957
36W	12	0.597	0.459	0.742
37	12	0.909	0.872	0.941
39	9	0.375	0.227	0.593
40	9	0.527	0.353	0.669
42	7	1.000	1.000	1.000
43	9	0.888	0.841	0.936
45b	6	1.000	1.000	1.000
46	7	1.000	1.000	1.000
47	12	0.699	0.631	0.768
49	12	0.425	0.264	0.573

Discussion

Our study is the first attempt to determine the genetic structure of colonies of an ant species of the *Cardiocondyla stambuloffii* group. According to our morphometric analysis and sequencing of mtDNA, specimens collected in Georgia, though similar, appeared to be different from *C. stambuloffii*, which several previous papers had listed for the region (Gratiashvili & Barjadze, 2008; Kiran & Karaman, 2012; Pashaei Rad et al., 2018; Bračko, 2019). Furthermore, the sequences from Georgia were also different from those from more eastern species of the *C. stambuloffii* group, *C. koshewnikovi*, and *C. tibetana*. Unfortunately, we did not have material from *C. gibbosa*, an ant recorded from Kazakhstan, Kyrgyzstan, and Xinjiang (Seifert, 2002; Schultz, Radchenko, & Seifert, 2006; Gulzar, Tang, Zheng, & Xu, 2014), but according to Seifert (2002), its head size (CS, arithmetic mean of cephalic length CL and cephalic width CW) is lower (518±9, range 508–526) and the ratio between CL and CW (1.191±0.020, range 1.176–1.219) is larger than in the specimens from Georgia (CS 546±13, range 519–562, CL/CW 1.120±0.026, range 1.071–1.161, Table 2).

Ruzsky (1905) had described material collected in Aralykh (today Aralık, Turkey) similar to *C. stambuloffii* as a new species, *C. bogdanovi*. As the type material was lost and the assigned neotypes (Radchenko, 1995) did not match Ruzsky's original description, Seifert (2002) synonymized *C. bogdanovi* with *C. stambuloffii*. Similarly, *C. montandoni* Santschi, 1912 from Lacu Sărat, Romania, and *C. stambuloffii taurica* Karava-

jev, 1927 from Enishary, Crimea, have been synonymized with *C. stambuloffii* (Pisar-ski, 1962; Arnol'di & Dlussky 1978). According to Seifert (2002), syntypes of both taxa are fully consistent with the intraspecific variability of *C. stambuloffii*. Interestingly, COI/COII sequences from Georgia were 100% identical to those obtained from three samples of *Cardiocondyla* recently collected near Tulcea, Crișan, and Caraorman in Romania, not far from the type locality of *C. montandoni* (A. Trindl, unpubl.).

As expected from the position of the *C. stambuloffii* group in the Palaearctic clade (Oettler et al., 2010), in which all colonies of the so far studied species are monogynous (Schrempf et al., 2005; Lenoir, Schrempf, Lenoir, Heinze, & Mercier, 2007; Schrempf, 2014), the genetic composition of the colony samples from Akhaltsikhe also suggests monogyny. The presence of multiple wingless queens in nests collected by Forel (1892) in summer in Bulgaria presumably reflects a transient phase between mating in late summer and dispersal in spring without laying eggs, similar to what has been observed in *C. elegans* (Lenoir et al., 2007). The high relatedness observed in our study stands in contrast to data from *C. elegans* and *C. nigra*, in which frequent multiple mating by queens results in relatedness estimates of 0.5 and less (Lenoir et al., 2007; Schrempf, 2014; M. Vidal & J. Heinze, unpubl.). Because of the low variability of microsatellites, results should be considered with caution, but worker genotypes suggested multiple mating by queens only for two colonies, while queens in eight colonies presumably were singly mated. The high inbreeding coefficient and the low variability of microsat-ellite loci – both typical for all hitherto studied species of this genus – make it difficult to determine exact mating frequencies: female sexuals may mate with multiple, closely related males, which share the same multilocus genotype.

So far, only three species of *Cardiocondyla* are known from Georgia (Gratiashvili & Barjadze, 2008). The number of species reported from neighbouring countries, such as Turkey and Iran (Kiran & Karaman, 2012; Pashaei Rad et al., 2018), is three times higher, and it is likely that future studies in Georgia will also reveal additional taxa. In the past, each newly studied species of *Cardiocondyla* has revealed novel aspects of its highly variable life histories and reproductive strategies (Heinze, 2017), and further investigations on *Cardiocondyla* from the Caucasus region of Eurasia will likely add new puzzle stones to better understand the evolution of these ants.

Disclosure Statement

No potential conflict of interest is reported by the authors.

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