



Socio- and population-genetic analyses of two West-African ponerine species (*Megaponera analis* and *Paltothyreus tarsatus*) with winged and wingless queens (Hymenoptera: Formicidae)

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Abstract

Depending on the reproductive strategy of a species, the same environmental barrier can affect gene flow differently. In this study, we analyzed the effects of a river on the gene flow of two ant species, one with wingless queens (*Megaponera analis*) and one with winged queens (*Paltothyreus tarsatus*), both with winged males. Colonies were sampled in the Comoé National Park, Côte d'Ivoire, from April to June, in 2017 and 2019. A detailed sociogenetic analysis corroborated monogyny and monandry for *M. analis* (74 of 78 colonies), including 29 colonies with evidence of recent fission events. In contrast, the sociobiological structure of *P. tarsatus* was more heterogeneous. Nine colonies were monogynous and monandrous, 14 colonies were either polygynous and / or polyandrous, and worker genotypes in seven colonies can only be explained by polygyny. For both species, we quantified gene flow between four sympatric subpopulations that are separated by the Comoé river. Comparisons of the different populations using two mitochondrial genes showed a clear substructure in *M. analis*, separating the respective river sides, while no substructure was found in *P. tarsatus*. Microsatellites, as likely neutral nuclear markers, showed, in contrast to mitochondrial DNA analyses, no significant substructure between any of the four subpopulations for both species. Even though microsatellites have been mostly replaced in population genetics by large-scale single nucleotide polymorphism analyses (e.g., based on restriction site associated DNA or whole genome sequencing), they are still the most efficient way to determine the social structure of social insect colonies based on thousands of samples (in our case, approx. 2000) or revealing atypical reproductive systems like genetic caste determination. These microsatellite analyses allowed us to show that gene flow in *M. analis* through wingless queens is restricted but compensated for by male dispersal on the nuclear DNA level. This underlines the importance of having at least one winged sexual alate in the reproductive strategy of social insects to allow for sufficient gene flow across minor environmental barriers.

Key words: Alates, ergatoid queens, dispersal ability, population genetics, Ponerinae, natural barriers, colony fission.

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Introduction

Limited dispersal ability and thus limited gene flow among populations can lead to increasing genetic differentiation and eventually the emergence of new species (MAYR 1947, FRANKHAM & al. 2002). Additionally, limited dispersal can have a major impact on the potential for adaptive evolution because it can reduce heterozygosity and genetic variability through inbreeding (WRIGHT 1931, LANDE 1995, KELLER & WALLER 2002) or foster local adaptations. In ant species with wingless, ergatoid queens, males are

always winged and could therefore compensate for any female-based restrictions in gene flow. This can result in sex specific population structures due to the different dispersal strategies of males and females (PUSEY 1987, SLATKIN 1987, PERRIN & MAZALOV 2000, BOWLER & BENTON 2005, GROS & al. 2008).

Limited female dispersal can be demonstrated using mitochondrial genes because mitochondrial DNA (mtDNA) is in most animals strictly maternally inherited (Ross &

SHOEMAKER 1997, PERRIN & MAZALOV 2000, LIAUTARD & KELLER 2001, SEPPÄ & al. 2004). At the same time, sex specific dispersal might not be obvious at the nuclear level of the same population or species if, for instance, males disperse widely during their mating flights and hence compensate for a restricted female dispersal (SEPPÄ & al. 2004, SUNDSTRÖM & al. 2005, JAFFÉ & al. 2009).

Insects, especially ants, evolved many different strategies to disperse and regulate gene flow among populations. The predominant and presumed ancestral type of colony organization and dispersal in ants is through winged, monandrous queens and haplometrosis, leading to monogyny (BOOMSMA 2007). But permanently wingless queens (BOLTON 1986, FRANKS & HÖLLDOBLER 1987, PEETERS 1993) have evolved convergently in several ant lineages (PEETERS 2012). Furthermore, new colonies can be established in ants by two different strategies, independent or dependent of the worker caste (HÖLLDOBLER & WILSON 1977). Commonly, species that spread through alate individuals produce many virgin queens and males, which both participate in nuptial flights (HÖLLDOBLER & WILSON 1990). Thereafter, newly mated queens shed their wings and found a new colony independently (KELLER & PASSERA 1989). Colony fission or budding is a worker-dependent founding strategy (LIAUTARD & KELLER 2001, SEPPÄ & al. 2006). During colony fission, a mated queen leaves her nest accompanied by workers to establish a new colony. That restricts queen and ultimately colony dispersal to “walking distance”.

Army ants and several other ant species including *Megaponera analis* reproduce exclusively by colony fission and have wingless (apterygote) queens (HÖLLDOBLER & WILSON 1977, 1990, KRONAUER 2009). Dependent colony foundation (DCF) is often associated with polygyny, whereas independent colony foundation (ICF) usually results in monogyny (BOURKE & HEINZE 1994, PEDERSEN & BOOMSMA 1999, SUNDSTRÖM & al. 2005). However, colony fission in social insects is, as far as we know, characterized by monogyny but high polyandry, for example in army ants or honey-bees (OLDROYD & al. 1997, KRONAUER & BOOMSMA 2007).

The population- and sociogenetic structure of ponerine ants (Ponerinae) (BOLTON 1990) is in comparison with other ant subfamilies poorly understood (CROZIER & PAMILO 1996, BOURKE 1999, GIRAUD & al. 2000). Most ponerines retain several ancestral traits, such as small colony size, a monomorphic worker caste, little morphological differentiation between workers and queens, and solitary foraging, and are generally thought to have a simple sociogenetic structure, that is, monandry and monogyny (BOLTON 1990, PEETERS 1997, WILSON & HÖLLDOBLER 2005). But at the same time, several ponerine species have undergone significant changes in life-history traits, in particular in terms of number and presence of reproductive individuals (repeated evolution of mated workers as principal reproductives, i.e., gamergates or polygyny), queen morphology (wing loss), and colony foundation strategies (colony fission) which resulted in a combination of ancestral and

specialized traits (PEETERS & CREWE 1985, PEETERS 1987, BUSCHINGER & al. 1989, PEETERS 1993, BRAUN & al. 1994, HÖLLDOBLER & al. 1994, PEETERS & HÖLLDOBLER 1995). Hence, although ponerines generally exhibit a quite simple colony organisation, some species are highly adapted and specialized to their ecological niche and are as complex as other ant subfamilies. The question is whether these “aberrant” species also show a derived sociogenetic structure. Socio- and population-genetic analyses using mitochondrial and microsatellite markers allow estimations about gene flow, migration rates, inbreeding, and the social structure within and among populations and sexes.

Here, we compare the socio- and population-structure of two sympatric West-African ponerine species, *Megaponera analis* (LATREILLE, 1802) and *Paltothyreus tarsatus* (FABRICIUS, 1798), which are similar in terms of morphology, distribution, and colony size (> 1000 workers), and belong to the same ant tribe, Ponerini, as well as to the *Odontomachus* genus group (SCHMIDT 2013) but differ in their colony founding strategy, behavior, and queen morphology (winged vs. wingless queens). Both genera are monotypic (SCHMIDT & SHATTUCK 2014) and prey on larger insects and termites. *Megaponera analis* shows army-ant-like traits, that include obligate group foraging (i.e., raids), male-biased sex-ratios, and wingless queens including DCF through colony fission (VILLET 1990a, BRADY 2003). Although *M. analis* colonies use a DCF strategy, they significantly differ in colony size. *Megaponera analis* colonies rarely exceed worker numbers bigger than 2200, and queens are suspected to be monandrous (VILLET 1990b, FRANK & al. 2017, see Results). In comparison, highly polyandrous army ants differ significantly in their colony size with over a million individuals (KRONAUER & al. 2007, KRONAUER 2009), while also using colony fission as DCF strategy (GOTWALD 1995).

Paltothyreus tarsatus has winged queens, using ICF as reproductive strategy. They predominantly exhibit haplometrosis and semi-claustral nest founding strategies, but reports of polygyny and our own results (see below) indicate that they might use a variety of colony foundation strategies (PEETERS 1993). We used mitochondrial sequences and microsatellite genotypes to determine mating frequency and queen number for both species. This allowed us to test whether the presence of wingless queens and colony fission in *Megaponera analis* has led to an increase in inbreeding and population substructure in comparison with *P. tarsatus*, which has winged queens and independent, semi-claustral colony founding. Since our four subpopulations are separated by the Comoé river or dense gallery forests, we expected to see significant population substructure in *M. analis*, but none in *P. tarsatus*.

Material and methods

Study site

The study was carried out at the Research Station in the Comoé National Park (Côte d'Ivoire, 8° 45' 36" N, 3° 46' 48" W). The station is located close to the gallery

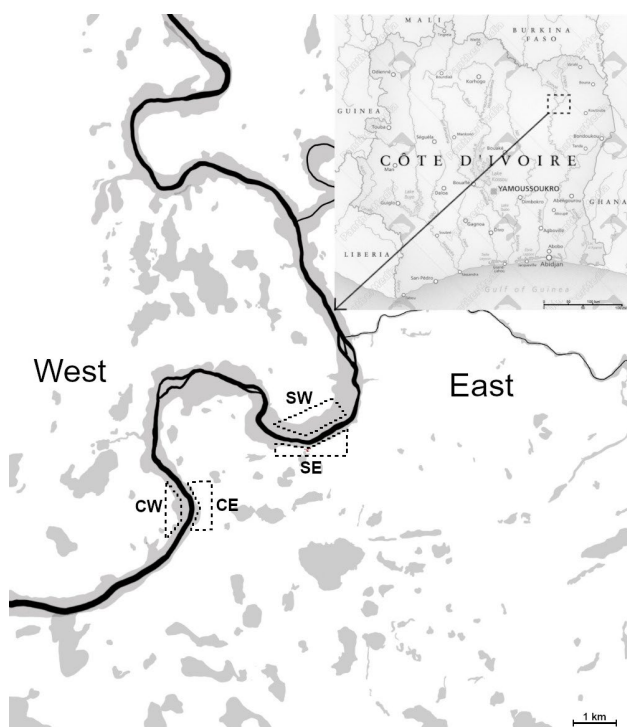


Fig. 1: Map of the research area and Ivory Coast (inset) and the respective population areas of the sympatrically distributed species *Megaponera analis* and *Paltothyreus tarsatus* (SW, SE, CW, CE) in Comoé National Park, Ivory Coast. Sample size in terms of colonies collected for our population analyses were identical for all subpopulations of *M. analis* ($n = 20$) and *P. tarsatus* ($n = 10$).

forest and the Comoé river. Both genera are monotypic and therefore easy to identify to the species-level using the most recent key to the ponerine genera (FISHER & BOLTON 2016). Ants were collected between April to June in 2017 and 2019. In total, 1775 ants from 110 colonies of *Megaponera analis* (2017: $n = 38$, 2019: $n = 40$) and *Paltothyreus tarsatus* (2019: $n = 32$) were collected. Worker samples from 19 *M. analis* colonies collected in August 2016, near the E.O. Wilson lab in the Gorongosa National Park, Mozambique, were included as an outgroup (MZ). Additionally, twenty-one *M. analis* colonies were excavated in the Comoé National Park in 2014 - 2019 for demographic analyses. The necessary collection permits were provided by the Directeur Général of the Office Ivoirien des Parcs et Réserves (OIPR), Côte d'Ivoire (permit number: N°018 / MINEDD / OIPR / DZ) and the Director do Departamento dos Servicos Cientificos, Parque Nacional da Gorongosa, Mozambique.

The vegetation in the region around the Comoé river is characterized by a dense gallery forest which can be several hundred meters wide (ADJANOHOUN 1964, KONATÉ & KAMPMANN 2010). Two collecting locations, the Research Station (S) and the former camp site (C) were further divided into two different areas each and named after the respective geographic orientation of the river sides (West = W, East = E). Thus, the respective abbreviations of the

four collected populations are SW, SE, CW, CE (Fig. 1). In addition, the abbreviations were extended with "17" if the colonies were collected in 2017, and no extension was added to colonies collected in 2019. The distance between the respective collection areas of the two riversides is approx. 3.5 km. For each collection site, GPS data (Tabs. S1 - 3, as digital supplementary material to this article, at the journal's web pages) were recorded and entered on a geographical map (Figs. S1 - 2).

Megaponera analis samples were collected mostly during raids on termites or whole colonies during excavations at active nest entrances. Samples were collected in the transition zone between savannah and gallery forest (*M. analis*, *Paltothyreus tarsatus*) or in the gallery forest itself (*P. tarsatus* only). Ten workers (2017) and 20 workers (2019) were collected, shock frozen at -20°C , and preserved in 100% ethanol, respectively. For *P. tarsatus*, 20 workers per colony were collected in 2019 at active nest entrances to ensure the nest affiliation of all workers. Based on the sampling experience (time to find and collect the same number of colonies per site), the population densities are assumably comparable between collection sites for each ant species.

DNA extraction and PCR

DNA was isolated using a modified Chelex extraction protocol (WALSH & al. 2013) from head and thorax of individual ants (gasters were removed). Samples were filled in a sterile tube with two metal beads, 100 μl $1 \times$ TE (10 mM Tris-HCL containing 1 mM EDTA * Na_2 , pH 8.0) and 2 μl Proteinase K (10 mg / ml) and ground in a Mixer Mill (Retsch, Haan, Germany) for 40 seconds at a frequency of 28 Hz. Thereafter, 100 μl Chelex solution (Bio-Rad, Feldkirchen, Germany) was added and incubated for 1 hour at 57°C , agitation intervals every 3 minutes for 60 seconds at a frequency of 800 rpm on an Eppendorf Thermomixer Comfort (Hamburg, Germany). The samples were centrifuged in an Eppendorf Minifuge for 10 minutes at 14,000 rpm, and 100 μl of the supernatant were transferred to a new sterile tube and stored at -20°C . PCR was conducted in the following volumes: 1 μl DNA solution (supernatant), 2 μl of $5 \times$ reaction buffer, 1 μl of 25 mM MgCl_2 , 0.6 μl of dNTPs in 1.25 mM concentration, 0.2 μl of each primer in 20 mM concentration, 0.06 μl of *GoTaq* DNA polymerase (Promega, Walldorf, Germany), and 4.94 μl distilled water for a total reaction volume of 10 μl . PCR cycles were as following: initial denaturation for 3 min at 94°C ; 30 / 38 cycles, 60 s each, at 94°C , 60 s at 45 - 58°C (primer-specific annealing temperatures in Tab. 1), and 90 s at 72°C ; and a final elongation step of 5 minutes at 72°C . Reactions were done in an Eppendorf Thermal Cycler. The species-specific microsatellites Meg1, Meg2, Meg3, Meg5, Meg17, and Meg25 were used for *Megaponera analis* and Pt1, Pt10, and Pt42 for *Paltothyreus tarsatus* for amplification. Universal primers were used to amplify cytochrome oxidase subunit I (COI, FOLMER 1994) and cytochrome B (cytoB, Tab. 1, CHIOTIS & al. 2000) for both species. The obtained PCR products were stored at -20°C .

Tab. 1: Microsatellite and barcoding primer pairs used for *Paltothyreus tarsatus* (Pt1, Pt10, and Pt42) and *Megaponera analis* (Meg1, Meg2, Meg3, Meg5, Meg17, and Meg25) and their annealing temperatures; for mitochondrial barcoding the same primers were used for both species (LCO / HCO), cytochrome oxidase subunit I, and cytochrome B. Cycle numbers for all microsatellite markers were 30 and for barcoding 38. The expected product size is based on the MinIon genomic sequence. *: first number 2017 / second number 2019.

Primer pairs (5'-3')	Annealing temperature (°C)	Expected product Size (bp)	Primer Sequences (5'-3')	Allele number*	H ₀ *	H _E *
Meg1 , Forward	57	225	CCGTACCTGTAATTATCCCTTCCC	8/7	0.76/0.73	0.75/0.77
Reverse	57		GACGAGGACGATTTCGACGTC			
Meg2 , Forward	57	162	GGCTGCATGGTCACGATTG	13/12	0.84/0.80	0.83/0.79
Reverse	57		GAATTCTAACGGCGGGAAGG			
Meg3 , Forward	57	175	TGCTTTTCCGTTCTCTTCG	11/15	0.90/0.91	0.82/0.81
Reverse	57		GGAATACACGCAGATCCCG			
Meg5 , Forward	57	191	CGAAGAATGTAGTCGCGAGC	10/11	0.88/0.87	0.85/0.82
Reverse	57		TGTACGAGAAGCTTGCTTGC			
Meg17 , Forward	50	176	GTACAATTCACAGTTTCTTGAC	3/5	0.52/0.40	0.49/0.32
Reverse	50		GGATATGTACCACTGTATTAATGC			
Meg25 , Forward	50	216	GCGATTACACATATTTTCGTCG	12	0.75/0.68	0.76/0.72
Reverse	50		GTTGTATTACTCTCAAGTTACTCGAG			
Pt 1 , Forward	58	198	CCGGTGTGGGTGTCAGG	26	0.81	0.87
Reverse	58		CAGGCAGGGGAAATAGAACG			
Pt 10 , Forward	51	196	GCTGATTGAACGAATGGTGC	23	0.88	0.95
Reverse	51		GTTCTCGCGCATCTATACG			
Pt 42 , Forward	58	288	CTAAGCGTTCTCAATACCGGG	22	0.75	0.83
Reverse	58		CTAAGATCCGTTAGTCCCGC			
HCO 2198 (FOLMER 1994)	45	710	TAAACTTTCAGGGTGACCAAAAAATCA	-	-	-
LCO1490	45		GGTCAACAAATCAAAGATATTGG			
CytoB (CHIOTIS & al. 2000)	47	459	TATGTACTACCATGAGGACAAATATC	-	-	-
CytoB Reverse	47		ATTACACCTCTAATTTATTAGGAAT			

Primer design

For both species, a MinIon sequencing run (Oxford Nanopore Technology, Oxford, UK) was conducted using genomic DNA from a single male. The resulting sequences that were longer than 500 base pairs were searched for dinucleotide microsatellite sequences using (GC)₁₀ as template. Primer pairs for 27 microsatellite loci in *Megaponera analis* and 45 for *Paltothyreus tarsatus* were designed based on the same number of independent DNA sequences. Primers were designed using the program Primer 3.0 (UNTERGASSER & al. 2012) (Tabs. S4 - 5). Standard settings were used, except a GC-Clamp was enforced and the product size limited to 150 - 400 base pairs. All 72 primer pairs were tested for amplification and polymorphism. The most polymorphic and reliable six and three primer pairs for *M. analis* and *P. tarsatus*, respectively, were used for this study.

Microsatellite genotyping

PCR products were individually genotyped using ABI 3130xl Genetic Analyzer (Thermo Fisher Scientific, Waltham, USA). PCR products (0.5 µl) were mixed with 9.375 µl formamide and 0.125 µl GeneScan™ 350 ROX™

(Thermo Fisher Scientific) size Standard. Thereafter, the mixtures were denaturalized for 3 minutes at 95 °C. Allele calling was performed manually with the help of the software Gene Mapper v4.0. (Thermo Fisher Scientific). Ten (*Megaponera analis* 2017) or 20 (*M. analis* and *Paltothyreus tarsatus* 2019) workers for each colony were genotyped (Tabs. S6 - S7). Information about the allele number, allele frequency, and the heterozygosity of colonies and populations are reported in Tab. S8 (*M. analis*) and Tab. S9 (*P. tarsatus*). If present, we also genotyped males.

COI and cytoB barcoding

COI (658 bp region, Tab. 1) DNA fragments were amplified using the stated polymerase chain reaction conditions. Standard PCR conditions were modified for cytoB (364 bp region, Tab. 1) as following: annealing temperature was set to 47 °C, and the number of cycles for cytoB was adjusted to 38 (Tab. 1). PCR products were purified with Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP-IT™) (Thermo Fisher Scientific). 5 µL PCR product was mixed with 0.5 µL Exonuclease I and 0.25 µL Shrimp Alkaline Phosphatase (ExoSAP-IT™). The mixture incubated for 15 minutes at 37 °C, followed by 15 minutes at 85 °C, and

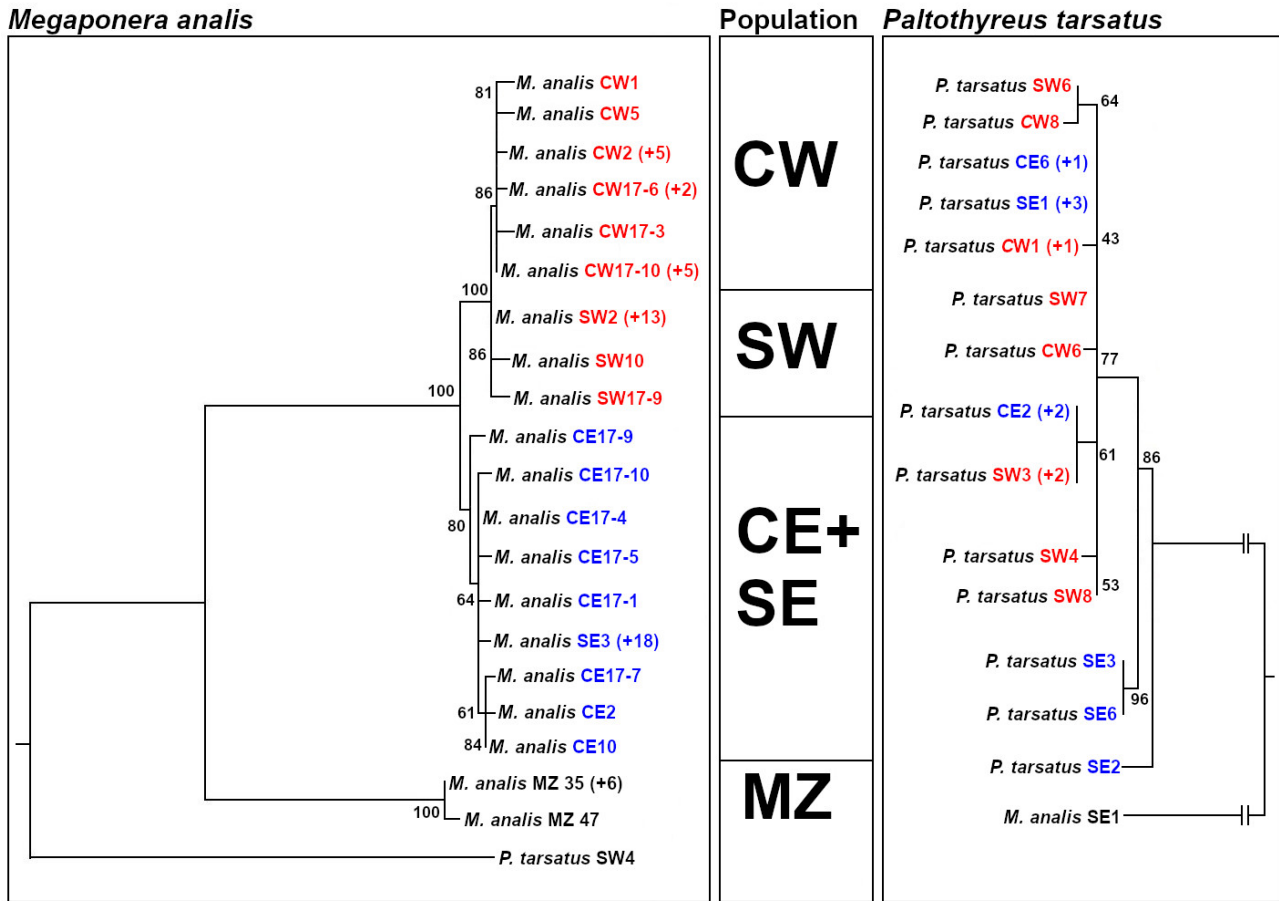


Fig. 2: Comparison of two neighbour-joining trees of the four sympatric populations of *Megaponera analis* and *Paltothyreus tarsatus* based on concatenated mitochondrial DNA. *Megaponera analis* populations CW and SW (red) form a clear cluster according to river sides (bootstrap 100% CW / SW and 80% CE / SE (blue)). In contrast, *P. tarsatus* shows no sub structure or population-specific clusters. Colonies that do not differ in their mtDNA sequence are represented by a single colony. Numbers in brackets indicate the number of colonies which share the same sequence. Whereas many *M. analis* colonies share the same mtDNA sequences, *P. tarsatus* shows much greater diversity at the mitochondrial level. This is most likely due to the fundamental differences in colony reproduction, that is, fission versus independent colony founding by queens.

finally cooled down to 4 °C. For Sanger sequencing, 5 µL of the mitochondrial purification was either mixed with 5 µL (5 mM concentration) of cytoB and COI forward or reverse universal primer, respectively. Sequencing was conducted by the GATC Biotech Centre Cologne. All sequences were manually inspected for quality using the software BioEdit 7.2.5 (HALL 1999). Primer sequences were removed manually, and sequences were aligned using MEGA-X 10.1.8 (KUMAR & al. 2018).

Phylogenetic analysis based on mtDNA

One individual of each colony was sequenced for the two mitochondrial gene regions, COI and cytoB. Obtained sequences were checked manually for quality. Only sequences with unambiguous assignments of base pairs were used for further phylogenetic analyses. Sequences were aligned using the multiple alignment program MEGA-X 10.1.8. Concatenated sequences of COI and cytoB were also created (COI 1 – 658 bp, cytoB 659 – 1022 bp, Tab. S10) in order to increase the number of phylogenetically informa-

tive sites and obtain phylogenetic trees with higher resolution. Sequence alignments were conducted using ClustalW (version 10.1.8). Phylogenetic relationships among the populations were inferred using neighbour-joining trees in MEGA-X 10.1.8 (Fig. 2). Branch support was calculated from 100 bootstrap replicates. Trees were rooted using one worker sequence of the respective other species as an outgroup. Voucher specimens are deposited at the Institute for Evolution and Biodiversity, University Münster.

Socio- and population-genetic analysis

Monogynous and monandrous colonies have at most three different alleles per locus. One of two alleles is inherited by a diploid female / queen, and one allele from a haploid male / mate. In terms of colony fission, an additional fourth allele is possible to be present, since a new queen, accompanied by full sisters, mates with an unrelated male, and therefore introduces one additional allele to the gene pool through her offspring (Tab. 2). The new offspring slowly replace the old worker force, which originated from

Tab. 2: Social structure of *Megaponera analis* (n = 78) and *Paltothyreus tarsatus* (n = 30) based on microsatellite genotyping of workers. The majority of *M. analis* colonies had one singly mated queen (monogynous / monandrous). Thirty colonies had workers from a previous queen (second matriline) after colony fission and 4 required additional patriline (monogynous / polyandrous). Since *Paltothyreus tarsatus* does not reproduce by colony fission, colonies with additional matriline were interpreted as polygynous. Hence only a minority of the *P. tarsatus* colonies have a simple monogynous/monandrous social structure.

Species	Monogynous/ monandrous colonies	Colonies with second matriline	Monogynous, polyandrous colonies	Polygynous, polyandrous colonies
<i>M. analis</i> (n=78)	44	30	4	-
<i>P. tarsatus</i> (n=30)	7	-	14	9

Tab. 3: Median and 95% confidence intervals (CI, bootstrapped over loci) for F_{IS} and F_{ST} of *Megaponera analis* colonies, collected in 2017 and 2019 and calculated from 10 and 20 randomized datasets (1 worker per colony), respectively (GOUDET 2003).

F-statistics	<i>Megaponera analis</i> 2017	95% CI range bootstrapped over loci	<i>Megaponera analis</i> 2019	95% CI range bootstrapped over loci
F_{IS} (median)	-0.036	-0.146 – 0.081	-0.0365	-0.144 – 0.098
F_{ST} (median)	0.019	-0.01 – 0.056	0.025	-0.010 – 0.071

the previous, old queen. Thus, the appearance of one additional allele is seen as remnants from the previous, old queen. If new queens would mate with their brothers, we would expect a significant inbreeding coefficient and many homozygous queens.

The most common method for determining population differentiation and inbreeding is the F-statistics (WRIGHT 1931, WEIR & COCKERHAM 1984, SLATKIN 1987). Based on worker genotypes, we calculated allele frequency, observed, and expected heterozygosity's (H_O and H_E , Tab. 1), genotypic diversity, F-statistics (F_{IS} and F_{ST} , Tab. 3), the number of reproductive queens, and mating frequency (Tabs. S11 - 13). Since *Megaponera analis* colonies relocate nest sites frequently over a short distance of max. 95 meters (LONGHURST & HOWSE 1979b, YUSUF 2010), sampled colonies with close proximity (geographical distances < 10 meters apart) and different collection dates, including identical genotypes for the respective queen and male, are assumed to be from the same colony. Initially, *M. analis* colony samples of SE1, SE3, SE7 and *M. analis* colony samples of SW2 and SW4 were thought to be separate colonies, but when taking into account the genotyping results and the geographical positions of the sampling events, the probability appears high that these data in fact represent colonies resampled. For all further analyses, we only used one colony sample of these suspected repeatedly sampled colonies (e.g., SE1 and SW2).

Statistical analyses were conducted using Matesoft Silver V1.0 (MOILANEN & al. 2004) and F_{STAT} 2.9.4. (GOUDET 2003). Median values of the respective data sets were calculated and are shown in Table 3. Matesoft Silver V1.0 was used for determining mating frequencies, excluding colonies that cannot be monogynous given the worker genotypes (Matesoft generates an error message if worker

genotypes of a colony are not compatible with monogyny). Workers with additional alleles, that require a second matriline, had to be excluded from the mating frequency analysis (MOILANEN & al. 2004). Colonies marked with “*” have several possible queen genotypes. Combination of alleles with highest probability are listed in Tables S11 - 13. As Matesoft accepts only monogynous colonies, additional alleles of single individuals, that violate monogyny, were excluded and marked with “+”. Colonies in *Paltothyreus tarsatus* that were not monogynous were excluded from mating frequency calculations. The obtained genotypes of queens and males for the individual colonies are listed in Tables S11 - 13. Allele frequencies, population genetic analyses for investigations of the inbreeding coefficient F_{IS} (NEI 1978) and H_E and H_O , as well as population sub-structure F_{ST} (WEIR & COCKERHAM 1984) were calculated using F_{STAT} 2.9.4.

To avoid any bias due to the intracolony relatedness of workers in our population genetic analyses, we generated 10 (2017) and 20 (2019) independent datasets which contained only a single worker per colony. Medians for F_{ST} and F_{IS} were calculated, based on the 10 and 20 datasets, respectively. 95% confidence intervals (CI) were obtained by bootstrapping over loci. The nominal level for multiple comparison in pairwise tests of differentiation was set to 0.0083 using Bonferroni correction (Tab. 3).

Results

We successfully amplified six and three polymorphic primer pairs for genotyping *Megaponera analis* and *Paltothyreus tarsatus* workers and males, respectively. *Megaponera analis* primer pairs Meg1, Meg2, Meg3, Meg5, and Meg25 were polymorphic with allele numbers between 7 (Meg1) and 15 (Meg3), but Meg17 was less polymorphic

Tab. 4: Counted colony composition of 21 *Megaponera analis* colonies. Queen number, major, intermedia, minor, cocoon, larva, eggs, males, juveniles date, and respective sums of ants and brood are indicated. In rows 23 to 25 we provide the averages, medians, and median absolute deviations (MAD).

Colony size of <i>Megaponera analis</i>												
#	Queen	Major	Inter-media	Minor	Total	Cocoon	Larva	Eggs	Males	Juveniles	All In	Date
1	1	390	257	595	1242	172	236	-	6	-	1656	18.VIII.2014
2	1	701	659	931	2291	819	578	255	132	-	4075	09.IX.2014
3	1	599	343	601	1543	709	443	310	7	-	3012	20.IX.2014
4	1	613	369	813	1795	991	807	934	2	-	4529	01.X.2014
5	0	310	249	406	1115	605	331	210	2	74	2337	31.VII.2015
6	1	205	265	315	919	228	241	449	3	85	1925	07.VIII.2015
7	1	333	332	572	1423	788	376	-	50	106	2743	19.VIII.2015
8	1	263	208	262	842	523	830	372	1	70	2638	04.IX.2015
9	1	682	399	688	1977	752	720	674	81	143	4347	17.IX.2015
10	1	264	222	300	964	580	501	484	3	132	2664	19.X.2015
11	1	240	119	185	544	260	451	732	22	84	2093	01.III.2016
12	1	-	-	-	1940	981	633	131	50	230	3965	10.III.2016
13	1	-	-	-	699	351	607	586	39	18	2300	18.III.2016
14	1	-	-	-	810	6	1128	768	25	83	2820	30.III.2016
15	1	99	116	161	376	0	749	130	47	47	1349	07.IV.2017
16	1	589	265	604	1458	923	870	186	2	139	3578	24.IV.2018
17	1	199	125	368	692	667	569	339	0	-	2267	10.II.2019
18	1	302	418	410	1130	745	539	-	2	169	2585	19.V.2019
19	1	440	211	393	1044	405	318	192	12	64	2035	23.IX.2019
20	1	335	264	354	953	289	412	426	4	36	2120	24.IX.2019
21	1	364	323	534	1221	504	333	9	1	214	2282	26.IX.2019
Average	1	384.9	285.8	471.8	1189.4	538.0	555.8	399.3	23.4	105.9	2729.5	-
Median	1	334	265	410	1115	580	539	372	6	85	2612	-
MAD	0	152	97	180	336	237	178	117	6	43	505	-

with 3 to 5 alleles (Tab. 1). Primer pairs for *P. tarsatus* were highly polymorphic with allele numbers of 22 (Pt42), 23 (Pt10), and 26 (Pt1).

Social structure and colony demography

The demographic structures were analysed and are shown in Table 4. Colony counts of alates in *Megaponera analis* indicate a highly male-biased sex ratio. Only one ergatoid queen was found in each completely excavated colony, whereas an average of 23.4 ± 7.2 SE males was present, with a maximum of 132 (Tab. 4). We genotyped 12 males from a single colony and all were sons of the resident queen, suggesting that males in a colony are usually sons of the resident queen and not potential mates. (Tab. S14).

Based on the genotypes and inbreeding coefficient F_{IS} , we have no indication that virgin queens mate with their brothers. Microsatellite genotyping showed that 45 out of 78 colonies of *Megaponera analis* were uniformly monogynous and monandrous. Colonies with no more than three alleles across all workers were determined to be monogynous and monandrous (45 and 7 colonies in *M. analis* and *Paltothyreus tarsatus*, respectively, Tab. 2). Furthermore, 29 colonies had worker genotypes not consistent with monandry because they display an additional fourth allele which could indicate that the queen mated with a second (haploid) male. Alternatively, since the new queen, accompanied by full sisters after colony fission, is

mated with an unrelated, haploid male, one new allele enters the gene pool that will replace the offspring of the old queen over time. Hence, we interpreted the presence of a fourth allele in a colony as a marker for recent fission events (see below, Tab. 2). Thus, the 29 colonies with no more than four alleles were also counted as monogynous and monandrous. Consequently, 74 out of 78 colonies of *M. analis* are monogynous and monandrous.

In 21 colonies, less than 20% of the workers display an additional allele and therefore originated from a second matriline based on genotypes at one or several loci. Only 4 colonies have a high occurrence of an additional, second matriline (30 - 40%). The remaining 4 colonies SE9, CE4, CE9, and SW17-6 have worker genotypes that are not compatible with two matrilines (Tabs. 2, S6). In colonies CE4, CE9, and SW17-6, there is only one single allele per locus not consistent with monogyny and monandry, which could be either a mutation or technical artefact. Since the old queen's offspring will become rarer over time and the new queen's offspring are rare at the beginning and we have no way of determining which genotype belongs to the old or new queen, we cannot determine the relative timing of a fission event from the frequency of the additional allele / genotype.

Seventy percent of all sampled *Paltothyreus tarsatus* colonies (21 out of 30) have worker genotypes consistent with monogyny. Of those, seven colonies could be explained by a monogynous and monandrous colony organization,

and 14 colonies required at least one additional patriline to explain the worker genotypes. The remaining 9 colonies were genetically much more diverse and were classified as polyandrous and polygynous.

Inbreeding

The inbreeding coefficient F_{IS} based on our microsatellites was not significantly different from zero in both years. Hence, our *Megaponera analis* populations are not inbred (*M. analis* 2017 $F_{IS(\text{median})} = -0.036$, range 95% confidence interval (CI) bootstrapped over loci = $-0.146 - 0.081$; *M. analis* 2019 $F_{IS(\text{median})} = -0.0365$; range 95% CI bootstrapped over loci = $-0.144 - 0.098$ (actual F_{ST} and CI distribution of all subsamples in Tab. S15)). Since we only had three microsatellite loci for *Paltothyreus tarsatus*, we could not calculate any meaningful inbreeding coefficient with confidence intervals.

Population structure based on mitochondrial DNA

Both parsimony and maximum likelihood trees did not differ from the neighbour-joining trees. For *Megaponera analis*, trees were rooted by an additional intraspecific outgroup of 19 *M. analis* colonies from Mozambique (*M. analis* MZ population). *Megaponera analis* colonies, collected in 2017 and 2019, cluster with 100% bootstrap support in accord with subpopulations of the same riverside CW / SW and CE / SE (Fig. 2; West = red and East = blue coloured, Figs. S3 - 6). The intraspecific outgroup from Mozambique also clearly differs from the Ivorian samples (Fig. 2, bootstrap support 100%). In contrast, the neighbour-joining tree of *M. analis* shows no substructure along river sides or subpopulations (Figs. S3 - 5). In comparison, *Paltothyreus tarsatus* has a significantly more homogenous distribution of the individual population, without any apparent sub structuring (bootstrap support between 43 and 96%, Fig. S6). As an example, we show one neighbour-joining tree of both species, which shows the differences in population structures of both species side by side, visualized by coloured assignment to the respective river sides (West = red and East = blue, Fig. 2).

Population structure based on nuclear DNA

All F_{ST} values, based on microsatellite genotypes, were not significantly different from zero (i.e., confidence intervals include zero), indicating no population substructure across our four populations for both species: *Megaponera analis* 2017 $F_{ST(\text{median})} = 0.0019$, range 95% CI bootstrapped over loci = $-0.01 - 0.056$ (actual F_{ST} and CI distribution of all subsamples in Tab. S15); *M. analis* 2019 $F_{ST(\text{median})} = 0.025$, range 95% CI bootstrapped over loci = $-0.010 - 0.071$. For *Paltothyreus tarsatus* 2019, CI bootstrapped over loci were not possible to calculate using only 3 loci (Tab. 3). The pairwise test of differentiation had an indicative adjusted nominal level (5%) for multiple comparisons with a significance level of $p\text{-value} = 0.0083$, also showing no significance in any population (Tabs. S15 - 16).

Discussion

We confirmed that natural barriers, like the river Co-moé, restrict the mtDNA gene flow among populations in *Megaponera analis* but not in *Paltothyreus tarsatus*. We show that male-biased sex ratio and males' dispersal ability compensate for the reduction in gene flow among populations due to the limited dispersal abilities of ergatoid queens in *M. analis*. Furthermore, male-biased sex ratios with obligate male dispersal prevent colonies with ergatoid, apterygote queens from inbreeding and possibly mitigate inbreeding depression (KELLER & WALLER 2002). Our result, that *M. analis* is monandrous but reproduces by colony fission, supports the idea that colony fission alone does not select for polyandry. Hence we argue that polyandry might rather be linked to large colony size in other social insects that also show colony fission (e.g., honeybees, army ants). In our study, we used two sympatric, closely related ponerine species, hunting for the same prey (SCHMIDT 2013) but differing in their life-history, dispersal strategy, and social structure. We used these two ponerine species to gain insights into the development and evolution of colony fission and how gene flow is secured with apterygote queens.

Sociogenetic structure and the usefulness of microsatellite markers

We used microsatellites to compare the different genotypes of our four tested populations. Although microsatellites have come out of fashion due to the availability of high throughput sequencing generating a bounty of single nucleotide polymorphism (SNP) markers, microsatellites are well established for relatedness / kinship and population genetic studies (QUELLER & al. 1993, GADAU & al. 1996, KRONAUER & al. 2003, WAGNER & al. 2006, KIM & SAPPINGTON 2013). The advantages of using microsatellites besides their reproducibility is their high polymorphism, which provides each locus and genotype with a higher information content than other genetic markers like SNP. This feature of microsatellites requires fewer markers to obtain a good estimate for several sociogenetic or population-genetic estimates in comparison with SNP markers. Furthermore, using microsatellites is much cheaper and makes it feasible to genotype > 1000 individuals if one wants to get a good estimate of the sociogenetic structure of several populations of the same species or compare it among species (POL & al. 2008, OVERSON & al. 2016). Additionally, microsatellites are arguably the best method to quickly identify unusual reproductive systems like genetic caste determination or thelytoky. This is because genotyping parents and offspring for a single locus or loci with high expected heterozygosity (heterozygosity for microsatellites is typically higher than 0.9) may alert a researcher that the genotype frequencies are not in Hardy-Weinberg-Equilibrium (HELMS CAHAN & KELLER 2003, SCHWANDER & al. 2007, DARRAS & al. 2013). Hence, microsatellites are still a competitive marker system if one is interested in the sociogenetic structure of social insects, and although they may not be ideal markers for population-genetic estimates, they

can provide a first glimpse. Moreover, areas where microsatellites are problematic have been described and hence can be avoided or highlighted (LEMPOULOS & al. 2019).

Monandry and monogyny are thought to be the ancestral condition in all major lineages of eusocial insects (BOOMSMA 2007, HUGHES & al. 2008). These conditions are thought to be favored for two main reasons: First, the relatedness among the offspring of the queen is high (inclusive fitness theory), supporting the evolution of eusociality (CROZIER & PAMILO 1996, BOOMSMA 2007, CROZIER 2008, HUGHES & al. 2008, BOOMSMA & al. 2009). Second, females minimize the cost of mating, time, and energy. Although the subfamily Ponerinae is usually described as having a simple social structure, they have adapted to various environmental factors over the course of evolution and have developed derived social traits, which can be as complex as in other ant subfamilies (BOLTON 1990, WILSON & HÖLLDOBLER 2005).

***Megaponera analis*:** *Megaponera analis* colonies / queens / workers have undergone strong morphological and ecological adaptations to their habitat (LONGHURST & al. 1979, CREWE & al. 1984, VILLET 1990a, FRANK & LINSENMAIR 2017a, b, FRANK & al. 2018a, b). For example, DCF and dispersal occur through colony fission (PEETERS & ITO 2001), queens are permanently wingless, and worker castes show monophasic allometry (LONGHURST & HOWSE 1979b, CREWE & al. 1984, VILLET 1990a), which is linked to a division of labor during raids on termite feeding grounds.

We confirmed monogyny and monandry unambiguously in 45 of 78 *Megaponera analis* colonies. Twenty-nine colonies display four or more than four alleles. In theory, additional alleles could be explained by polygyny, polyandry, fusion, or oligogyny. Polygyny and oligogyny would be clearly visible in microsatellites with several different alleles per loci (> 5). In excavations of whole colonies, multiple inseminated queens were also never observed in one colony (Tab. 4). Colony fusion is not likely due to distinct chemical cuticle hydrocarbon profiles, and aggression assays showed a clear hostility towards foreign profiles (YUSUF & al. 2010).

We found a second matriline in 29 *Megaponera analis* colonies, but we interpreted these as remnants of previous fission events because in all cases we found a maximum of four alleles, that is, three alleles from the previous (monandrous) queen and one additional from the mate of the new queen. Under this assumption, we confirm monogyny and monandry in 74 of 78 *M. analis* colonies, which was further supported by nest excavations finding only one queen in all cases (Tab. 4).

Not much is known about the frequency of fission events in *Megaponera analis*. Based on estimated colony demography parameters, for example, mean estimated birth rate of 13.3 ± 3.8 per day and matching mortality rate for a colony in equilibrium (FRANK & al. 2017), a fission event could occur at least once a year. We found a second matriline in approximately 40% of the worker genotypes of our colonies. Smaller proportions of individuals per colony from a second matriline indicate either a very recent fission

event in which the workers of the new queen just started to appear or an older fission event where the majority of workers from the previous queen have already been mostly replaced (Figs. S7 - 8).

All species known to have colony fission have a significant male biased numerical sex ratio (honey-bees, army ants, stingless bees (BRIAN 1965, SCHNEIRLA 1971, MICHENER 1974)). *Megaponera analis* also shows a male biased sex ratio (average of 23.4 ± 7.2 SE males / colony, $n = 21$, Tab. 4) and males seem to stay within their natal colony for a while but do not mate with their sisters (no significant inbreeding coefficient F_{IS} , Tab. 3). Hence, winged *M. analis* males leave their natal colony to find mates and thus compensate for the restricted dispersal abilities of their wingless queens.

DCF through colony fission is already well studied in bees, wasps, and stingless bees and fairly well documented in ants (WEST-EBERHARD 1982, PEETERS 2001, KRONAUER & al. 2004, GROZINGER & al. 2014). In comparison with *M. analis*, army ants (e.g., in the genus *Eciton*) show some similarities in terms of DCF, monogyny, and highly male-biased sex ratios but differ significantly in queen mating frequency and colony size. Army ant colonies can include millions of individuals, driven by a single, highly polyandrous queen and a high population turnover (KRONAUER & al. 2004). There are several theories about the advantages of polyandry: firstly, the most prominent “genetic variance” hypothesis, supporting the overall colony fitness in aspects like increasing colony productivity, tolerance to variable environments and pathogen resistance (KELLER & REEVE 1994, CROZIER & FJERDINGSTAD 2001, BOOMSMA & al. 2009); secondly, the sperm-limitation hypothesis, that one male cannot provide the necessary number of sperm for species with a high number of individuals in long-lived colonies like army ants or honeybees (COLE 1983, KRAUS & al. 2004); thirdly, it could be interpreted as counteracting inbreeding, inbreeding depression, and population fragmentation (KELLER & WALLER 2002, JAFFÉ & al. 2009, BARTH & al. 2013) that *M. analis* have significantly smaller colonies with 900 - 2200 individuals and a low population turnover (VILLET 1990a, YUSUF & al. 2013, FRANK & al. 2017). This could suggest that the main driver for polyandry in army ants is driven by sperm limitation rather than genetic variance, inbreeding or lifestyle changes (like colony fission), but this remains to be tested.

***Paltothyreus tarsatus*:** There are only three studies on the social and population structure of *P. tarsatus* (HÖLLDOBLER 1984, BRAUN & al. 1994, PEETERS & al. 2013). Initially, *P. tarsatus* was thought to have a simple monogynous and monandrous colony structure (PEETERS 1993, BRAUN & al. 1994). However, these studies have also shown that *P. tarsatus* colonies can vary greatly in the number of dealate queens, number of workers and brood, and nesting strategy (polydomy) (PEETERS & al. 2013). Our results support the notion that *P. tarsatus* has a wide range of sociogenetic organization, which include both mono- and polygyny and mono- and polyandry (Tab. 2). Hence, *P. tarsatus* might switch from monogyny

to polygyny as colonies grow or start colonies already with multiple queens, but the exact mechanisms (queen adaptation, pleometrosis, colony fusion) and whether there are differences among populations are unknown. Supporting the finding of PEETERS & al. (2013) that large colonies are polygynous and polydomous, we found two cases where nest samples which we initially assumed to come from different colonies could originate from the same polydomous colony. For example, colonies SE3 and SE6 (with a distance of ~ 75 m between them) and *P. tarsatus* SE2, SE3, SE4, SE5, and SE7 (with a distance of ~ 146 m between them) had several overlapping genotypes indicating that they may come from the same polydomous / polygynous colony. Alternatively, this may indicate that new reproductive queens form satellite or new colonies close to their parental colony. Due to highly polymorphic microsatellite loci (allele numbers 22 - 26, Tab. 1) and the limited sample size of 20 individuals per colony, we can only estimate the size and organization of *P. tarsatus* colonies. However, each sample also had unique genotypes, showing that we are far from an exhaustive sampling of all genotypes / matriline / patriline in these colonies. In our study, 9 out of the 30 sampled *P. tarsatus* colonies have a clear monogynous and monandrous colony structure. In 14 other colonies, genotypes could not be unambiguously assigned to either polygyny or polyandry. However, the remaining 7 *P. tarsatus* colonies have a clear polygynous colony structure, which is atypical for ponerines. To get a better picture of the sociogenetic structure of *P. tarsatus*, one would need to genotype many more workers for more microsatellites because our results show that large colonies could have a rather complex sociogenetic organization including both polyandry and polygyny. Overall, our results on the sociogenetic structure of both species demonstrate that the social structures of ponerines can be as diverse as in other ant subfamilies.

Inbreeding

The observation that males are often present in larger *Megaponera analis* colonies (Tab. 4) has led to the question whether new virgin queens mate with their brothers or avoid inbreeding and only mate with foreign males. Observations of single males entering *M. analis* colonies support the foreign male hypothesis although it is unknown whether these were foreign males or brothers (LONGHURST & HOWSE 1979a). Brother-sister mating would give a highly significant, positive F_{IS} value and generally low heterozygosity. All F_{IS} values for *M. analis* colonies sampled in 2017 and 2019 are negative or not significantly different from zero, and the observed and expected heterozygosity did not differ significantly (see results, Tab. 3). Thus, we could not detect any significant inbreeding in the investigated *M. analis* populations, demonstrating that virgin queens of *M. analis* do not or very rarely mate with their brothers.

In *Paltothyreus tarsatus*, the inbreeding coefficient F_{IS} could not be calculated due to limited sample sizes. However, since *P. tarsatus* has winged alates, which mate during extensive nuptial flights, it is unlikely that a sig-

nificant degree of inbreeding is present. Additionally, the amount of genetic diversity observed both at the population and colony level argue against widespread inbreeding in this species.

Population genetics (mitochondrial DNA)

Mitochondrial DNA (mtDNA) is only maternally inherited, hence mtDNA serves as an indicator for maternal gene flow. We expected a significant population substructure in *Megaponera analis* due to wingless queens in this species but not in *Paltothyreus tarsatus*.

***Megaponera analis*:** The mitochondrial neighbour-joining tree clusters *M. analis* colonies of the western and eastern river side with high bootstrap values ($\geq 99\%$, Figs. 2, S3 - S5), whereas populations from the same river side seem to be less isolated (bootstrap values $< 86\%$). This confirms our hypothesis that the river Comoé acts as a natural barrier and restricts the maternal gene flow since wingless females are not able to overcome such natural barriers.

***Paltothyreus tarsatus*:** In contrast to *Megaponera analis*, the mitochondrial neighbour-joining tree of *P. tarsatus* (Figs. 2, S6) shows no substructures correlated with the river sides. Hence, the river Comoé does not act as a limiting factor regarding the mitochondrial / maternal gene flow in *P. tarsatus*, and queens and males seem to easily overcome this natural barrier. This difference is probably due to the fact that in *P. tarsatus* both male and female reproductives are winged and perform nuptial flights, and hence alate females of *P. tarsatus* have a significantly larger dispersal range than *M. analis* queens.

Population genetics (nuclear DNA)

Although sub-structuring of individual populations in *Megaponera analis* can be observed based on the concatenated mtDNA sequences, microsatellite genotype analyses yielded a different outcome. The 95% CI bootstrapped over loci for the estimated F_{ST} value did include zero indicating no significant population substructure (Tab. 3). This suggests that the gene flow among *M. analis* populations across further distances and natural barriers like rivers is based on males, which seems to be enough to avoid population substructure, at least across the distances we are investigating.

Conclusion

The mtDNA gene flow among monogynous and monandrous *Megaponera analis* populations is severely restricted over short distances by natural barriers such as the river Comoé. No significant restrictions could be detected in *Paltothyreus tarsatus*, most likely because this species has winged queens which can overcome those natural barriers. As there are no significant F_{ST} values for all populations of *M. analis*, it can be concluded that *M. analis* males, like winged males in other ergatoid species, are mainly responsible for the gene flow among populations. Highly male-biased production and outbreeding of new founding queens prevent populations from inbreeding depression (KELLER & WALLER 2002). Interestingly, our

results support the occurrence of monogynous and polygynous *P. tarsatus* colonies in the same population. How polygyny in *P. tarsatus* originated (pleometrosis, adoption, or fusion) requires further investigation.

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Declarations

Consent for publication: All authors have consented to publication.

Conflict of interest: The authors declare no competing interest.

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Ethics approval: The research was carried out under research permit number N°018 / MINEDD / OIPR / DZ from the Office Ivoirien des Parcs et Réserves and adhered to the requirements of the relevant research guidelines. The experiments detailed here comply with the current laws of the country in which they were performed.

Data availability statement: The authors confirm that the data supporting the findings of this study are available within the article [and / or] its supplementary materials.

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