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One tree, many colonies: colony structure, breeding system and colonization events of host trees in tunnelling *Melissotarsus* ants

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Ants exhibit a striking variety of lifestyles, including highly specialist or mutualist species. The minute blind workers of the African genus *Melissotarsus* chew tunnels in live trees to accommodate their obligate partner scale insects. Their modified legs are adapted for tunnelling, but are unsuited for walking outside, confining these ants to their initial host tree. Here, we investigated whether this unique lifestyle results in complex patterns of genetic diversity at different scales, from the same tree to different populations. Using 19 microsatellite markers, we assessed their mating strategy and colony structure among and across populations in South Africa. We showed that only one queen reproduces within a colony, mated with up to three males. However, several inseminated dealate queens are present in colonies; one probably replaces the older queen as the colony ages. The reproduction of a single queen per colony at a given time results in genetic differences between colonies, even those located on the same tree. We discuss how the slow process of colony digging under the bark and the lack of workers patrolling above the bark might result in reduced competition between colonies and allow several secluded colonies to cohabit the cramped space on a single tree.

ADDITIONAL KEYWORDS: ant – myrmecophyte – population structure – queen turnover – tree-living.

INTRODUCTION

Ants are one of the most ecologically dominant insects due to a striking variety of diets (live and dead insects, fungi, honeydew and other sweet secretions), and represent a huge biomass resulting from large numbers of workers that sustain their perennial colonies. Among 13 000 known species with colony sizes ranging over six orders of magnitude (less than ten to millions of workers), some genera stand out for their extreme lifestyles, such as army ants, social parasites, fungus farmers and intimate mutualists with plants, sap-feeding insects or other organisms (Holldobler & Wilson, 1990; Dill *et al.*, 2002; Brady *et al.*, 2006; Oliver *et al.*, 2008; Schultz & Brady, 2008). In addition to having profound ecological consequences, these extreme lifestyles can have major influences on the population genetic structure of the species. This is especially true for specialist or mutualist species because their dispersal abilities, hence the amount of gene flow within and between populations, are intrinsically linked to the local distribution of the partner organism (Herre *et al.*, 1994; Thompson, 1999; Hoeksema & Bruna, 2000; Thompson & Cunningham, 2002). Consequently, disruptive effects of geographical distance and/or barriers may be amplified in specialist and mutualist species by the patchy distribution of suitable habitats.

In addition to extreme lifestyles, ants are also characterized by a large diversity of mating strategies and colony composition, resulting in a range of genetic structure patterns. The number of reproductive queens in a colony varies greatly, together with the number of matings for each queen. The level of polygyny (multiple queens per colony) may also vary across populations within a single species (Ross & Keller, 1995; Seppä *et al.*, 2004; Purcell *et al.*, 2015; Eyer *et al.*, 2017), as well as the degree of polyandry (multiple matings per queen) to a lesser extent (Boomsma & Van Der Have, 1998). These strategies strongly affect the amount of genetic diversity within colonies. In addition, ant species may differ in their modes of dispersal. In dependent colony foundation (DCF), new queens and nestmate workers disperse on foot to establish a colony

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nearby (Cronin *et al.*, 2013). Such short-range dispersal reduces the level of gene flow and can enhance the genetic differentiation between populations (Liautard & Keller, 2001; Clémencet *et al.*, 2005; Leppänen *et al.*, 2013). In contrast, independent colony foundation (ICF) is often associated with long-range dispersal of new queens through either nuptial flights or female-calling (Cronin *et al.*, 2013; Peeters & Aron, 2017) and usually decreases population genetic structure. In addition to distinct mating strategies, ants also exhibit different colony structures. A colony can occupy a single nest, which is referred to monodomy, or a colony can comprise several nests exchanging workers, brood and reproductive queens, which is called polydomy (Chapuisat *et al.*, 2005; Jackson *et al.*, 2007; Steiner *et al.*, 2007; Helanterä *et al.*, 2009; Eyer *et al.*, 2018a).

The African genus *Melissotarsus* can be included in this compendium of extreme lifestyles as it combines a number of unorthodox traits: these minute blind ants (workers are 2 mm in length) chew tunnels in live trees in order to accommodate diaspidid scale insects that are their obligate partners (Delage-Darchen *et al.*, 1972; Prins *et al.*, 1975; Schneider *et al.*, 1999; Ben-Dov & Fisher, 2010; Peeters *et al.*, 2017). Adaptations of the workers for tunnelling under the bark include highly modified middle legs that are incompatible with walking outside host trees (Khalife *et al.*, 2018). Consequently, these highly specialized ants depend entirely on the diaspidids for food, even though they produce no honeydew. Instead, *Melissotarsus* ants probably feed on the exuviae and excretions from the Malpighian tubules, together with the wax and proteins secreted by the diaspidids as material to construct their protective shield (Peeters *et al.*, 2017). Furthermore, and unique among the Formicidae, adult queens and workers produce silk (unique among the Formicidae) to secure their tunnels against arboreal ants (Fisher & Robertson, 1999; Billen & Peeters, 2020), a crucial adaptation because they have no morphological defence, being stingless, without poison glands and with mandibles made for chewing wood, not combat. In contrast, to workers, queens can fly and retain normal leg morphology, which suggests independent foundation of new colonies (ICF).

Because *Melissotarsus* workers cannot walk outside their tunnels, DCF is unlikely, although it may still occur through intentional or accidental tunnel blockage. Once initiated by dispersing queens, colonies are therefore confined to the initial host trees and cannot relocate or expand to another tree. Because workers cannot chew tunnels in branches lacking sufficient bark thickness, colony foundation is restricted to established trees. Several incipient colonies may consequently inhabit the same tree. As the different colonies grow larger over the years, workers extend the network of tunnels throughout the host tree up to the highest branches, concurrent with tree growth. Twenty-three botanical families of trees have been recorded with *Melissotarsus* in Africa (Peeters *et al.*, 2017), and these exhibit a substantial diversity of growth forms. In *M. beccarii* and *M. weissi* in Cameroon, behavioural observations suggested that the absence of aggression between different colonies may lead to colony merging, resulting in a single huge polygyne colony covering an entire tree (Mony *et al.*, 2007). This hypothesis is based on conjecture and it remains unclear whether expanding colonies can mix freely within a tree, or whether strict colonial boundaries are maintained. Similarly, the cryptic lifestyle of *Melissotarsus* hampers our ability to determine colony boundaries, the number of queens per colony, whether all queens reproduce after putative merging of colonies, and whether additional queens are recruited as colonies age. If the lattermost, are these queens related to the founding queen? Or are they unrelated queens coming from other colonies? Likewise, several dealate queens are mated in a colony (Mony *et al.*, 2002; Peeters *et al.*, 2017), and it is unclear whether these queens mated with their brothers as the colony grows.

In this study, we investigated mating strategy and colony structure of the wood-chewing *Melissotarsus* ants across five populations in southern Africa. We questioned whether their confined foraging strategy results in complex genetic patterns among and between colonies. More specifically, we investigated whether a single colony can monopolize a branch or a host tree, and whether different colonies inhabiting the same tree merge or maintain a strict colony boundary. We sampled 35 nests of *Melissotarsus* in four localities in South Africa and one locality in Mozambique, and genotyped their workers using 19 microsatellite markers. We assessed the reproductive system and population genetic structure by exploring patterns of genetic diversity at different scales. We investigated genetic difference at the local scale (i.e. nests located on different parts of the same branch and nests located on different stems of the same tree) to genetic differentiation at the regional scale (i.e. populations). We inferred the social structure and breeding system of each colony, determining the number of queens, the number of matings per queen and their mode of dispersal.

MATERIAL AND METHODS

Colony sampling

Ants of the genus *Melissotarsus* were studied in four localities across South Africa (2017–2019): uMkhuze (MK), St Lucia (SL), Eastern Cape (EC) and Cederberg (CE) (Fig. 1; detailed sampling information is provided

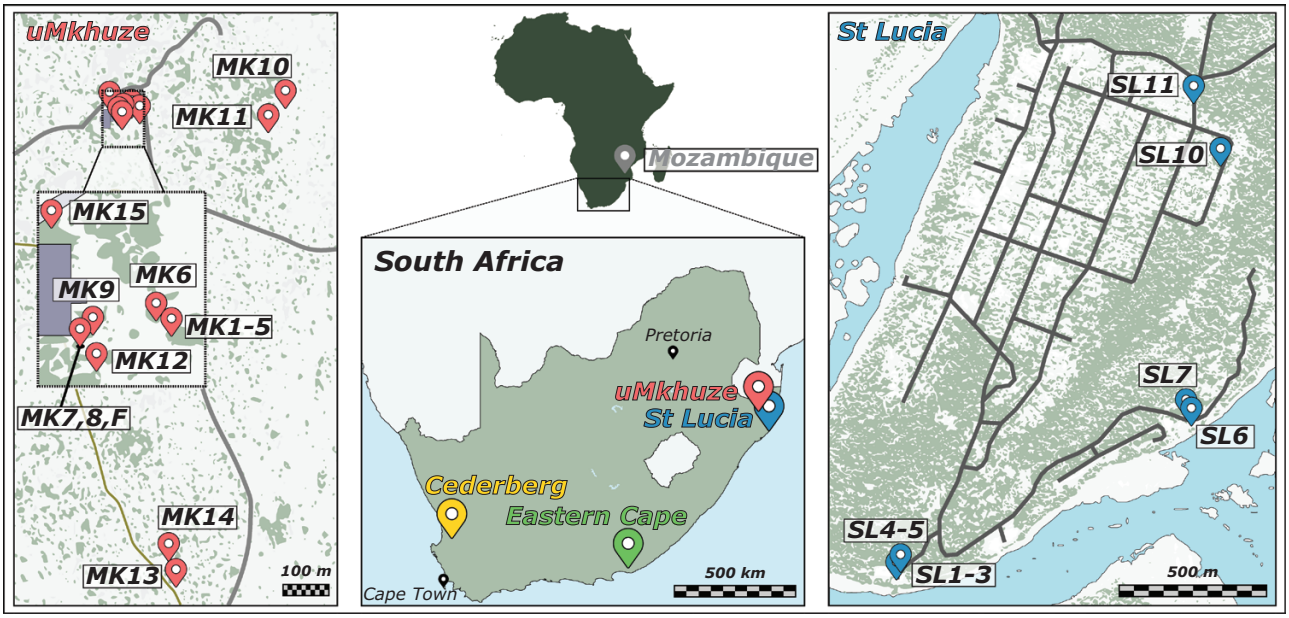

Figure 1. Geographic positions of the 34 nests of *Melissotarsus* sampled in four localities in South Africa, and one pooled sample from Mozambique. Insets indicate sampling positions of nests within the localities of uMkhuze (left) and St Lucia (right). Nests located on the same branch or tree are indicated with the same label.

in Supporting Information, Table S1). Nests were found in different host tree species. However, the same diaspine species, *Morganella conspicua,* was identified in almost all the nests sampled. Inhabited trees were identified by vein-like markings on the bark, revealing the presence of tunnels under the surface. For each nest, small areas of bark were shaved off and at least 20 adult workers and larvae were collected in ethanol for genetic analyses. All trees sampled were mapped and documented with Mapit GIS.

The uMkhuze and St Lucia localities in KwaZulu-Natal Province are about 100 km distant from each other. In uMkhuze, 17 nests were sampled, including six nests located on six different stems of the same *Strychnos madagascariensis* tree (*MK1-MK5*), and three nests located on different parts of the same stem (*MK7+MK8+MKF*; Supporting Information, Fig. S1). *MKF* denotes an incipient founding colony consisting of three adults and five larvae. In this locality, all but one nest was found in' inhabited *S. madagascariensis* trees (*MK15* was collected in *Ziziphus mucronata*). For four trees, sections of branches (50–60 cm long) were sawn off and taken to Paris (Sorbonne University) for behavioural observations and to search for reproductive queens. In St Lucia, nine nests were sampled out of five *Erythrina lysistemon* trees, including some nests (*SL1-3* and *SL4-5*) located on different stems of the same tree. For two trees, a branch was removed and brought to Paris (Supporting Information, Fig. S1). In the Eastern Cape population, four nests were sampled from *Olea capensis* and *Pterocelastrus tricuspidatus* trees. In the Cederberg (Western Cape), Four nests were sampled in the Cederberg population (Western Cape) from *Leucospermum praemorsum* and *Maytenus oleiodes* trees. Additional samples were collected in Mozambique (Mo; 2016): Cabo Delgado Province in the north (Namoto Forest; *Olax dissitiflora*) and Sofala Province (Gorongosa National Park; *Piliostigma thoningii*). However, the Mozambique workers were pooled and therefore were not used to characterize the mating system and colony structure.

Molecular analyses

DNA was extracted for eight randomly chosen workers per nest, all mother queens available (N = 15) and up to eight alate (i.e. winged and young) queens per nest (N = 25), following a modified Gentra-PureGene protocol (Gentra Systems, Inc. Minneapolis, MN, USA). Every individual was genotyped using 19 microsatellite markers previously developed for all species of ants (Butler *et al.*, 2014; Supporting Information, Table S2). Genotyping was performed using the M13-tailed primer method (Boutin-Ganache *et al.*, 2001), which separately dyes each marker with a 5'-fluorescently labelled tail (6-FAM, VIC, PET or NED dyes). PCR reactions were carried out in a volume of 15 µL including 0.25–1.0 U MyTaq HS DNA polymerase (Bioline), 2 µL MyTaq 5 × reaction buffer (Bioline), 0.08 µL each primer, 0.08 µL each M13 dye and 1 µL DNA template using a

Bio-Rad thermocycler T100 (Bio-Rad, Pleasanton, CA, USA). PCR products were analysed on an ABI 3500 genetic analyser and sized against the LIZ500 internal standard (Applied Biosystems, Foster City, CA, USA). Allele calling was performed using Geneious software v.9.1 (Kearse *et al.*, 2012).

In order to estimate population differentiation and confirm species identity, two workers per nest were sequenced for a fragment of the cytochrome oxidase 1 marker (*COI*) using the *LF1* and *LR1* primer pair (Hebert *et al.*, 2004; Smith *et al.*, 2005). PCR products were purified using the EXOSAP-it PCR purification kit (Applied Biosystems) and sequenced with the ABI BigDye Terminator v.3.1 cycle sequencing kit (Applied Biosystems). Base calling and sequence pairing were performed using CodonCode Aligner (CodonCode Corporation, Dedham, MA, USA). Fifty-four new sequences were generated for our samples and combined with 27 additional sequences of *Melissotarsus* species obtained from GenBank [*Melissotarsus insularis*, *Melissotarsus weissi*, *Melissotarsus beccarii* and *Melissotarsus emeryi* (Smith *et al.*, 2005)].

Population and colony structure

Allele frequencies, observed and expected heterozygosity, and *F*-statistics were assessed using FSTAT (Goudet, 1995). Population and colony structure were determined using only worker genotypes. For each locality, genotypic frequencies were compared between every pair of nests using log-likelihood (G)-based tests of differentiation using GENEPOP ON THE WEB (Rousset, 2008), to determine whether they belonged to the same colony. A Bonferroni correction was applied to account for multiple comparisons of all pairs. The genetic clustering of individuals within nests and populations was visualized by plotting individuals on a Principal Component Analysis (PCA) using the *Adegenet R* package (Jombart, 2008). The clustering of nests into distinct colonies was also assessed by Bayesian assignments of individuals into genetic clusters using STRUCTURE v.2.3.4 (Pritchard *et al.*, 2000). For each locality, the most likely number of genetic clusters (K) was estimated using simulations run with values of K ranging from one to the total number of nests encountered within each data set and repeated ten times for each value of K. Each run included a 5×10^4 burn-in period followed by 1×10^5 iterations of the MCMC. The most likely number of genetic clusters was evaluated using the ΔK method (Evanno *et al.*, 2005) implemented in Structure Harvester v.0.6.8 (Earl & von Holdt, 2012). Different nodes of clustering were determined using CLUMPAK (Kopelman *et al.*, 2015). PCA and STRUCTURE analyses were first performed at the entire population scale (i.e. when all localities were grouped together) and then run for each locality separately.

In addition, the phylogenetic relationship among mtDNA haplotypes was investigated using Maximum Likelihood implemented in the PhyML online web server (Guindon & Gascuel, 2003). Nodal support was assessed by bootstrap resampling (1000 pseudoreplicates). Trees were visualized using FigTree v.1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

Reproductive system and breeding strategies

For the colony fragments brought back to the laboratory, both alate and dealate queens were found while opening branches. For each colony, workers, alate and dealate queens that were genetically analysed were taken from the same network of galleries to ensure they belonged to the same colony. The latter showed various degrees of gaster enlargement, with some being highly physogastric (i.e. a fully enlarged gaster denoting high production of eggs). The abdomen was dissected to inspect the condition of the ovaries and spermatheca in a subset of these queens. For all colonies, the presence of several reproductive queens was estimated using microsatellite markers, inferring whether all workers could be assigned to a single queen (carrying one of the two alleles of the mother queen at each microsatellite locus studied). The reproductive contribution of multiple queens was deduced when at least one worker per colony could not be unambiguously assigned to a single queen. The number of matings per queen was determined for each monogyne colony based on mother-offspring analyses using the maximum-likelihood method implemented in the software COLONY v.1.2 (Wang, 2004). This analysis infers the number of males from the workers' genotypes and assigns each worker to a given patriline. The probability that additional patrilines were not detected due to two fathers sharing the same alleles at all loci was calculated for each population studied (Boomsma & Ratnieks, 1996). For each monogyne colony, the effective mating frequency (M_{ep}) was calculated for each queen following Nielsen *et al.* (2003). This estimator takes into account potential unequal contribution of the different fathers to offspring production. The effective number of patrilines equals the absolute mating frequency when all males contribute equally.

For all colonies, relatedness coefficients (r) among nestmate workers were calculated using the program COANCESTRY v.1.0 (Wang, 2011), according to the algorithm described by Queller & Goodnight (1989). Relatedness coefficients were weighted equally and standard errors (SE) were obtained by jack-knifing over colonies. Relatedness coefficients were calculated separately for each locality to account for the strong genetic structure (and differences in allele frequencies) between populations (see *Results*).

RESULTS

POPULATION AND COLONY STRUCTURE

Overall, eight new haplotypes were found on the 622 bp fragment of the *COI* mitochondrial marker for the 54 samples sequenced in this study. GenBank samples were included, although the species identifications may be ambiguous (*Melissotarsus* alpha taxonomy awaits a thorough revision). The overall data set comprised 81 sequences for which 200 nucleotide positions were variable (175 parsimony-informative). Surprisingly, samples from different localities clustered into clearly separated clades (Supporting Information, Fig. S2). The Cederberg population clustered with the GenBank samples identified as *M. emeryi.* However, the localities of Eastern Cape and St Lucia segregated away from *M. emeryi*, separated by one putative species (*M. insularis*), and the locality of uMkhuze clustered away from *M. emeryi*, separated by two putative species (*M. insularis* and *M. beccarii*). These findings suggests either that our sampling encompasses several undescribed species, or that previously described species represent geographical variants of a single species. In this study, we do not attempt to delimit different species; we hereafter refer them as different populations of *Melissotarsus*.

Nineteen microsatellite loci were successfully genotyped for up to eight workers per nest (mean ± SD = 7.49 ± 0.94; N = 262) and were found to be polymorphic with allele numbers ranging from 3 to 27 (mean ± SD = 11.26 ± 6.59). For each locality, the number of alleles for each marker, as well as observed and expected heterozygosities are provided in Supporting Information (Table S3a, b). Strong genetic differentiation was found among localities of the overall population, with F_{ST} = 0.44 ± 0.08 (F_{ST} values for each pair of localities are given in Supporting Information, Fig. S3a). Accordingly, STRUCTURE and PCA analyses showed a clear separation of the localities. When all localities were analysed, STRUCTURE revealed the occurrence of at least three genetic clusters (most likely K = 3), corresponding to the different localities sampled (i.e. EC, MK and SL+CE+Mo; Fig. 2). However, the localities of SL, CE and MO are also separated in minor nodes of clustering for K = 3 (Supporting Information, Fig. S4a) and at higher values of K (Fig. 2). Similar results were obtained when STRUCTURE analyses were performed using a single individual per colony (Supporting Information, Fig. S4b). Therefore, the microsatellite results at the overall population scale reflect the strong genetic differentiation observed between localities, which is consistent with the clear segregation observed on the mtDNA (Supporting Information, Fig. S2).

Within each locality, clear genetic differences were observed among nests (mean F_{ST} = 0.29, 0.32, 0.32

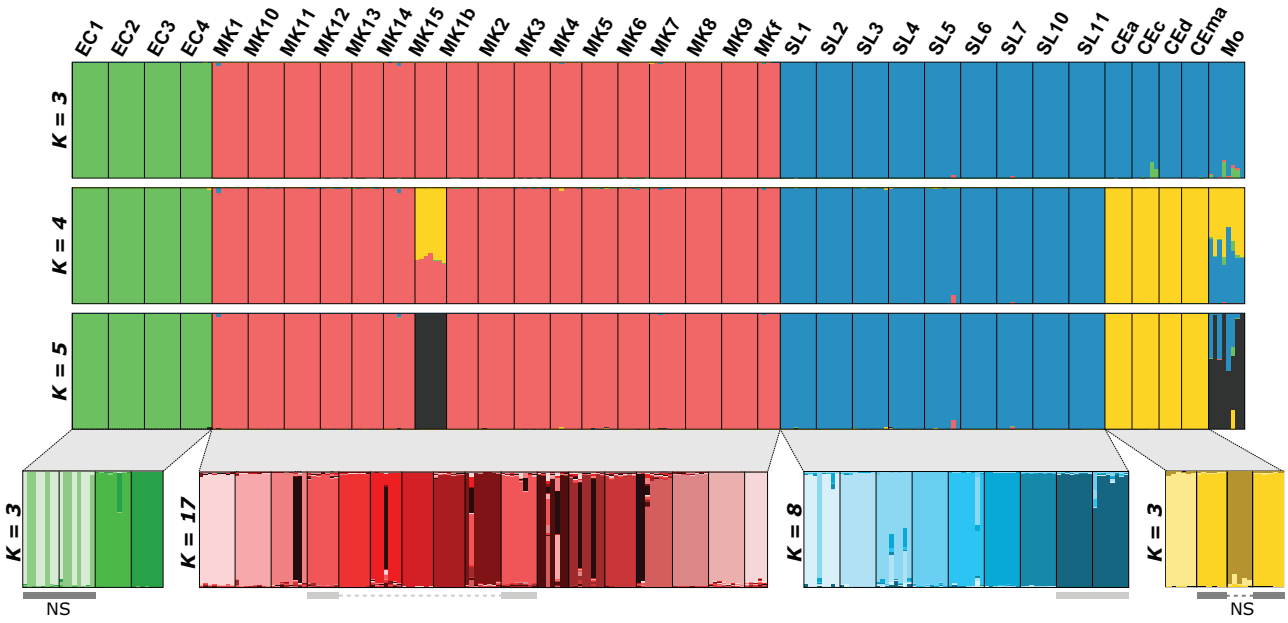

Figure 2. Graphical representation of STRUCTURE results determining the number of genetic groups in the overall dataset for different values of K. Each genetic group is characterized by a colour; and each individual is represented by a vertical bar according to its probability of belonging to each group. Distinct simulations were subsequently run for the four populations, separately. In each population, grey bars below the plot indicate different colonies assigned to a single genetic group by STRUCTURE (only the pairs EC1/2 and CEc/ma are not significant using the G-test of differentiation).

and 0.27 for EC, MK, SL and CE, respectively; F_{ST} values for each pair of nests are given in Supporting Information, Fig. S3b). Therefore, all but two pairs of nests were genetically different from each other (G-tests significant for each pair of nests; $P < 0.001$; $P = 0.994$ and 0.998 for the pairs EC1/2 and CEc/ma, respectively), even those located on the same host trees.

When each locality was analysed separately, STRUCTURE mostly segregated the different nests as distinct genetic clusters finding optimal K = 3 (out of 4 nests) for the EC population, K = 17 (out of 17) for the MK population, K = 8 (out of 9) for the SL population, and K = 3 (out of 4) for the CE population. Only four pairs of nests were not separated using STRUCTURE (MK12/3, SL10/11, EC1/2 and CEc/ma; with only the last two pairs being not significant in the G-test). This lack of difference most likely results from their genetic similarity rather than their actual belonging to a common colony, as the nests from each pair were located on different trees, separated by hundreds of metres. The PCA analysis showed clear separation of nests within localities, while nestmate workers clustered together (Fig. 3). Overall, the results at the locality scale indicate that nests mostly segregated from one another (Fig. 4), confirming that every nest, even those sampled on the same host tree, represents its own separate colony. Interestingly, colonies *MK1* to *MK5* in the uMkhuze population were located on different stems of the same host tree; however, the genetic difference between those colonies (pairwise $F_{ST} \pm SD = 0.32 \pm 0.07$) was similar to the average F_{ST} (= 0.33) observed within the uMkhuze population. However, the host tree *Strychnos madagascarensis* has an unusual growth pattern since new stems originate from the roots, and the stems never get tall (Supporting Information, Fig. S1). A clear difference between those colonies was expected as workers cannot walk between stems, hence colonies cannot merge together. However, similar differences were also found between colonies *MK7*, *MK8* and *MKF* (pairwise $F_{ST} \pm SD = 0.34 \pm 0.02$; Supporting Information, Fig. S3) inhabiting a single branch of *S. madagascarensis*, and between colonies *SL1* to *SL3* (pairwise $F_{ST} \pm SD = 0.23 \pm 0.09$) and colonies *SL4* and *SL5* ($F_{ST} = 0.43$) inhabiting single 'conventional' trees in the St Lucia population ($F_{ST} = 0.32$).

Reproductive system and breeding strategies

A single reproductive queen was inferred genetically in all but one of the 34 colonies analysed (*SL11* was polygyne). For monogyne colonies, mother-offspring inferences suggested that half of the queens were mated with a single male (16 out of the 33 queens analysed), while 13 queens were mated with two males, and only two queens were mated with three males. No queen was found mated with more than three

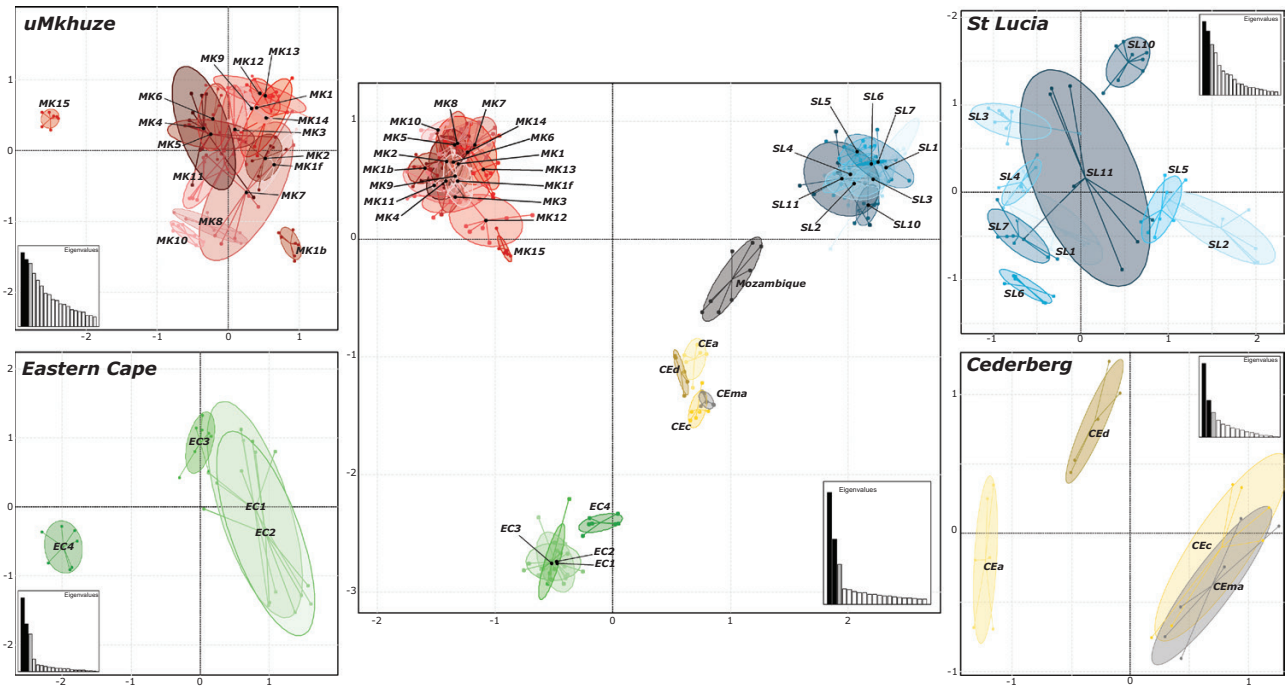

Figure 3. Clustering of nests in the overall sampling using principal component analysis of the microsatellite markers. Clustering analyses were subsequently run for each of the four populations of nests.

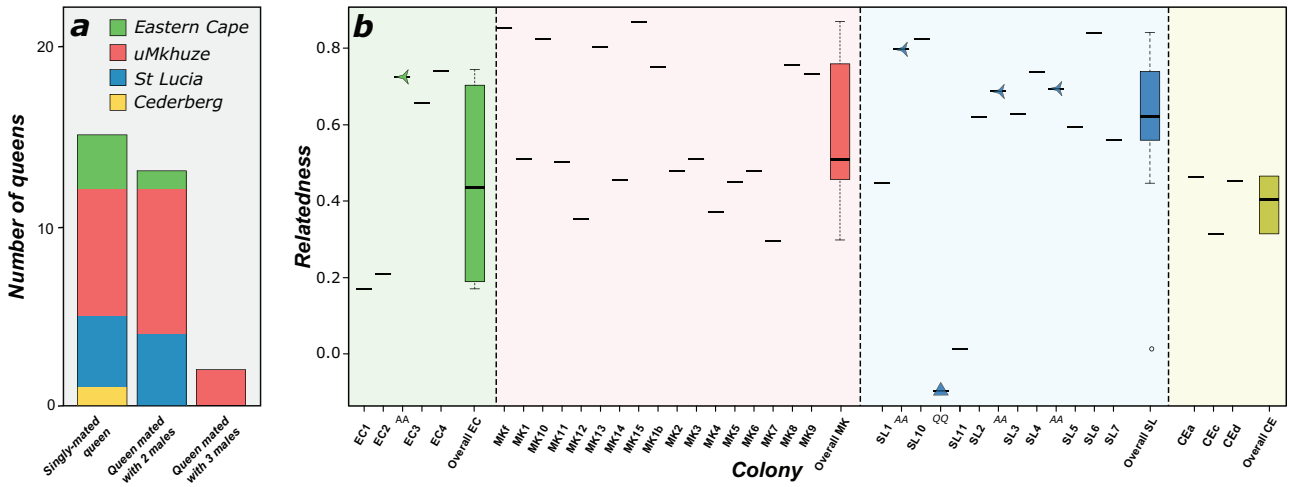

Figure 4. a, number of matings per queen for each monogyne colony in each population. b, relatedness values among nestmate workers for each colony. Arrows indicate relatedness values between alate queens (rA-A) and the triangle indicates relatedness value between queens in the *SL11* polygyne colony.

males (Fig. 4a). Non-detection error due to two fathers sharing the same alleles at all 19 loci was very low for all localities (*Pnon-detection* = 0.00044 for CE and < 0.0001 for EC, MK and SL). Consequently, the average number of matings per queen (± SD) was 1.68 (± 0.67). A similar result was found when the contribution of the different males was taken into account, with the effective number of matings of each queen (M_{ep} ± SD) being 1.53 (± 0.53).

Although a single reproducing queen was inferred in all colonies except *SL11* (see below), the genotypes of four out of six queens sampled were incompatible with being the mothers of nestmate workers. Of these four colonies, the queens in *SL3* and *SL10* were related to the workers in their respective colonies ($r_{q\text{-}w}$ ± SD = 0.18 ± 0.03 and 0.79 ± 0.0 for *SL3* and *SL10,* respectively), whereas the queens in the *SL6* and *MK15* colonies were not ($r_{q\text{-}w}$ ± SD = -0.26 ± 0.01 and -0.28 ± 0.0 for *SL6* and *MK15*, respectively). In contrast, the queens sampled in colonies *SL2* and *SL5* were the mothers of the workers from their colonies. Interestingly, different degrees of physogastry were observed among dealate queens (Fig. 5). Ovarian inspections revealed that several non-physogastric queens were inseminated, but only some had active ovaries.

The occurrence of more than one reproductive queen was found in only one colony (*SL11* in St Lucia). Three mother queens were found while sampling a large branch in the tree housing colony *SL11*; however, we cannot rule out the possibility that different adjacent colonies were pooled unintentionally. The genotypes of nominal *SL11* queens indicated that they were not related ($r_{q\text{-}q}$ = -0.09). Notably, the increased genetic

Figure 5. Dealate queens of *Melissotarsus* ants with different degrees of physogastry.

diversity resulting from the presence of multiple unrelated queens in *SL11* may explain the inappropriate clustering of this colony with *SL10* in the STRUCTURE analysis. Interestingly, the inbreeding coefficient for the overall data set was negative (F_{IS} ± SE = -0.32 ± 0.02) for the localities analysed separately (F_{IS} = -0.23, -0.33, -0.34 and -0.31 for EC, MK, SL and C, respectively), as well as for all colonies (except *SL11*; Supporting Information, Fig. S5). This absence of inbreeding reflects the non-independence of genotypes from a single family. This indicates that mother queens mated with unrelated males, and that daughter queens and

sons do not mate within the nest. Overall, within-colony relatedness was accordingly medium to high in most colonies, ranging from 0.17 Except for *SL11*, within-colony relatedness was accordingly medium to high in most colonies, ranging from 0.17 to 0.87 ($r_{w\text{-}w}$ ± SD = 0.55 ± 0.21; Fig. 4b). Four nests had more than one alate queen. The genotypes of all alate queens analysed (mean ± SD = 6 ± 2.1) was consistent with those of workers from the same colony, indicating that alate queens and workers have the same parents. Consequently, the relatedness among alate queens within these four nests was also high, ranging from 0.69 to 0.87 ($r_{A\text{-}A}$ ± SD = 0.72 ± 0.04; Fig. 4b).

DISCUSSION

Our study provides valuable insights into the mating system and colony structure of wood-chewing *Melissotarsus* ants. Most colonies are headed by a single reproducing queen, mainly mated with a single male but occasionally mated with up to three males. In four out of six colonies for which a queen was sampled, we found that the resident queen did not produce the workers of the colony. This finding suggests relatively frequent queen turnover by related (N = 2) or unrelated queens (N = 2) that replace the older queen in established colonies. We showed that colonies differ genetically from each other, even those located on different stems or branches of the same tree. We also highlighted that the four populations across South Africa exhibit strong genetic differentiation, based on both microsatellite and mtDNA markers.

These results may potentially denote the presence of several undescribed species in South Africa or geographical variants of a single species. In some ant species complexes, close species may differ in their mating frequencies or social organization (Eyer & Hefetz, 2018; Cordonnier *et al.*, 2020), while in some cases there can be differences present between different populations of the same species (Purcell *et al.*, 2015; Eyer *et al.*, 2017). In our study, the possibility that there were multiple *Melissotarsus* species represented does not impact our results, as the different localities all exhibit a similar colony structure and breeding system. This absence of variation in mating strategies therefore suggests that the main evolutionary force reducing gene flow and enhancing speciation in this unorthodox genus is not related to differences in the breeding system. The strong genetic differentiation found across populations of this obligate tree-living ant species may result from the scattered distribution of available host trees throughout the region studied. Most of South Africa is spread over the arid region of the Central Plateau where no trees are available for *Melissotarsus* ants. As a consequence, available habitats are patchily distributed and restricted along the shore, potentially increasing population structure of this tree-living ant species. A large-scale phylogeographic study of this group over Africa, from arid and high-elevation South Africa to tropical and lowland Cameroon, will help decipher species delimitations and will surely provide insights into how host availability shapes the patterns of genetic distribution in this group.

The monodomous and monogynous colony structure observed in *Melissotarsus* ants in South Africa strongly differs from previous reports in Cameroon. In *M. beccarii* and *M. weissi*, numerous egg-producing physogastric queens were hypothesized to belong to the same colony because they were all found on the same tree, despite being located more than 1 m away from each other (Mony *et al.*, 2002). Low aggression between colonies was suggested to favour colonies merging as tunnels expand beneath the bark resulting in a very populous, polygyne colony spanning an entire tree (Mony *et al.*, 2007). In contrast with these observations, our genetic results clearly show that all nests sampled belong to distinct colonies, even those inhabiting the same branch. These findings therefore suggest that colonies maintain strict boundaries, casting doubt on the hypothesis that the multiple physogastric queens found on a tree (1 m apart) actually belong to the same colony. However, our study was performed on *Melissotarsus* in South Africa, and we therefore cannot rule out the possibility that distant populations or species of this genus exhibit different mating strategies.

Interestingly, in addition to the physogastric reproducing queen, numerous inseminated, but non-physogastric, queens were reported within Cameroon colonies of *M. beccarii* and *M. weissi* (Mony *et al.*, 2002). In contrast with previous observations (Mony *et al.*, 2002), our study reveals that some of the non-physogastric queens have active ovaries, and therefore seem to reproduce. However, our results indicate that all workers from all colonies studied can be assigned to a single mother (except that the mother queen can be physogastric or not), suggesting that these supplementary non-physogastric queens do not contemporaneously participate in worker production. In Cameroon, new alate queens are present within colonies and seem to swarm the year round (Mony *et al.*, 2002). Our results showed that, within a colony, alate queens are produced by the same mother as that of the workers. This result challenges the possibility that non-physogastric queens are opportunistic and produce exclusively female reproductives, whereas the physogastric queen produces the numerous workers. Our sampling did not include enough males to conclude whether the non-physogastric queens with active ovaries may produce males within the colonies. Additionally, it is also possible that the current workers,

through policing, cannibalize the eggs they produce, regardless of which caste they would develop into.

The presence of these additional non-physogastric queens also questions whether they originated from the same physogastric mother, and whether they mate with their brothers in the nest to extend the colony lifespan after the mother queen dies. Mony *et al*. (2002, 2007) previously suggested that newly-inseminated queens are accepted by foreign colonies to perform worker-like tasks, and do not produce eggs until they have the chance to dominate their section of the colony. Our results partially support this hypothesis, as the queen sampled in four out of six colonies did not mother the workers of the colonies. For two of them, despite not being their mother, the new queens were related to the workers present in the colonies. The absence of inbreeding in any of the colonies sampled can rule out the possibility that these queens originated from the same mother and mated with their brothers (Trontti *et al.*, 2005; Foitzik *et al.*, 2011; Eyer *et al.*, 2018b). However, we cannot exclude the possibility that these queens may originate from the same mother and mate with foreign males before reintegrating into their natal colony. This may happen through female-calling syndrome, whereby queens stand close to their nest entrance and release sex pheromones to attract neighbouring males (Hölldobler & Haskins, 1977). Although this mating strategy leads to reduced gene flow compared to a nuptial flight of both sexes (Peeters & Aron, 2017), it still prevents inbreeding, as males disperse from their colonies before mating (Bourke *et al.*, 1988). For the two other colonies containing queens who were not the mothers of their nestmate workers, the new queens were not related to the workers present in the colonies. Overall, the finding of reproducing unrelated queens that did not mother the workers of the colonies suggests queen turnover by foreign queens sometimes being accepted within the colonies, a finding previously suggested by Mony *et al.* (2002). These findings raise questions regarding the forces driving queen turnover in this group, and call for further investigation into the chemical and behavioural mechanisms determining queen acceptance, dominance and replacement in *Melissotarsus* ants (Keller & Nonacs, 1993; Koedam *et al.*, 1997).

The social organization of *Melissotarsus* strongly contrasts with other tree-living ant species. In *Myrmelachista schumanni*, each colony may contain millions of workers and up to 15 000 queens inhabiting hundreds of trees that offer specific shelters (i.e. domatia) (Frederickson *et al.*, 2005). Colonies still maintain high relatedness through the reintroduction of daughter queens mated with related males (Malé *et al.*, 2020). Colonies of some species of *Pseudomyrmex* ants are established on swollen-thorn acacias by a single queen. As the colony grows, workers aggressively patrol the outside of the plants eliminating any new foundresses and competing colonies. Consequently, a single monogyne colony can expand and monopolize up to 20 neighbouring acacias [up to a hundred trees for polygynous colonies (Janzen, 1966)]. In ants of the genus *Azteca*, several founding queens establish colonies, together or individually, on saplings of *Cecropia* trees (Davidson *et al.*, 1989; Yu & Davidson, 1997). After the emergence of a first brood, competition between queens restores monogyny within colonies, and the competition between colonies ends up with a single colony occupying each tree (Longino, 1989, 1991). Compared to these other tree-inhabiting ants, the foraging strategy of *Melissotarsus* ants results in sharply contrasting colonization and cohabitation outcomes. Young trees are unsuitable for *Melissotarsus* ants because the bark is too thin to chew tunnels (likely to vary among tree genera). Only trees of a certain age are therefore suitable for colonization, these trees also produce more branches that are appropriate over the years. In addition, the inability of workers to walk and patrol outside the host trees allow new foundresses to establish additional colonies in already in habited trees, as long as they select 'empty' branches. Because chewing tunnels takes time, it is likely that empty branches are often available, and young queens can succeed to found new colonies. Overall, the foraging strategy of *Melissotarsus* ants allows multiple colonization events over many years on a single large tree, while the lack of encounter and direct competition between colonies allows several colonies to establish and cohabit the same tree. Consequently, in contrast with other tree-living ant species, as well as with previous suggestions for this genus, *Melissotarsus* colonies of the populations investigated in this study never reach colossal sizes (Mony *et al.*, 2002); each tree rather contains a myriad of small secluded colonies of various ages.

Overall, these findings show that different phenologies of the host plant may select for distinct mating strategies and colony structures of the tree-living ant partner (Mayer *et al.*, 2014). *Melissotarsus* species have been reported to inhabit at least 31 genera of host trees in 21 botanical families (Peeters *et al.*, 2017), showing different growth and branching patterns. The availability and the distribution of host trees and scale insects seem crucial for ants of the genus *Melissotarsus*. Investigating how the distribution of these partner organisms influences the breeding systems and population genetics structure of the ants of the *Melissotarsus* genus therefore clearly deserve further study.

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C.P. designed the study and collected the samples. P.-A.E. performed the genetic analyses and analysed the data. P.-A.E. and C.P. wrote the paper with contributions from E.L.V.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Information on sampling locality and number of individuals analysed for each colony sampled.
Table S2. PCR multiplexing and number of alleles for each of the markers used in our study. This also includes the methods used to estimate detection of null alleles and linkage disequilibrium for the microsatellite marker analyses.
Table S3. a, number of alleles for each of the 19 microsatellite markers for every locality. b, observed and expected level of heterozygosity for each of the 19 microsatellite markers for every locality.
Figure S1. *Strychnos* and *Erythrina* trees that were sampled showing the difference in growth patterns.
Figure S2. Maximum Likelihood tree of the mitochondrial *COI* marker. Each sample is coloured according to its population of origin. Numbers indicate branch support through bootstrap values. Accession numbers and their described species are indicated for the samples from GenBank.
Figure S3. a, pairwise F_{ST} values between each pair of localities. b, pairwise F_{ST} values between each pair of nests.
Figure S4. Major and minor nodes of clustering using (a) STRUCTURE (at K = 3) and (b) results obtained (at K = 3, 4 and 5) when a single individual per colony was used.
Figure S5. Inbreeding coefficients (F_{IS} values) for each colony.

SHARED DATA

The microsatellite data reported in this study are deposited in the Open Science Framework [https://osf.io (doi:10.17605/OSF.IO/MSJCD)]. The mtDNA sequence data generated in this study have been deposited in GenBank.