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Outnumbered: a new dominant ant species with genetically diverse supercolonies in Ethiopia

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Abstract A *Lepisiota* (Hymenoptera: Formicidae: Formicinae) species in Ethiopia has been observed forming supercolonies spanning up to 38 km. *L. canescens* occurs at very high densities where there is sufficient moisture or herbaceous cover and dominates the local ant community, traits reminiscent of an invasive species. The supercolonies are genetically diverse, however, indicating they have not gone through the population bottleneck usually characteristic of species invasions. We conclude that the species is native to this region, though expanding its range locally into areas of human disturbance, where it is exploding in numbers. The lack of aggression across a genetically diverse population suggests that mitochondrial genetic variation is decoupled from variation relating to colony recognition cues like cuticular hydrocarbons. All in all, *L. canescens* could have the makings of an invasive species at an international scale and may represent a novel system to study the evolution and spread of supercolonies in ants.

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Introduction

Certain ants form complex societies that allow them to achieve ecosystem dominance (Hölldobler and Wilson 1990). The most extreme species form extensive cooperative units called supercolonies with the capacity to expand their nest sites over large areas, in some cases thousands of square kilometers, yet maintain a clear separation from other [super]colonies. Supercolonies are almost exclusive to ants (but see Leniaud et al. 2009). As in ants generally (Hölldobler and Wilson 1990; Tsutsui 2004), these polydomous ants identify their colony by specific cuticular hydrocarbon profiles. This identification is retained following budding (Torres et al. 2007) such that individuals may move freely among nests where the environment permits, intermixing and thereafter cooperating indiscriminately with any other portion of that colony [in some instances low-level aggression may occur, at least temporarily (Roulston et al. 2003)]. Furthermore, in species like the Argentine ant (*Linepithema humile*), colony growth seems to continue as long as suitable unoccupied space is available (Buczkowski and Bennett 2008; Van Wilgenburg et al. 2010). Therefore, a colony can take over huge expanses but nevertheless have a clear membership and distinct boundaries (Torres et al. 2007). The capacity of a

colony to extend its range without constraints is the strongest basis for describing such ants as having supercolonies (Moffett 2012). Supercolonial ants have multiple queens (they are polygynous), which permit their exponential growth rate and spatial expansion to occur not only through budding, but also through jump-dispersal potentially over long distances. Because of their nesting and dietary generalism, invasive ants with supercolonies are especially successful as stowaways in human cargo (Holway et al. 2002). Over the past century, jump-dispersal has expanded the natural range of numerous species of invertebrate (e.g., Ascunce et al. 2011; Booth et al. 2011; Saenz et al. 2012; Gotzek et al. 2012) and in the ants has led to the very same supercolonies ranging between continents (Vogel et al. 2009; Sunamura et al. 2009; Vogel et al. 2010).

Compared to the 14,000 described ant species, supercolonialism is rare, found in less than 1% of all ants, and finding new examples is uncommon. While studying patches of forest scattered across the Ethiopian highlands, called *church forests* because they surround Orthodox churches, DMS and MWM noticed that a *Lepisiota* since identified as *L. canescens* (Peter Hawkes, pers. comm.) within Zhara Church Forest (8 ha) reached densities of several thousand individuals per square meter over wide tracks of ground. During our work in the dry season in this part of Ethiopia, we found the species was also prevalent, although at less extreme densities, in open disturbed habitats where there was herbaceous cover or other signs of moisture. Foraging and nesting over wide areas, it exhibited possible supercolony formation based on a lack of aggression between individuals transferred to distant (up to 38 km) localities. Being a small and innocuous-looking monomorphic ant, *L. canescens* at least superficially resembled the invasive supercolony-forming Argentine ant. *L. canescens* is unstudied, but has been previously recorded "invading" an oasis in Algeria, where nests were found to make up 50–85% of total ant nests present (Bernhard and Cagniant 1962).

Many supercolonial ants have turned into invasive species (e.g., Van Wilgenburg et al. 2010) although some notable exceptions mainly in the genus *Formica* exist (e.g., Kümmerli and Keller 2007). The genus *Lepisiota* is broadly distributed in Africa, with its greatest species richness there, and also occurs in southern Europe and southern Asia (Janicki et al. 2016). This distribution suggests an African origin of the genus; however, recent evidence of translocations and subsequent establishment have been reported in Australia (West Australian Department of Agriculture and Food 2015) and South Africa (Sithole et al. 2010), suggesting that members of this genus may have the potential to become a global nuisance. The taxonomy of the genus *Lepisiota* is currently lacking satisfactory resolution and hence previous species identifications in the literature should be considered with caution.

In this study we examine the genetic variability across the sampled range using data derived from the mitochondrial cytochrome oxidase I subunit and compare these data with pairwise aggression assays performed on colony pairs along a 100-km transect to assess the presence and extent of supercolonies and their population structure. We evaluate whether the colony boundaries determined by behavioral identity correspond to genetic identity (i.e. whether locations with no aggression exhibit the same haplotypes), as well as determine genetic diversity within the sampled range which, in the case of an established invasion, may reveal if it resulted from a single or multiple introductions.

Methods

Natural history

The behavior of species of this genus is unknown. DMS and MWM observed the ants for several hours a day over 10 days, mostly at Zhara Church Forest, and to a lesser extent at other locations, to build a general description of *L. canescens* activities relevant to assessing its ecological dominance as a species.

Sample collection and bait transects

Lepisiota canescens was collected from 16 locations along a ∼100-km transect following the road between Bahir Dar and Debre Tabor, Ethiopia, in January 2012. Additional samples were collected at Zhara Church Forest (8 ha), Debresena Church Forest (11.5 ha), Gelawdios Church Forest (100 ha), and in the towns of Lalibela and Gonder resulting in a total of 28 sampling locations (Fig. 1, Table S3). Samples were stored in 95% ethanol for genetic analysis, and a subset was kept alive for behavioral tests. Seven bait transects were installed at three of the Church Forests (Zhara, Gelawdios, and Debresena) to assess habitat selection. Each transect was 100–200 m long with bait stations every 10 m. At Zhara Church Forest, we started the transect at a stone wall designed to fence in the forest and leading 200 m into the forest (although the transect led through several open areas and sunny patches), with a second transect starting at the outside of the same wall leading through an open field outside of the forest into a patch of Eucalyptus forest ∼150 m away. At Gelawdios Church Forest one transect was installed in the forest interior, one in the open field outside the forest (an open area with many rocks), and one along the edge of the forest (each 100 m). At Debresena Church Forest, both a forest and a field transect were installed (both 100 m). The field transect consisted of five stations in a harvested straw field and five stations in a plowed fallow field. Baits consisted of ca. 2 cm^3 of sardines
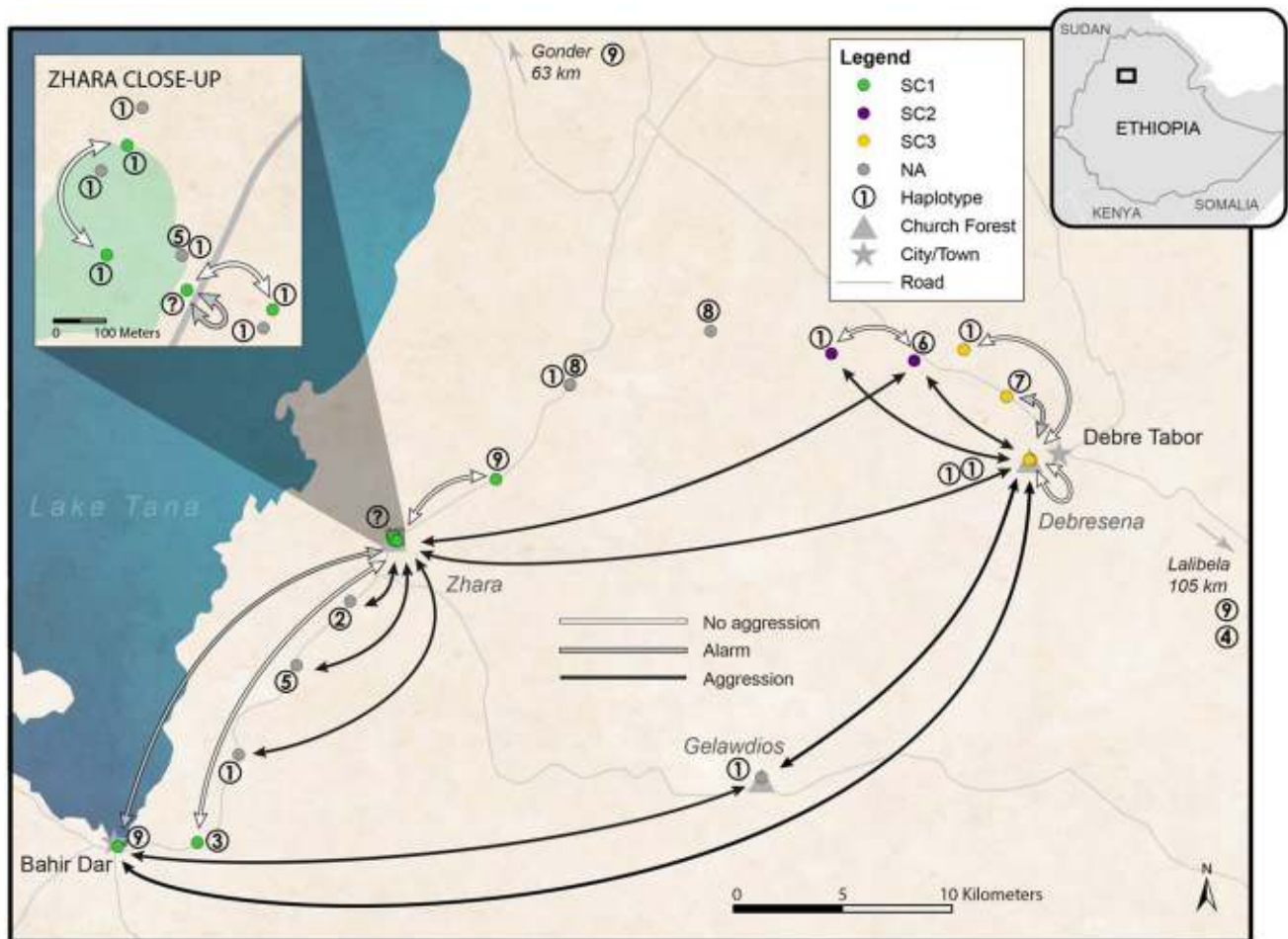
Fig. 1 Sampling map including results from aggression assays and haplotypes. *Arrows* indicate results from aggression assays between locations. Locations colored the same show supercolony identity indicated by no aggression. See Table S3 for more information on sampling locations

in oil placed directly on the ground. Each bait station was marked and visited after 1 h. All the ant species at the bait were recorded and voucher specimens were collected (see Table S1). Voucher specimens of *L. canescens* are held at the AfriBugs Collection in Wolmer, Pretoria North, South Africa (AFRC), the North Carolina State University Insect Museum in Raleigh, North Carolina, the D.M. Sorger collection in Vienna, Austria (CSW) and the Natural History Museum Vienna, Austria (NHMW), and have been imaged and uploaded to AntWeb (casent0251850).

DNA extraction and genetic analysis

A total of 28 workers from 27 locations of *L. canescens* were analyzed (Table S3, Fig. 1). We also sequenced one queen from colony ZS02 (ET040) and ten alate queens from colony ZL02 (ET010) (Table S3). Total genomic DNA was isolated from whole specimens using the Qiagen DNeasy blood and tissue extraction kit (QIAGEN, Valencia, CA). From each specimen, a 669-bp fragment of the mitochondrial

Cytochrome Oxidase subunit I (COI) gene was amplified by PCR using primers LepF1 and LepR1 (Hebert et al. 2004; Hajibabaei et al. 2006). PCR and sequencing protocols followed Menke et al. (2010). Sequence variation was calculated with the Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 software (Tamura et al. 2013). Diversity parameters, including nucleotide diversity (π) and haplotype diversity (h), were computed with DNaSP 5 (Librado and Rozas 2009). Genetic distances between haplotypes were reconstructed using a minimum-spanning network algorithm implemented in PopART 1.7 (epsilon = 0) (Bandelt et al. 1999). Trends in the demographic history of *L. canescens* were investigated using Tajima's D and Fu's F_s statistics. A significant negative value in these two tests indicates a recent population expansion event.

Aggression assays

While not all pairs could be tested due to logistical constraints, 22 pairwise experiments were conducted to

assess aggression between ants from different localities. Several hundred ants were collected from each location using aspirators and kept alive in plastic collection vials. Aggression was then assessed in the field by locating ant trails and positioning a vial containing ants from the testing location near the ant trail. Ants were allowed to exit the vial and interactions with ants from the trail were observed for 10 min. The response was recorded into three categories: (1) aggression—fights, pulling appendages and pointing abdomens (Fig. 2a); (2) low-level aggression, described here as alarm—raised abdomens and close examination of the introduced workers, occasional pulling on them, with subsequent acceptance by the resident ants (Fig. 2b); and (3) no aggression—the ants show no signs of aggression and intermix freely with the resident group.

Results

Natural History

At Zhara Church Forest *Lepisiota canescens* nests with multiple queens (e.g., 50–100 queens in a nest area of 0.5 m^2), alates and brood were found under rocks. The ants also entered unadorned holes in the ground, suggesting subterranean nesting. There were no dirt piles to indicate excavation; thus the species may be using available cavities. Queens often ran on surface trails alongside the workers, some of which carried brood. The species was common in an urban setting at the Ghion Hotel in the city of Bahir Dar. The ants did not enter rooms in a noticeable way, though some came to the windowsills and onto the concrete paths. They were particularly abundant in parts of the garden most watered by the hotel staff. In fact, *L. canescens* often came

to the surface when water was poured on the earth, suggesting there was a reserve population belowground across a broad area.

DMS identified several *L. canescens* workers provided by Neville Winchester, which he reports were collected in litter and moss 12 m high in the Zhara forest canopy in 2010. In our experience the workers often climbed herbaceous plants, notably African senna (*Senna didymobotrya*), a native to Eastern Africa but a recent arrival to the study area, showing up soon after the road had been paved and also associated with disturbance. On many plants, particularly senna, the ants were observed tending froghoppers (*Tettigometra* sp.), and in one instance a caterpillar. They also carried freshly killed insect prey such as caterpillars and termites and efficiently dispatched other kinds of ants, among them species with workers larger than themselves such as *Pheidole* and *Camponotus*. To do so, they gathered around to pull the workers apart, much as they did the foreign-colony ants of their own species that we had transferred from other areas.

Bait transects

In general, ant presence at baits was low (no ants at 47% of all bait stations) probably due to the scarcity of ant foragers during the dry season and the placement of some baits along transects extending into bone dry areas (Fig. 3, Table S1). Nevertheless, *L. canescens* was abundant on the ground surface at Zhara, especially in the forest and a few meters out into the adjacent fallow field. In fact it was the only species found at any of the bait stations except for one occupied by a *Monomorium* species in the field transect where *L. canescens* was seen nearby. The other two church forests sampled had no *L. canescens* anywhere except along a transect extending into a field next to Gelawdios, where it

Fig. 2 Example of *aggression* response: Multiple ants attack a conspecific transferred from the territory of another supercolony (**a**). Example of *alarm* response: ant raises abdomen when encountering a

conspecific taken from a site within the territory of the same supercolony (**b**). Photo credit: M. Moffett
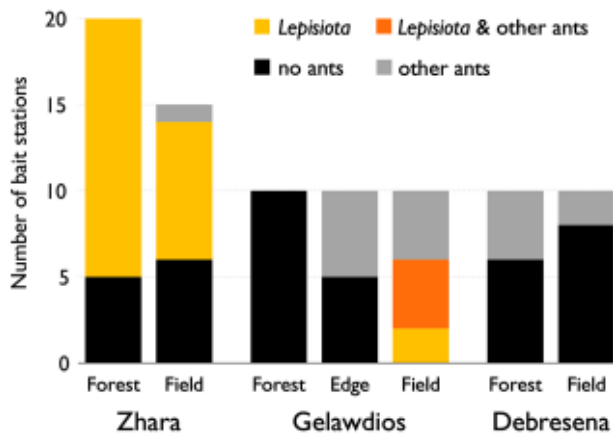
Fig. 3 Number of bait stations occupied by no ants, *L. canescens* alone, *L. canescens* and other ants, and just other ants in three church forests. *L. canescens* was the dominant ant in Zhara compared with two other forests

was present at 60% of bait stations. All told, *L. canescens* occupied the bait in high numbers (presence of 100 + workers) at 16 of the 45 stations that had ants at them (three transects) and was highly aggressive towards other ant species, whether encountered near baits or elsewhere.

Genetic analysis

Nine unique COI haplotypes were identified across the sampled region (Fig. 1, Table S1). Eight haplotypes were found to be present in the main sampling area encompassing Bahir Dar and three church forests, while a single haplotype (4) was only found in an individual from Lalibela (Mt. Asheton) (Fig. 1). The additional workers and queens sequenced possessed the same haplotype as the first worker sequenced from the location and were excluded from subsequent analysis to avoid inflation of haplotype frequency at a given site. Of the ten polymorphic sites, six were parsimony-informative. All haplotypes were joined in a minimum-spanning network with one haplotype (1) comprising 37% of all individuals (Fig. 4). Overall mean

sequence divergence across the nine haplotypes was found to be 0.3%. Haplotype diversity (h) amounted to 0.718 (SD 0.087) and nucleotide diversity (π) was 0.003. Tajima's D (-0.657, $p \geq 0.10$) and Fu's F_s (-2.301, $p = 0.056$) indicate that the population has not undergone a recent expansion.

Aggression assays

Our behavioral experiments revealed a pattern of non-aggression (with an initial alarm response in three instances) between sampling locations of up to 38 km apart (Fig. 1). The transferred ants intermingled with the local population just as they would have if they had been released exactly where they had been collected. We found no aggression between locations encompassing the area from Bahir Dar until 8.6 km beyond Zhara Church Forest, and only three locations within this area showed an aggressive response towards samples from Zhara (SC1, see Fig. 1). In addition, we found no aggression over 6 and 9 km for SC2 and SC3, respectively (Fig. 1). Locations sharing haplotypes were not different regarding their aggression response compared to locations not sharing haplotypes (Fisher's exact test, $p = 1$).

Discussion

Our results indicate that *Lepisiota canescens* forms expansive supercolonies like those seen in invasive species such as the Argentine ant.

A lack of aggression was observed in transfers of *L. canescens* over distances of up to 38 km, with the transplanted ants merging seamlessly with the local population, in a few instances after a brief inspection. Other transfers resulted in clear and extreme aggression towards the intruders. This is evidence for a unified identification of the ants across wide areas, as expected when there are supercolonies with definite territorial boundaries and

Fig. 4 Minimum-spanning haplotype network for *L. canescens*. Size of nodes represent number of individuals per haplotype, *tick marks* between nodes indicate number of mutations between haplotypes
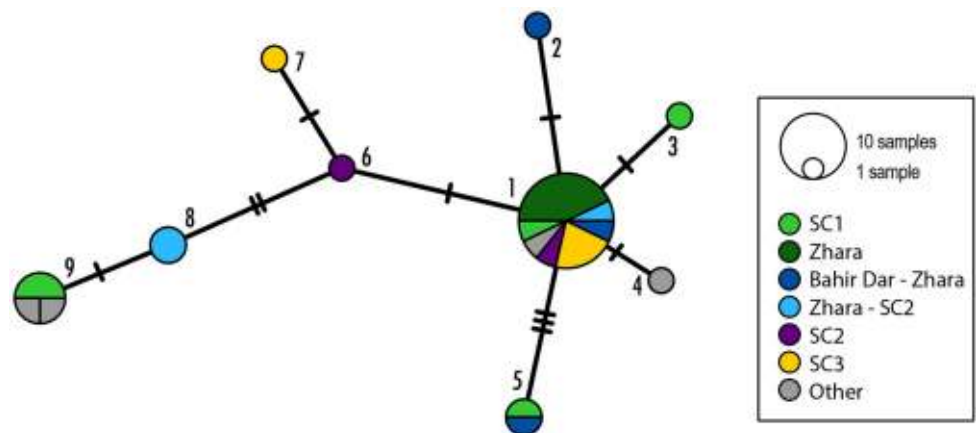
memberships (Pedersen et al. 2006; Heller et al. 2006; Moffett 2012). However, the considerable genetic variation of the ants did not correspond with supercolony boundaries. How is this possible? Colony membership in ants is based on behavioral responses to cuticular hydrocarbons. It could be that the patterns of mitochondrial genetic variation have been decoupled from variation at the loci encoding for, or influencing, the hydrocarbons and the behaviors required to recognize them. This could permit the significant local population structuring found, for example, by Ingram and Gordon (2003) within California's large Argentine ant supercolonies, which are nevertheless unified, with lethal aggression only along supercolony borders.

In the red imported fire ant (*Solenopsis invicta*), the yellow crazy ant (*Anoplolepis gracilipes*), and the Argentine ant (*Linepithema humile*), non-aggression within supercolonies is coupled with low genetic diversity. This was not the case in *L. canescens*. Haplotypes did not match across non-aggressive sites. A similar pattern was found in the native range of *Formica exsecta,* where six haplotypes were recovered within a single supercolony over a distance of 400 m (Seppä et al. 2012). It has been claimed that low genetic diversity, the outcome of their propagule pressure, would be pivotal to a supercolony staying together as a cooperative unit over large areas (e.g., Tsutsui and Suarez 2003). *L. canescens* confirms that an ant society (supercolony) with a diverse population can remain united as long as the members continue to accurately identify each other as belonging to the same society (also see Moffett 2012).

Bait transects indicated the ecological dominance of *L. canescens* wherever the species was present, which was in moist areas with some level of disturbance. Dominance of the species was most pronounced at Zhara Church Forest; the smallest and most disturbed of the three forests sampled. In addition to the church itself, there is a human settlement in the forest until recently with no latrines, and undergrowth had grown dense and weedy due to the recent exclusion of grazing animals. *L. canescens* was entirely absent within the other two forest sites, which were less overgrown. However, *L. canescens* was found in the cleared landscape and fields between the forests where there was moisture. Pouring water on the ground sometimes brought up ants, suggesting that in this, the dry season, the colonies were present widely but hidden, a dependence on water also seen in Argentine ants (Holway and Suarez 2006; Heller et al. 2008). Moreover, *L. canescens* was the only ant species to control bait stations except for a *Monomorium* species that took over a single station in a bare open field.

Invasive ant species share multiple characteristics that appear to promote establishment in foreign environments. Though exceptions exist [e.g., the original monodomous and monogynous form of *S. invicta* that first colonized the southeastern United States (Tschinkel 2013)], these traits, which make up the invasive ant syndrome (Cremer et al. 2008), include (a) indiscriminant nesting, (b) generalist diet, (c) polygyny (with successive generations of queens staying within their natal colony), (d) ecological dominance, (e) polydomous colonies, and (f) expansion through budding, by which means a colony may span large areas (Holway et al. 2002; Tsutsui and Suarez 2003). *L. canescens* exhibits all these traits. However, our *L. canescens* samples had a mtDNA diversity greater than that found in the continent-wide distributions of many invasive supercolonial species (Ascunce et al. 2011), and more in line with what would be expected for a native ant (Booth et al. 2011; Saenz et al. 2012). We propose that *L. canescens* is native to this general region of Africa but has been expanding locally into areas disturbed by humans. Many invasive species must have begun by evolving the means to efficiently move around the native environment before human commerce gave them the chance to "go international" and before habitat degradation opened up opportunities for them to become ecologically dominant. Although *L. canescens* has not yet achieved an invasive status globally, it will be a significant species to watch.

Other species of the genus *Lepisiota* have already revealed this potential to be invasive. In 2005, the Browsing ant (*L. frauenfeldi*) was discovered at the airport in Guam and subsequently eradicated (Hoffmann et al. 2011). In 2013, the same species was found covering a 60-ha area around Perth airport, where it may have arrived 5 years earlier, and a year later a 10-ha outbreak was located 6 km away in the suburb of Belmont. While these Perth populations have been eradicated, this year another infestation of the same species led to the partial shut-down of the port of Darwin, Australia (Mark Widmer, pers. comm.). Similarly, *L. incisa* has been reported from Kruger National Park, South Africa, where it is thought to have arrived in the 1990s (Sithole et al. 2010). There the species is most abundant in habitats associated with human disturbance and development, where it is behaviorally dominant to all other ants, including endemics. Judging from the slight, if any, aggressive response toward ants transferred between sites hundreds of kilometers apart around Kruger (Caldera 2004, cited in Sithole et al. 2010), it appears that Kruger is occupied by a single supercolony—presumably derived from the initial invading colony that arrived and spread widely within a region previously uninhabited by the species. At a single site, however, the workers were hostile to con-specifics from elsewhere in the park (Sithole et al. 2010), indicating that a second supercolony may have become established. The similarities between the *Lepisiota* species at Kruger and the species described in the present study indicate that supercolony behavior, and the concomitant potential to invade new terrain, may be a common attribute of the genus, making *Lepisiota* a potentially informative model system to study invasiveness and supercolony-formation in ants.

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