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Testing the effect of a nest architectural feature in the fire ant *Solenopsis invicta* (Hymenoptera: Formicidae)

Walter R. Tschinkel

Abstract

When natural fire ant colonies (*Solenopsis invicta*) are sampled over five orders of magnitude of colony size, their production efficiency (new biomass per current biomass) remains constant, whereas in laboratory colonies, it declines. A striking difference between laboratory and natural nests is the subdivision of natural nests into hundreds of small chambers that limit the size of work groups. I tested the effect of nest subdivision on brood-rearing efficiency in laboratory nests with a single chamber or many small chambers of equal total area. Nest subdivision had no significant effect on any measure of brood rearing efficiency or final colony size. Experiments are still needed to test cause-and-effect relationships between specific features of ant nest architecture and specific colony functions. The results are discussed in the context of the superorganism.

Key words: Ant biology, ant nest architecture, production efficiency, colony development, brood production, colony growth rate, experimental myrmecology.

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Introduction

The function of subterranean ant nests as shelter and as a defensible space is obvious, so why does nest architecture vary so much among species? It stands to reason that ants with large colonies and/or large individuals would build large nests, and vice versa, but across species, nests vary not only in size, but in most of their features. Even for ants of similar colony and individual size, nests may differ in any or several features, including the number and size of chambers, spacing between chambers, chamber shape and volume, number and size of connecting shafts and maximum depth (Tschinkel 2015). This raises the question, do particular features best serve particular functions, that is, do they affect fitness, or is at least some of this variation neutral with respect to natural selection? Not only do these questions have theoretical importance, but practical as well, for in the research laboratory, ants are usually housed in simple nests that have little or no resemblance to the natural nest that was the colony's original home. What artifacts in colony function this produces is unknown.

Most subterranean ant nests are composed of the same basic units, namely, more or less vertical shafts connecting more or less horizontal chambers (the shish-kebab unit). All of the elements of these units, including nest depth, chamber shape, size and spacing, seem to evolve independently of each other, creating the range of species-typical nests (Tschinkel 2015). Some species build nests composed of multiple units packed side-by-side with variable spacing between units. Most conspicuous and extreme among these are the fire ants *Solenopsis invicta* and *S. geminata* whose nests consist of many shish-kebab units packed so close together that many chambers coalesce (Tschinkel 2006). Nests are enlarged by adding more chambers and shish-kebab units as well as deepening the nest, so that a large nest contains hundreds of chambers that average about 5 cm^2 in area and hold about 200 ants (Cassill & al. 2002). As a consequence, the colony is divided into hundreds of small "work groups" with unknown effects on colony functions but radically different than the typical large, single-chamber nests in which they are housed in the laboratory.

The study of nest architecture naturally raises the question of whether particular architectures, or architectural features affect colony functions, that is, whether some architectures are favored by natural selection over others, thus leading to the observed variation between species. Many phenomena in biological studies invoke the concept of "efficiency", and many authors have used this concept in biological explanations of social insect behavior and colony function. Thus, one can also ask whether the particulars of nest architecture promote efficiency. However, the definition of "efficiency" is often vague and varies from author to author. The rigorous physical definition is the unitless ratio of output to input, for example the percent of input energy that is transformed to work or some other form of energy, and the percent that is lost as heat. In social insect biology, "efficiency" is typically actually a rate, for example, new workers per initial worker per week or food collected per unit time. The energetic version of efficiency would require the estimation of kcal of new ants produced per kcal of food eaten (or collected), and this has almost never been done for any social insect (but see Offenberg 2011 for an exception).

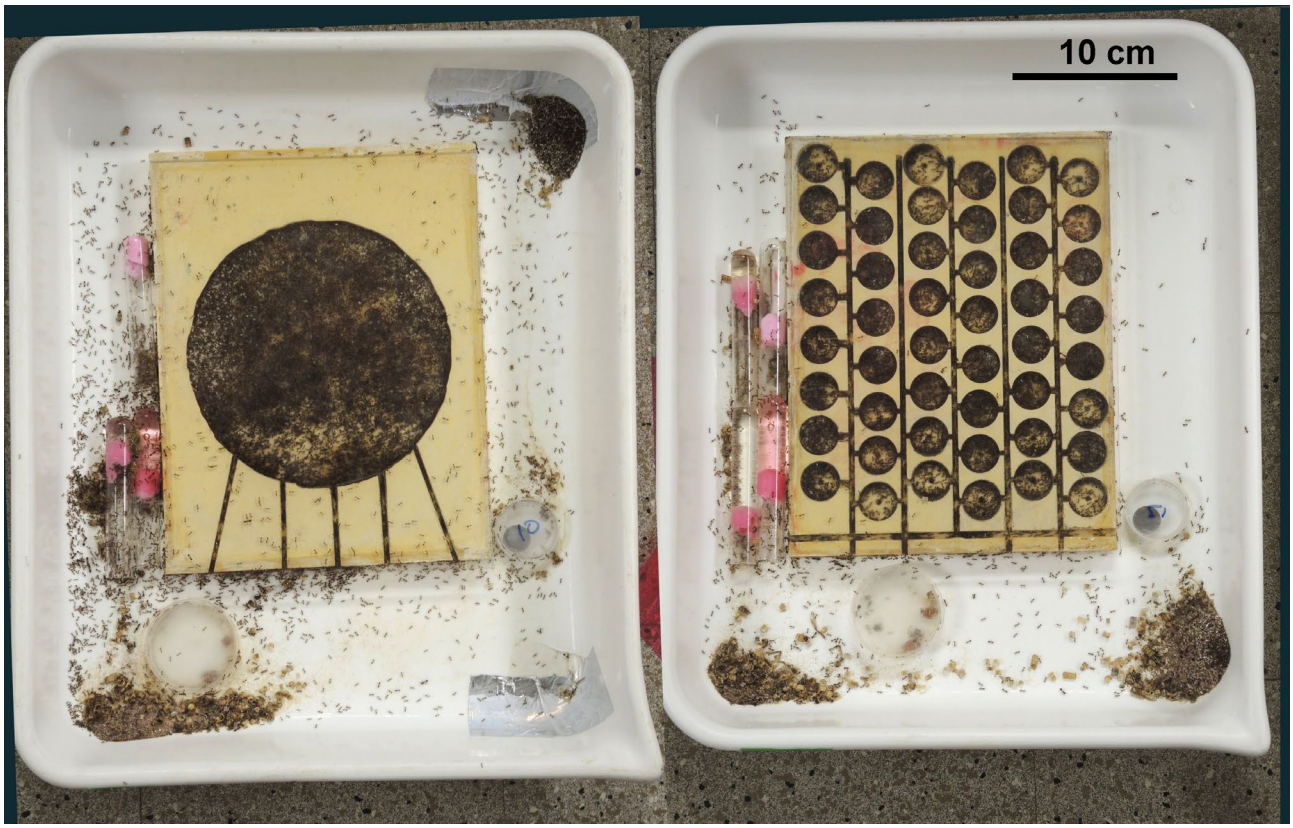

Fig. 1: The experimental nests. Nests were cast as plates of dental plaster, and the 5 mm deep chambers and tunnels formed with a router. The nests were covered with glass, and dampened by squirting water along their edges. The total chamber area of the two treatments was the same.

Several authors have reported that larger colonies of social insects produce new individuals less efficiently (Brian 1956a, b, Michener 1964, Porter & Tschinkel 1985, Vargo 1988) (but Cole (1984) found no such decline in *Leptothorax allardycei*). Several pieces of evidence suggest that colony subdivision may have important consequences for production in fire ants. Porter & Tschinkel (1985) found that the larger the number of workers in simple laboratory nests, the less efficient they were at rearing brood. Notably, their smallest and most efficient group (0.75 g or about 750 workers) was still far larger than the average work group in real nests (200). This falling size-related efficiency suggested that large fire ant colonies are less efficient at production than small ones. However, Tschinkel (1993) showed that at any one sample time during the year, production efficiency (calculated from the pupa to worker ratio) was independent of colony size over four to five orders of magnitude. Because this finding contradicts the laboratory studies, it begs for an explanation. One of the many glaring differences between colonies in nature and in the laboratory is the extreme subdivision of natural colonies into many small work groups, a condition that remains constant no matter how large the natural colony because mean chamber size remains constant. Could this subdivision into small, efficient groups be the cause of the constant efficiency as the colony grows? I tested this hypothesis in the laboratory because single and multiple chamber nests that partly mimic the subdivision of natural nests are readily constructed.

Materials and methods

Collection of colonies: During the cool season (January - March), colonies bring the brood and queen into the above-ground mound to warm, and can be readily captured there (Tschinkel & Howard 1978). One to three trowels full of mound soil were spread evenly in the bottom of large photo trays, and a few minutes allowed for the panic to settle down. If the queen was present, she was easily recognized by her larger size and her attractiveness to workers. The queen, along with a few thousand workers and brood from 20 or more nests were returned to the laboratory for experimental setup.

Brood and workers were separated from each other by etherizing colonies until the workers had just stopped moving, then pouring the colony onto construction paper, and as the workers woke up, tipping the brood off the paper. Workers in the process of waking up do not usually pick up brood, but do hang onto the paper. The proportion of larvae, pharate pupae and pupae in the brood was estimated by counting samples. Once brood and workers were separated, each experimental nest was initiated with the queen, 3000 workers, 1500 larvae and 1500 pupae. Numbers were computed from the weights of counted samples, and the total weight of an item.

Experimental treatments: Nests were made of rectangular 26 × 20 cm plates of orthodontal plaster about 2 cm thick with two different kinds of 5 mm deep recesses routed into their tops (Fig. 1), constituting the two experimental architectural treatments: (1) a single large chamber with a

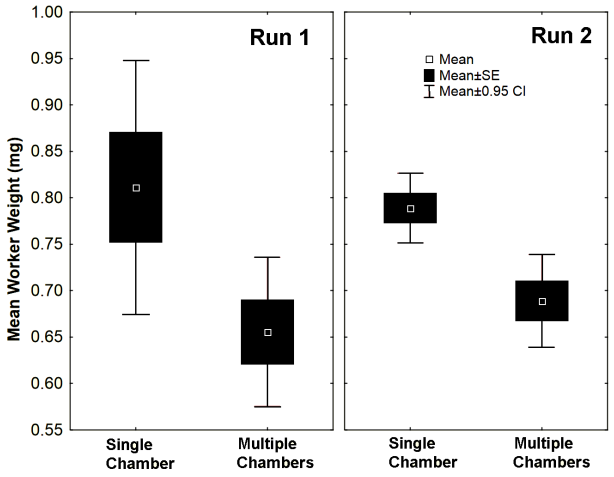

Fig. 2: Worker size by treatment and run. Multiple-chamber nests produced significantly smaller workers in both runs.

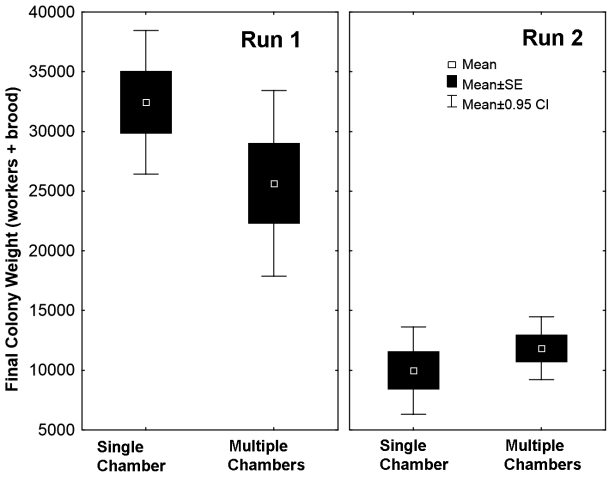

Fig. 3: Final colony weight by treatment and run. Final weight was the sum of live workers and brood, and represented colony production minus worker mortality. Single and multiple chamber treatments were not significantly different, but run 2 was significantly lower than run 1.

diameter of 16.5 cm and an area of 213 cm^2; or (2) 48 small chambers with a diameter of 2.4 cm totaling the same area. Both types of nests had 5 exits to the foraging arena. The multiple chambers were connected with five 3 cm-wide tunnels running the length of the nest and connecting chambers into three rows with exits at one end of the nest. Chambers near the exits were also cross-connected to simulate the cross-connections in the upper regions of natural nests. Each nest was covered with a 26 by 20 cm plate of glass, which in turn was covered with red cellophane to darken the nest interior for the ants.

Each nest was housed in a 38 by 44 cm photo tray whose sides had been treated with Fluon to prevent escape, and provided with sugar water and water in cotton-plugged test tubes. The same food was provided to both treatments ad libitum in the form of frozen tenebrionid beetle larvae, crickets, Spam, and occasional pieces of meat. The plaster nests were moistened as needed, and were kept in an insect room at about 27.5°C and constant light.

Treatment switching: At the end of the first brood cycle (about a month), all nests were disassembled and the number and weight of workers and brood estimated. From these, 3000 workers and 3000 brood were retained to set up the opposite treatment, that is, colonies initially in single-chamber nests were set up in multiple chamber nests, and vice versa. At the end of the second brood cycle, the number and weight of workers and brood were once again estimated, and the experiment terminated.

Data and analysis: For each brood cycle and nest treatment, data consisted of the initial number and weight of workers, larvae and pupae, and the final number and weight of each. These basic data produced the number or weight of initial workers that produced the number and weight of new workers and brood at the end of each brood cycle. The most meaningful measure of efficiency is a unitless ratio of input to output, or a factor of increase of number and / or weight. Given the identical starting condition of both treatments, we can assume that the maintenance and work energy did not differ, and dropped out of the equation. Data were analyzed by analysis of variance (ANOVA) using Statistica 13 (Statsoft Inc.). An alpha of 0.05 was applied in all tests.

Results

No measure of production or efficiency differed between the two chamber-type treatments (ANOVA, nest type, $F_{1,33} = 0.022$; $p > 0.05$). For example, in the first run, the new biomass produced per initial biomass in single-chamber nests was 4.73, while in multiple-chamber nests it was 4.40. In the second run, these values were 1.03 and 1.53, respectively. This lower performance in the second than the first run was significant (ANOVA, run; $F_{1,33} = 32.9$; $p < 0.0001$), with means of 4.55 and 1.03. The reason for this poorer performance in the second run is unclear.

The number of new workers (including those still in the form of brood) per initial worker (including those that were pupae at start-up) was also not different between nest treatments ($F_{1,35} = 0.0014$; $p > 0.05$). In run 1, the average was 7.68 (single chamber) and 6.92 (multiple chamber). In run 2, these were 2.75 and 3.45, respectively. Although production seemed higher in the multiple nest treatment, workers produced in these nests in both runs were smaller than those in the single chamber treatment (Fig. 2; $F_{1,32} = 11.9$; $p < 0.002$), so that the new biomass per initial biomass did not differ by nest treatment (see above). As before, there were large differences in biomass production between runs ($F_{1,35} = 31.8$; $p < 0.0001$) for unknown reasons.

The same pattern necessarily pertained to the total weight of colonies at the end of each brood period (Fig. 3); nest treatment showed no difference ($F_{1,35} = 2.24$; $p > 0.05$) while runs showed a large difference ($F_{1,35} = 51.3$; $p < 0.0001$). Queen weight at the end of each run also did not differ by treatment ($F_{1,35} = 2.25$; $p > 0.05$), but did by run, decreasing from 15.8 mg in run 1 to 13.2 mg in run 2 ($F_{1,32} = 7.39$; $p < 0.01$). An earlier version of this experiment in 2016, but at a lower rearing temperature, produced similar results, including the lower production rate by all colonies in the second run, no matter what the treatment (W.R. Tschinkel, unpubl.).

Discussion

Although the tested nest architectures had no significant effect on brood production efficiency, the experiment was one of the first to test a specific aspect of ant nest architecture on a specific colony function. To the extent that this experiment mimicked the subdivision of field colonies, nest subdivision does not explain the constant size-related efficiency of natural colonies (Tschinkel 1993) vs. the declining size-related efficiency of laboratory colonies (Porter & Tschinkel 1985).

This result could have been anticipated by perceptive extension of the results of Cassill & Tschinkel (1995) who found that the number of workers patrolling brood piles for hungry larvae never varied much from a saturation value of 85% coverage. As a result, larvae were assessed for hunger 200 - 800 times / h, but fed only 2 - 50 times / h (depending on their hunger and size). This high excess of assessment rate over feeding rate assured that larvae were reliably fed upon demand, making the production of new ant biomass independent of work group size (at least above a certain worker per larva threshold). This same phenomenon may also explain why the biomass production efficiency of field colonies did not change with colony size (Tschinkel 1993), but it conflicts with the observation that production efficiency declined with colony size in the laboratory (Porter & Tschinkel 1985). Given the vagaries of laboratory experimentation, it seems more likely that the laboratory, not the field result, is the artifact. In laboratory nests that are more similar to natural nests (e.g., the cavity nester *Leptothorax allardycei*) the finding that production efficiency was not related to colony size may be less tainted by laboratory artifact (Cole 1984).

Other possible sources of laboratory artifacts come to mind. Husbandry and selection / elimination may bias outcomes, for colonies that do not prosper in the laboratory die or are eliminated from the experiment. In ours, as in all such experiments, the reduction of the captured natural colony to a small size at the beginning of each run risks distorting the division of labor in ways that affect multiple colony functions. At the very least, foragers were probably less represented in the sample, as many were probably afield at the time of capture. Such possibilities must be addressed in future experiments. It is also possible that workers organized themselves into efficient groups even in the single-chamber nests. Given these problems, it is likely that the field assessment of Tschinkel (1993) is subject to fewer distortions, and more likely to represent reality.

The most direct test of the nest subdivision hypothesis would have been (and is) to test sexual production by colonies in single and multiple chambered nests, but such tests were of a scale not achievable in my laboratory. Perhaps another laboratory will undertake such experiments.

The multiple chamber treatments did significantly reduce the size of workers, but with respect to biomass production, this effect was compensated by the production of more such smaller workers. The importance of this effect is unclear, but could be related to either chamber size, or nest subdivision. This result could also be seen in the light of a trade-off between worker number and size, a trade-off that is almost universal in founding and incipient ant colonies. Why such a trade-off should be produced by chamber size or nest subdivision is open to speculation. A mechanical explanation is also possible in that the small chambers reduced access and flow of food, so that larvae grew more slowly and pupated at a smaller size (a trade-off of size and developmental time). In essence, workers in divided nests spread their care and food across more larvae.

The difference between runs could be explained by rearing environment. Brood in the second run had experienced life in the laboratory nests, whereas those in the first run had not. While possible, the larvae had pupated within less than a week of setup, so that for the remaining three weeks of each run, brood had experienced only the treatment conditions. This difference could be controlled for in future experiments by starting with field-collected brood for both runs.

These experiments implicitly view the ant colony as a superorganism (Hölldobler & Wilson 2009), a higher-level entity with functions, regulation and processes whose goal is the production of more superorganisms, and in which the individual ants and their behaviors / physiologies are the moving parts of a larger machine. Seen in this light, the importance of individual level biology is primarily in how it contributes to the emergence of colony level outputs, in particular, to sexual production and founding success. Because ants build species-typical subterranean nests, the obvious question is how do particular architectures serve each species of superorganism. This paper is a first attempt to answer such a question.

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Natural history observations and kinematics of strobing in Australian strobe ants, *Opisthopsis haddoni* (Hymenoptera: Formicidae)

James S. Waters & Terrence P. McGlynn

Abstract

The strobe ants of Australia (*Opisthopsis* spp.) move with a rapid staccato gait, appearing as if they are under a strobe light. This extraordinary behavior has long caught the attention of natural historians, but the mechanics of strobing locomotion are as enigmatic as its function. We used high-speed video to track the movements of strobing *Opisthopsis haddoni* Emery, 1893 and *O. haddoni rufonigra* Forel, 1910 ants to develop plausible explanations for the phenomenon. We found that strobing involves periodic bursts of rapid acceleration and deceleration. The ants engage in walking with an alternating tripod gait, punctuated by pauses, with a strobing cycle frequency of 5 - 7 Hz. While stopped, ants distinctly tap their antennae on the ground and raise them again before resuming their gait. The peak speeds of strobe ants, at 50 - 60 body lengths per second, are impressive but are only sustained for an infinitesimally short period of time, and overall average speeds are slower due to the prolonged pauses between strobe cycles. We posit that strobing behavior may have evolved as a form of camouflage to move without easy detection or as a tradeoff to maximize high-speed locomotory behavior within the constraints imposed by the spatial and temporal demands for neurosensory processing.

Key words: Ants, locomotion, biomechanics, tracking, evolution.

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Introduction

A year's worth of tropical rain falls over five months in the Top End of Australia's Northern Territory. The wet season had not yet exhausted itself, and during a brief respite from the torrents, the strobe ants burst from their nests fitfully. A glance at their stunning staccato motion, distinct from other more continuous movements of ants in the undergrowth, calls the attention of the observer. Before the rains chased the strobe ants underground, we took the opportunity to bring some of them into the laboratory, to understand how they strobe.

One century ago, William Morton Wheeler visited Australia and characterized the integrative biology of strobe ants, *Opisthopsis* spp. (Wheeler 1918). Since that time, to our knowledge, no aspect of these charismatic animals has attracted further investigation until the present. Without any prior characterization of the biomechanics or behavior of this kind of movement, we entered this project without a working hypothesis to explain the function of strobing. To develop hypotheses for why strobe ants strobe, we seek to understand how they strobe.

Strobing behavior, involving rapid movement punctuated by intermittent brief pauses, is a relatively uncommon mode of locomotion among the ants. Strobing is most conspicuous in all *Opisthopsis* species, endemic to Australasia. Two other lineages that overtly strobe are the pantropical subfamily Pseudomyrmicinae (*Pseudomyrmex* and *Tetraponera)* and the South American species *Gigantiops destructor* (Fabricius, 1804). These ants often perform brief pauses during forward movement without an apparent cause or function. These movements are sometimes referred to colloquially among ant biologists as wasplike.

When ants and other terrestrial hexapods walk, they generally use the standard alternating tripod gait (Hughes 1952, Full & Tu 1990, Zollikofer 1994b). When ants run quickly, they may briefly "trot" with brief intermittent aerial phases, and one extremely fast species, the Sahara silver ant (*Cataglyphis bombycina* (Roger, 1859)), sometimes runs with just four of its six legs (Zollikofer 1994a). The gait that produces the unusual strobing effect is undescribed. By quantifying the mechanics of strobing in *Opisthopsis*, we endeavor to describe an important aspect of their natural history, reveal relevant environmental and physiological constraints that can account for this behavior, and make inferences about the potential function of strobing.

Methods

Field and laboratory work was conducted in Darwin, Australia, in the CSIRO Tropical Ecosystems Research Centre. Ants were identified using the TERC collection and verified by A.N. Andersen. Most of the work was conducted with

Fig. 1: Photograph of an *Opisthopsis haddoni* worker from the Northern Territory in Australia. Image by Alexander Wild and used with permission.

Opisthopsis haddoni Emery, 1893, but *O. haddoni rufonigra* Forel, 1910 individuals were also included for comparative purposes (Fig. 1). Colonies of *O. haddoni* were observed in the field for ca. four hours in varying weather conditions and times of day. Live workers were collected near the laboratory, by placing glass vials over stationary workers outside the nest. Workers were rested for a minimum of five minutes before recording their movements.

Recordings of ant movements were conducted in an apparatus fashioned from a cardboard box, about 400 cm per side. The box was lined with white paper, open on the top and the front, and illuminated with four white LED flashlights, one per corner of the box. Ants were placed adjacent to the focal area near the bottom of the box, and movements were recorded with the high-speed function of an iPhone 6 Plus (Apple Inc., Cupertino, USA). The high-speed video recording feature of this consumer electronics device (including frame rates of 120 and 240 Hz) works surprisingly well in relatively low-light conditions and has been validated as a useful tool to track locomotory and projectile motion (Hechter 2013, Balsalobre-Fernández & al. 2015). Video files were recorded at 240 Hz in 1280 × 720 resolution, and with the camera 12 - 13 cm above the arena, the image of individual ants (~ 4 mm long) measured approximately 50 pixels in length. In two trials, the antennal funiculi from *Opisthopsis haddoni* workers were experimentally ablated, while leaving the scape intact, to evaluate the role of the antennae in the strobing process. Video clips in which individual ants were exhibiting strobing behavior were selected and analyzed to determine the kinematics of their movement. The positions of specific body elements (including thorax center, legs, and antennae) were tracked using ImageJ 1.46r (Rasband 1997) and ant trajectories were tracked as a moving point mass using Physlets Tracker 4.92 (Brown 2009). Lengths and camera perspective were calibrated using the dimensions of a microscope slide in the field of view.

The strobing movements of 13 *Opisthopsis haddoni* workers and three *O. haddoni rufonigra* workers were digitized, with a total of 330 strobe cycles analyzed for timing over 16,602 frames (Fig. 2). Analysis of variance was used to compare the timing of the mean duration of the stationary phases and the mean cycle time. The visualization and analysis of body movement patterns in ImageJ was conducted on a single representative individual using 2007 manually digitized coordinates.

In some trials, gaits of ants were recorded on soot-covered slides which bore the markings where ants contacted the surface with legs and antennae (Hangartner 1969). These marks on the slides were used to observe the tracks left by legs and antennae contacting the surface, to verify that legs and antennae touched the surface where they appeared to do so in the recordings.

Results

The walking gaits of *Opisthopsis* can be classified into three distinct categories: slow, strobing, and flight. Strobing is the most frequent mode of movement for *O. haddoni*. If the ants are traveling from one place to another, then they may be expected to move with the characteristic strobing gait (see the video in Appendix S1, uploaded as digital supplementary material to this article, at the journal's web page).

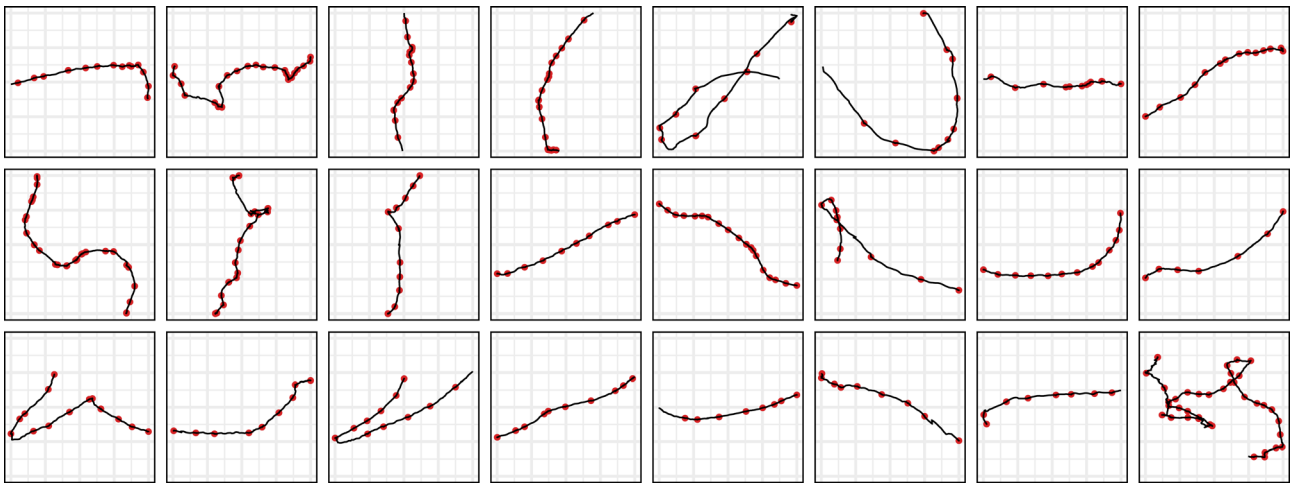

Fig. 2: Tracking the strobing behavior of *Opisthopsis*. Each of these panels illustrates the x - y trajectory of an individual strobing *Opisthopsis* ant. Red dots indicate positions of the paused stage of strobing cycles, as determined by using a drop in their velocity to below 1% of their maximum as a classification threshold. The duration of the displayed tracks spanned 0.53 - 5.6 s, and for clarity the tracks have been scaled to fit the available space, maintaining a fixed 1:1 coordinate system.

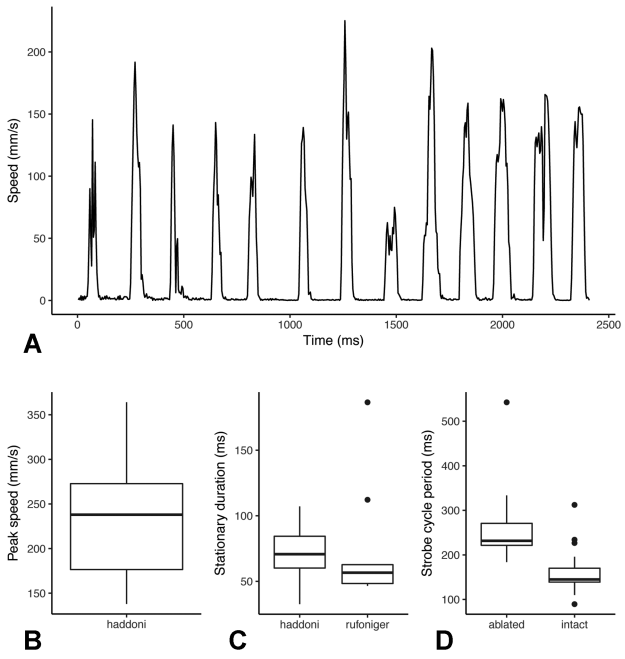

Fig. 3: Kinematics of *Opisthopsis* strobing. Panel (A) shows the speed time series data for a 2.4 s sequence of an individual *O. haddoni* strobing at approximately 5 Hz. The panels below summarize the characteristics of individual strobe cycles across multiple individuals, including their average peak speeds (B), the duration of the stationary period between movements (C), and the duration of the entire strobe cycle period (D).

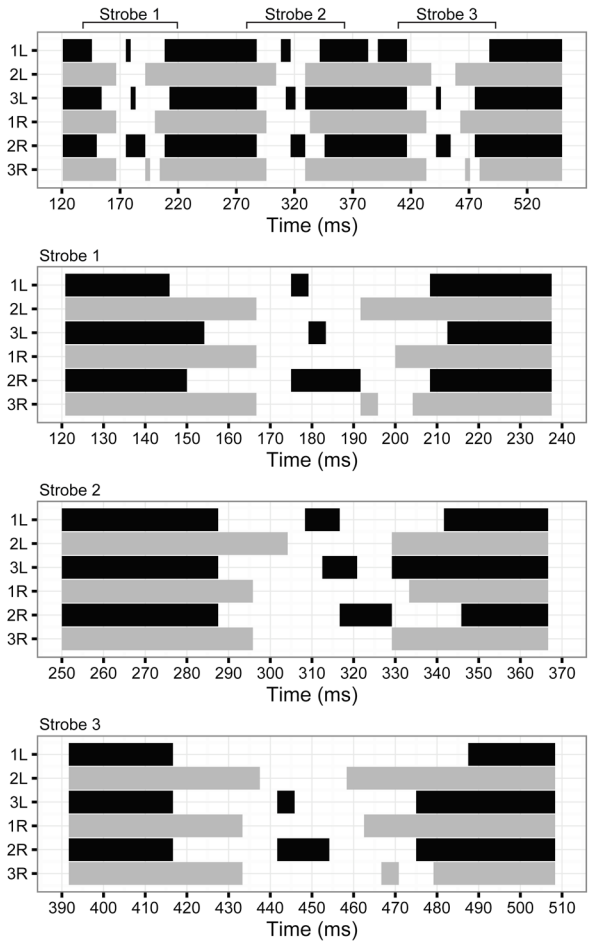

Fig. 4: Alternating tripod gait. By tracking the distal tips of each leg over a strobing sequence for a single ant, we were able to identify its gait pattern based on the classification of leg motion into two distinct tripod groups. Three strobe cycles are illustrated in top panel with a zoom for each depicted in the panels below. Solid shaded horizontal bars indicate periods of time during which a leg is stationary; gaps represent periods of time when a leg is moving. The coordination pattern is consistent with a tripod walking gait with two groups of three legs each. The first group (1L - 2R - 3L, in black) initiates the strobe and is followed by the second (1R - 2L - 3R, in grey).

However, when *O. haddoni* workers are moving slowly, such as loitering around a nest entrance or with an aggregation of workers at a rich food source, then they will not strobe, but walk more slowly with steps that are characteristic of other ants. Flight gait occurs when *O. haddoni* runs at a very high speed but without strobing. Flight typically happens when the vicinity of an ant is directly disturbed, for example, with a shoe, vial, or vertebrate predator. Flight gait lasts for only a few seconds before switching to a strobing gait. When ants are observed undisturbed in the field at a distance, flight gait is rarely observed.

Analysis of the tracking data indicate that the strobing gait of *Opisthopsis haddoni* can be quantitatively characterized as a high frequency intermittent locomotory behavior that involves stationary pauses and bursts of movement (Figs. 2 - 3). Close inspection of the individual leg movements for a single ant's strobing behavior reveals a timing pattern relatively consistent with the alternating tripod insect walking gait (Fig. 4). The general movement pattern between stationary phases involves three steps per strobe, with most instances involving the following sequence: tripod-1, tripod-2, tripod-1, followed by a relatively long pause. Although there appear to be moments during which all six legs are moving (e.g., at times 170, 305, 440, and 455 in Fig. 4), these durations are relatively short and too close to the limit of our temporal resolution (4.2 ms) to make it possible to definitely classify strobing as a true aerial (e.g., jumping or skipping) gait.

The peak speed we recorded, in *Opisthopsis haddoni*, was 364 mm s^{-1}. Comparing individuals with intact antennae, the duration of the stationary phase in the strobe cycle was not different between species ($F_{1,12} = 0.01$, $p = 0.92$) and averaged 63.4 ms ± 21.2 standard deviations (SD). The period of the strobe cycle was significantly different between species ($F_{1,12} = 6.01$, $p = 0.03$), with the average cycle time of *O. haddoni rufonigra* (189.9 ms ± 40.9 SD) greater than that for *O. haddoni* (143.6 ms ± 26.0 SD). While *O. haddoni* is strobing, it is paused (not moving forward) for 43% of the time. In the trials with ants subjected to ablation of antennal funiculi, compared with *O. haddoni* with intact antennae, there was no difference in the duration of the stationary phase ($F_{1,11} = 1.18$, $p = 0.30$), but the period of the strobe cycle was longer ($F_{1,11} = 33.64$, $p = 0.0001$). The strobe cycles of ants with ablated antennae were 271.5 ms ± 47.9 SD long, nearly twice as long as those with intact antennae. Since the stationary durations were the same, the ablated antennae ants were running in longer continuous bouts. During the stationary phase of a

strobe, the ablated antennae ants continued to wave their antennal scapes downward as if the antennal funiculi were still attached.

The antennae of the workers are touched to the surface during the stationary phase of the strobe cycle, and the distance between the antennae is unchanged, and greater when the ant is surging forward. Individual plots of velocity time series show there is individual variation among trials with different individuals, though the generalized alternating pattern of acceleration and full-stop is consistent for each individual during the observation of strobing gait in a trial.

Discussion

The strobing gait of *Opisthopsis* is among their most distinctive features. As described above, the uniqueness of this behavior can be characterized by its discontinuous and high frequency (~ 6 Hz) bursts of speed. Although the average walking speed of a strobing ant (~ 36 mm s^{-1}) is not remarkable, the peak speeds during the active phase of strobing (~ 225 mm s^{-1} or 50 - 60 body lengths per second) are on par with running *Cataglyphis* and rank below some of the fastest documented insect runners, such as cockroaches and tiger beetles. They are sustained however, only momentarily for small fractions of a second (Fig. 3). One additional distinguishing feature of *Opisthopsis* are their extraordinarily large eyes (Fig. 1). While there are no clear functional hypotheses why some ant lineages have evolved relatively large eyes, at least a few of these genera (e.g., *Pseudomyrmex* and *Gigantiops*) have also been observed moving with a degree of intermittent walking akin to that of *Opisthopsis*. Large eyes might facilitate predator avoidance or prey detection, but the ant lineages with large eyes are not exceptionally unique in predation risk or foraging habits. The rapid bursts of acceleration and deceleration, as in other examples of intermittent locomotion, may be energetically costly, may play a role in predator-prey dynamics, and / or may be associated with svisual or chemosensory information processing (Kramer & McLaughlin 2001).

When tiger beetles, *Cicindela repanda*, are in pursuit of prey, their default mode of pursuit involves fast movements punctuated by brief pauses. While they are capable of continuous pursuit of a moving target, this discontinuous motion is hypothesized to be necessary for the beetle to discriminate the image of the moving target against the image of the background which itself is changing due to the beetle's own high linear and angular velocity. Tiger beetles move so quickly that they lose the ability to have clear vision, and briefly stop to regain a visual image of prey while in pursuit (Gilbert 1997). When tiger beetles lack the ability to visually navigate the landscape, they use antennae to compensate for the lack of information (Zurek & Gilbert 2014). The mean duration of runs between pauses in tiger beetles was 164 ± 25 ms (Gilbert 1997), which is similar to the duration of the individual strobing runs of *Opisthopsis haddoni*.

An alternative and non-exclusive hypothesis for the discontinuous predatory behavior of the tiger beetles is that they themselves are often the object of predation by animals (e.g., lizards) that pursue moving targets but not stationary ones (Gilbert 1997). One of us (TPM) has observed multiple predation events by a jumping spider that mimics *Opisthopsis haddoni*, presumably *Myrmarachne rubra*, which closely associates with the nests of *O. haddoni* (see Ceccarelli 2010). If *Opisthopsis* is subject to high rates of predation by specialized jumping spiders, it is conceivable that strobing has evolved to detect this predator using a combination of visual and olfactory signals. However, since the natural history of *Opisthopsis* is heretofore undescribed, we are not able to put this hypothesis into context.

If *Opisthopsis* spp. are particularly susceptible to a predator that leaves a trace signal on the surface, the stereotyped antennation behavior during strobing events might be the primary function of strobing. In every strobe cycle we observed, the ants discretely came to a full stop, lowered their antennae to the surface, lifted their antennae, and after having antennae in an elevated position, commenced forward movement. The antennation may, or may not, be the primary function of the strobing movement, but it is integral to strobing. Strobing, and the concomitant antennation, occurs both on foraging paths as well as in novel environments. As nearly all ants that follow pheromone trails do not strobe we doubt this can account for strobing. Without antennae, we found that the duration of the stops was unchanged, but the duration of forward movement doubled. This suggests that while sensory feedback may be involved, it is not strictly necessary for the action of a potential strobing central pattern regulator. Intriguingly, similar constraints associated with sensory information processing have also been proposed to explain the strobing behavior exhibited by phorid flies which walk in remarkably similar 5 - 8 Hz stop and go bursts (Miller 1979).

In his account of trying to collect running *Opisthopsis*, W. M. Wheeler reported (1918) that they are "very difficult to catch, as they look backward as well as sidewise and forward and dodge about with such adroit zigzag movements that it is almost impossible to seize them with the tweezers or fingers. I finally resorted with greater success to slapping them with the hand, but this is apt to crush them or to make them fall from perpendicular surfaces."

Although in our analysis there were no apparent systematic changes in walking direction between strobe cycles and we did not find evidence for strobing being associated with a particularly "zigzag" movement (Fig. 2), we concur with Wheeler that field sampling of foraging *Opisthopsis* is more difficult than nearly any other kind of ant. Once *Opisthopsis* are disturbed, they often switch from a strobing gait to the faster flight gait, but only for one or two seconds before returning to a strobing gait. While our current study only considered strobing by individual ants, future fieldwork examining the kinematics of strobing in collective groups will be particularly informative for learning how this behavior varies within dynamic social, chemical, and visuospatial contexts.

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Differential responses of ant assemblages (Hymenoptera: Formicidae) to long-term grassland management in Central Germany

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Abstract

Biodiversity decreases in response to either intensive management or land abandonment in permanent grasslands of Central Europe. Here, we evaluated the long-term impacts of different management regimes on ant richness, nest abundance, assemblage structure, and food resource use in three experimental grassland sites in Thuringia, Germany. Each experimental site comprised identical management regimes established in 2000/2001. Grassland sites differed with respect to plant community type and abiotic conditions. Ants were assessed in four treatments representing a gradient in management intensity: intensive mowing (four-five cuts per year), traditional mowing (two cuts per year), mulching (mulched once per year), and abandonment (no management). A total of fourteen species belonging to three genera were recorded. Overall, ant responses to management treatments were site dependent. Mean species richness did not vary across treatments but sites. Nest abundance was high in the intensive and traditional treatment but strikingly low in the mulching treatment. Assemblages were more diverse in the traditional and abandonment treatment in sites representing semi-dry conditions, while the intensive treatment enhanced ant diversity under mesic site conditions. Higher rates of food monopolization were detected in the intensive and traditional treatment in drier conditions. Our results show that long-term management affected ant assemblages in different ways, but these effects were strongly related to local climate and soil conditions. The responses of ants to grassland management, that is mowing, can be explained by how these management practices (or their absence) affect the microclimatic conditions under a local context. Hence, the interplay of these factors along with the species requirements is of key importance to determining the impact of the land management on ant assemblages in German and Central European grasslands.

Key words: Community structure, microclimate, mowing regimes, monopolization, mulching, nest abundance, traditional management, upland meadows.

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Introduction

Permanent grasslands are key elements of European agricultural landscapes, highly relevant for the establishment of a sustainable bio-based economy (Osoro & al. 2016). About 40% of the agricultural area in Europe and at least 13% in Germany constitute permanent grasslands, providing fodder for livestock, renewable raw materials for industry and energy production (FAO 2014, Statistisches Bundesamt 2016). Permanent grasslands are important in regulating climate and hazards like erosion and flooding, function as recreational areas, and shelter a high proportion of Europe's endangered biodiversity (Peeters 2009). Nevertheless, grasslands have been threatened by intensification of management or land abandonment in the past 50 years across Europe (Poschlod & al. 2005, Peeters 2009). These processes are known to cause changes in plant community composition and a loss of biodiversity in grasslands (Klimek & al. 2007, Poláková & al. 2011, Socher & al. 2013).

Ants are an important and omnipresent component of biodiversity in Central European grasslands (Curry 1994), being the major generalist predators in meadows (Kajak & al. 1972, Sanders & van Veen 2011). They are considered ecosystem engineers, directly or indirectly controlling many ecosystem processes by altering physical, chemical, and biological soil properties at their nesting sites (Dauber & al. 2008, Frouz & Jilková 2008, Sanders & van Veen 2011). From a nature conservation perspective, ants are key elements in the life cycle of flagship species of European grasslands such as endangered *Maculinea* butterflies (Elmes & al. 1998). Although ants are sensitive to anthropogenic disturbance and land-use changes (Crist 2009), the effect of different management regimes on the myrmecofauna of European grasslands is still a matter of debate (Dahms & al. 2005, Grill & al. 2008, Pihlgren & al. 2010).

The relationship between different mowing regimes and ant assemblages provides a paradigm to investigate the impact of grassland management (or lack thereof) on species richness and community structure. Prolonged and frequent mowing leads to habitat homogenization as a consequence of uniform plant height, cover, and the reduction of topographic structures such as mounds and grass tussocks (Curry 1994, Morris 2000). In contrast, the complete abandonment of

management practices allows natural succession leading to the establishment of tall grasses, litter accumulation, and shrub encroachment in the long run (Öckinger & al. 2006). Although the initial stages of natural succession may promote habitat heterogeneity and benefit grassland biodiversity (Öckinger & al. 2006), further advancement of succession can result in the loss of specialized and thermophilic ant species (Dahms & al. 2010, Dekoninck & al. 2010). Therefore, the effect of abandonment of grassland management on ant diversity is time dependent and further driven by site specific characteristics such as management legacy and grassland types (Dauber & Simmering 2006, Dahms & al. 2010, Dekoninck & al. 2010, Wiezek & al. 2011). In any case, both management extremes cause changes in habitat structure, microclimate, and food resources, affecting ant diversity and interspecific interactions within the assemblages (Curry 1994, Braschler & Baur 2005, Dahms & al. 2010). It has been proposed that regular use of traditional low-intensity mowing or livestock grazing is essential to preserve grassland biodiversity in Central Europe (Isselstein & al. 2005, Poláková & al. 2011). Traditional mowing can maintain high plant species richness by suppressing tall-statured dominant plant species, thereby decreasing light competition and enabling short-statured species to coexist (Hautier & al. 2009). Additionally, mulching has also been suggested as low-cost alternative management for the conservation of species-rich grasslands (Doležal & al. 2011). This management involves cutting the grassland without herbage removal, where the plant biomass subsequently decomposes and releases mineral nutrients into the ecosystem (Doležal & al. 2011, Gaisler & al. 2013).

Previous studies investigating the effects of grassland management on ants report ambiguous results (Dahms & al. 2005, Grill & al. 2008, Wiezik & al. 2011, Pech & al. 2015), perhaps because the intensity gradients of the considered management practices were not distinct enough (e.g., Dahms & al. 2005). In this study, a well-defined mowing gradient on permanent grasslands was investigated with intensive mowing and land abandonment as the outer limits. We evaluated the effects of different long-term grassland management regimes (intensive mowing, traditional mowing, mulching and abandonment) on ant fauna in three experimental grassland sites in Thuringia, Central Germany. The long-term experimental grasslands were established in 2000 / 2001 by the Thuringia Institute of Agriculture and covered different grassland plant community types and abiotic conditions representative of a large fraction of Germany and Europe. We hypothesized that ant diversity would be significantly reduced in both intensively managed and abandoned grasslands compared to traditionally managed or mulched grasslands. Furthermore, since land use imposes changes in habitat structure and alters community composition (Braschler & Baur 2005, Hillebrand & al. 2008, Philpott & al. 2009), we would also expect that such practices alter the biotic interactions within ant assemblages by promoting either dominance at food resources in intensively managed grasslands or co-existence in less disturbed habitats such as the abandoned grasslands. Hence, the following questions were addressed: i) Do different management regimes have an impact on ant assemblages in terms of species richness, nest abundance and assemblage structure?, ii) Are ant responses to management regimes consistent among grassland sites?, iii) Do the species associated with food resources differ between management regimes? Considering bait monopolization as a measurement of ant dominance and bait sharing as a measurement of species coexistence (Stuble & al. 2017), the additional question was addressed: iv) Do the management regimes affect competitive interactions by promoting changes in the monopolization or sharing of food resources? Here we focus on potential effects of management regimes on the resource usage rather than on establishing activity pattern, behavioral categorization or hierarchies within ant assemblages (*sensu* Cerdá & al. 2013, Stuble & al. 2017).

Materials and methods

Study area: The study was carried out in Thuringia, Central Germany, a region with heterogeneous landscapes, strong gradients in environmental conditions, management, and agricultural productivity. Its elevation ranges from 100 to 982 m, with annual precipitation between 500 mm and 1000 mm (Profft & al. 2009). Mean annual temperatures vary between 4 and 10 °C. Soils in the region include Brown soils, Rendzinas and Luvisols (Profft & al. 2009). Traditional and low input farming practices have created a range of different grassland types in the region. Long-term experimental grasslands at three different sites were selected: Wechmar, Hessberg and Oberweissbach (Tab. 1). The experimental grasslands were established in 2000 / 2001. The grassland sites are representative of the geological and climatic variation of Thuringia, and comprise different plant community types (Tab. 1). In Wechmar, the plant community consists of tall oat-grass meadows dominated by *Arrhenatherum elatius* with *Poa pratensis*, *Dactylis glomerata*, and *Trisetum flavescens*, and the herbaceous species *Galium mollugo* and *Geranium pratense* being also important. The site at Hessberg is characterized by foxtail meadows dominated by *Alopecuretum pratensis*, with *Ranunculus repens*, *P. pratensis*, *P. trivialis*, and *Elytrigia repens* as important species. The site at Oberweissbach contains golden oat meadows dominated by *T. flavescens*, with high abundances of *D. glomerata*, *Holcus lanatus*, *P. pratensis* and the herbaceous species *Taraxacum officinale*.

Long-term grasslands experimental design: At each grassland site, four different management regimes were applied representing a gradient in management intensity. Experimental treatments were: intensive mowing (intensive), traditional mowing (traditional), mulching once per year (mulching) and no management (abandonment) (Tab. 1). The intensive treatment represents intensively used grasslands with four to five cuts per year from mid-May until late-September. The traditional treatment represents extensively used grasslands with two cuts per year, the first in June-July and the second in September. The mulching treatment represents an alternative management for nature conservation with one cut in July without hay removal. The abandonment treatment includes grassland plots with natural succession and without mowing or fertilization. Mineral fertilizers (NPK) were only applied in the intensive treatment to balance the continued biomass removal by frequent mowing (Tab. 1). All treatments reflect common management regimes in temperate European grasslands. The experimental grasslands were rectangular vegetation plots of 20 to 30 m^2 in size (Tab. 1), isolated and separated by regularly mown extensions of 0.5 to 2 m distance. A randomized block design was applied with four plots per treatment at each site, except for Oberweissbach, where

Tab. 1: Characteristics of the sites, grassland experiments and management treatments in Thuringia, Central Germany. NPK indicates fertilization [kg / ha] with nitrogen (N), phosphorus (P) and potassium (K).

	Wechmar	**Hessberg**	**Oberweissbach**
Site features			
Bedrock	upper Muschelkalk	alluvial clay	Argillite
Elevation [m a.s.l.]	350	380	690
Mean annual temperature [°C]	8.0	7.4	5.7
Annual precipitation [mm]	541	773	861
Soil moisture regime	dry	moist	moderately moist
Soil type (German classification)	Mountain clay (Bergton-Rendzina)	Meadow / alluvial clay (Auenton-Amphigley)	Mountain loam (Berglehm-Braunerde)
Soil pH	6.8	6.2	6.1
Grassland experiments			
Plant community type	Tall oat-grass meadows (*Arrhenatheretum*)	Fox tail meadows (*Alopecuretum*)	Golden oat meadows (*Trisetetum*)
Site area (km^2)	2.9	2.7	2.8
Plot size (m^2)	25	30	20
Year of establishment	2001	2001	2000
Geographic location	50° 52' N, 10° 45' E	50° 25' N, 10° 46' E	50° 34' N, 11° 8' E
Management treatments			
Intensive	Annual cuts: 4 NPK: 200N 30P 220K	Annual cuts: 5 NPK: 260N 30P 220K	Annual cuts: 4 NPK: 200N 25P 200K
Traditional	Annual cuts: 2 NPK: None	Annual cuts: 2 NPK: None	Annual cuts: 2 NPK: None
Mulching	Annual cuts: 1 NPK: None	Annual cuts: 1 NPK: None	Annual cuts: 1 NPK: None
Abandonment	Annual cuts: None NPK: None	Annual cuts: None NPK: None	Annual cuts: None NPK: None

only two replicates were established for the mulching and abandonment treatment.

Ant sampling: Since all the plots constitute ongoing grassland experiments, non-intrusive methods had to be applied for the ant survey. Therefore, ant assemblages were assessed by direct sampling and baiting within a 6 m^2 rectangular area at each replicated plot (Bestelmeyer & al. 2000, Seifert 2017). The sampling areas were set at least 1 m from the plots' borders to avoid edge effects (Dauber & Wolters 2004). Direct sampling consisted of an intensive search of ant workers and nests on the soil surface, in the litter and the vegetation (turf) during 30 minutes per sampling area at each plot (one person). All surface-active ants were collected and fixed in 80% ethanol, and only workers were considered for relative abundance analyses. All ant nests were recorded and marked during the direct sampling, and up to 10 workers were collected from each nest after the sampling period. The time invested in direct sampling per plot corresponds to the sampling effort of other ground-dwelling ant surveys in Central European grasslands (Wyhnhoff & al. 2011, Seifert 2017). Direct sampling was performed between 09:00 and 17:00 under standard weather conditions (air temperature over 15 °C without rain; Seifert 2007). The direct sampling was performed twice, in May – June and July – August 2016, and data from both surveys were pooled for further analyses.

Baiting procedures were employed in order to complement the direct sampling and to evaluate the food resource use by ants among treatments. Two bait stations, each consisting of a plastic platform of 8 cm in diameter with tuna (~ 4 g) or honey-rum (4 ml solution in cotton balls) bait, were installed at 2 m distance in each plot. The numbers of species, individuals and dominance-coexistence events were recorded during a 30-s period at each bait station. Dominance was measured as events where one species drove another away from the bait by aggressive behavior or numerical displacement; while coexistence was measured as events where two or more species shared the resource without engaging in antagonistic interactions (Cerdá & al. 2013, Stuble & al. 2017). Each observation was repeated six times (after 10 min and then at 20 min intervals) in a baiting session. Bait sessions were evenly distributed between 09:00 and 17:00, and repeated eight times per plot with a minimum separation time of 18 hrs from late-May to early-August 2016. A total of 64 baits were used per treatment at each site, 32 baits for the mulching and the abandonment treatment in Oberweissbach. All individuals collected were determined to species level according to Seifert (2007), and the nomenclature of Radchenko & Elmes (2010) was followed for the *Myrmica* species.

Since microclimate for ants strongly depends on height and density of the vegetation (Seifert 2017), temperature and humidity were assessed within management treatments. At each plot, the daily mean, maximum, and minimum temperatures (T_{mean}, T_{max} and T_{min}), and relative humidity (%RH) were recorded using Tinytag Plus 2 TPG-4500

Tab. 2: Subsets of candidate models, global and null model explaining ant species richness and nest abundance. The table shows the Akaike's Information Criterion (AIC), the delta AIC values (ΔAIC), the Akaike's weight (w_i) and the relative importance of predictor variables ($w + (j)$) in the whole subset (Burnham & Anderson 2002). *Treat* = management treatment; *Site* = grassland site; %*RH* = percentage of relative humidity; "x" indicates interaction between two predictor factors, "X" indicates variable included in the model, and "-" parameter not included in the model. Only models with ΔAIC < 4 are considered as candidates.

Models	**Predictors**	**AIC**	**ΔAIC**	w_i	**Variables' relative importance**		
					Site	***Treat***	***%RH***
Species richness							
Candidate 1	*Site*	144.2	0	0.57	X	-	-
Candidate 2	*Site* + %*RH*	145.9	1.63	0.25	X	-	X
Candidate 3	*Treat* + *Site*	147.9	3.63	0.09	X	X	-
Global	*Treat* + *Site* + %*RH*	149.8	5.63	0.03	X	X	X
Null	*1*	160.2	16	< 0.01	-	-	-
Distribution family: Poisson				$w + (j)$	**0.95**	**0.13**	**0.29**
Nest abundance							
Candidate 1	*Treat* + *Site*	136.2	0	0.40	X	X	-
Global	*Treat* + *Site* + %*RH*	136.7	0.46	0.32	X	X	X
Candidate 2	*Treat* x *Site*	138.7	2.47	0.12	X	X	-
Candidate 3	*Treat* x %*RH* + *Site*	139.7	3.55	0.07	X	X	X
Null	*1*	159	22.8	< 0.01	-	-	-
Distribution family: Negative binomial				$w + (j)$	**0.90**	**0.90**	**0.38**

sensors installed 10 cm above the soil from May to August 2016.

Statistical analysis: All data analyses were performed using the statistical program R 3.3.1 (R Development Core Team 2016). Pearson correlation coefficients were calculated to explore the relationships among the microclimatic measurements T_{mean}, T_{max}, T_{min} and %RH. Positive correlations between T_{max}, T_{min}, and T_{mean} were found ($r > 0.69$, $p < 0.001$), but no significant relationship between T_{mean} and %RH was detected ($r = 0.12$, $p = 0.453$) when the whole data set was compared. However, negative correlations between T_{mean} and %RH were found in Wechmar ($r = -0.68$, $p = 0.004$) and Hessberg ($r = -0.73$, $p = 0.001$), but not in Oberweissbach ($r = -0.17$, $p = 0.58$). Linear models were used to evaluate the variations of T_{mean} or %RH among grassland sites and management treatments. Based on the correlations mentioned before and depending on the scope of the analysis (between or within sites), either T_{mean} or %RH was used as an explanatory variable in further analyses.

Generalized linear models (GLMs) were used to evaluate the effects of treatment, site and microclimatic variables on species richness and nest abundance. Species richness was calculated as the total number of species recorded by direct sampling and baits sampling per plot. Models were constructed using treatment (henceforth *Treat*) and grassland site (henceforth *Site*) as categorical explanatory variables and the mean temperature (henceforth T_{mean}) or percentage of relative humidity (henceforth %*RH*) as continuous explanatory variable. For each response variable a subset of candidate models was generated by fitting i) a global model that contained all explanatory variables or factors, and ii) candidate models with all possible factor combinations and interactions. The models selection was based on the delta AIC values (ΔAIC; Akaike's Information Criterion) and the Akaike weights (w_i) estimation (Burnham & Anderson 2002). The w_i values can be interpreted as the probability that the selected model is the best model of those considered. The ΔAIC procedure extracts delta AIC values of each model (global and candidates) against the best-fitted model, the one with the lowest AIC value (Burnham & Anderson 2002). The candidate models subsets were defined as models within a range of ΔAIC < 4. In addition, the relative importance of each explanatory variable (j) was calculated by summing the w_i values across all the models within the subset where such variable occurs ($w + (j)$). Therefore, the larger $w + (j)$, the more important is the variable compared to other variables (Burnham & Anderson 2002). Models were fitted using Poisson distribution family since the response variables were counts. In case of obtaining overdispersed Poisson models, negative binomial distribution was used. Multicollinearity among continuous (T_{mean} and %*RH*) and categorical variables (*Treat* and *Site*) was evaluated by calculating the variance inflation factor (VIF) for each model (Fox & Weisberg 2002). Model assumptions were visually inspected through diagnostic plots of residuals and normal QQ-plots (Zuur & al. 2010). All analyses were performed with the MASS version 7.3 - 45, car version 2.1 - 6, and MuMIn version 1.15.6 packages (Venables & Ripley 2002, Fox & Weisberg 2002, Barton 2009).

Cluster heat maps were used to explore the variation of ant assemblages between treatments and sites based on the workers and nest relative abundance data (ln[x + 1] transformed) recorded by direct sampling. The hierarchical clustering was done with Euclidean distances and the complete agglomeration method using the heatmap.2 function of the gplots version 3.0.1 package (Warnes & al. 2016).

The incidence of ant species on baits was measured as the proportion of baits visited by ants during the whole observation period. Monopolization (Mo) was calculated as the proportion of times (n = 6) a single species was dominant over the bait session. Sharing (Sh) was defined as the proportion of times (n = 6) more than one species coexisted without antagonistic interactions over the bait session. GLMs were fitted using treatment (*Treat*) and microclimatic data (T_{mean} or %*RH*) as explanatory variable with the ΔAIC procedure of Burnham & Anderson (2002). Binomial distribution was assumed for the incidence of ant species on baits (presence, absence) and Poisson distribution for Mo and Sh counts with an offset term equal to the total number of observations per bait session (log transformed). *Post-hoc* comparisons were performed using least-square means with Tukey's HSD (LSM Tukey) at $\alpha = 0.05$. Pairwise contrasts were calculated for treatment within each site. LSM Tukey was performed using the lsmeans version 2.25 - 5 package (Lenth 2016).

Results

The microclimatic conditions of the plots varied between treatments and grassland sites (Fig. S1 as digital supplementary material to this article, at the journal's web page). A general decrease of T_{mean} was observed from Wechmar to Hessberg and Oberweissbach experimental grasslands. The highest T_{mean} records were generally observed in the traditional treatment while the lowest values were always detected in the abandonment treatment (Fig. S1). The variation of T_{mean} was explained by a joint effect of grassland site and treatment ($F_{11, 32} = 49.22$, $P < 0.001$, $R^2 = 0.93$; Fig. S1). Further GLM analyses revealed collinearity of T_{mean} with the grassland sites (whole data set) and the treatments within each site (VIF > 3 to 10). Therefore, T_{mean} was not considered within the GLM analysis in order to avoid redundancy in the results. On the other hand, the variation of %RH values was also associated to the treatments of each site ($F_{11, 32} = 5.29$, $P < 0.001$, $R^2 = 0.52$; Fig. S1). Hessberg showed the highest values of %RH in almost all treatments (95%; Fig. S1), while Oberweissbach showed the highest variation of %RH (53 - 94; Fig. S1).

Overall, 14 species from three genera were recorded with a total of 11 species detected in Wechmar, five in Oberweissbach, and four in Hessberg (Tab. S1). The mean number of species per plot varied significantly between sites but not between treatments (Fig. 1a). Nest abundance within treatments varied between sites (Fig. 1b). The intensive and traditional treatment showed the highest nest abundance in Wechmar and Hessberg, while the number of nests was generally low in all treatments of Oberweissbach (Fig. 1b). The mulching treatment showed the lowest number of nests at all grassland sites, and in some mulching plots no nests were found (Fig. 1b). In agreement with the model approach, the model containing *Site* as a single factor was better in explaining the variation in species richness among treatments (Fig. 1a, Tab. 2). Although the models subset suggested an additional effect of %*RH*, *Site* showed the highest relative importance value among the predictor variables for species richness (Tab. 2). In the case of nest abundance, the models with higher w_i indicate an independent effect of *Site*, *Treat*, and %*RH* on the response variable (Candidate 1 and Global, Tab. 2). The most important factors to predict nest abundance were *Site* and *Treat* (Tab. 2; Fig. 1b). A list of all candidate models fitted for both variables is provided in the supplement (Tab. S2).

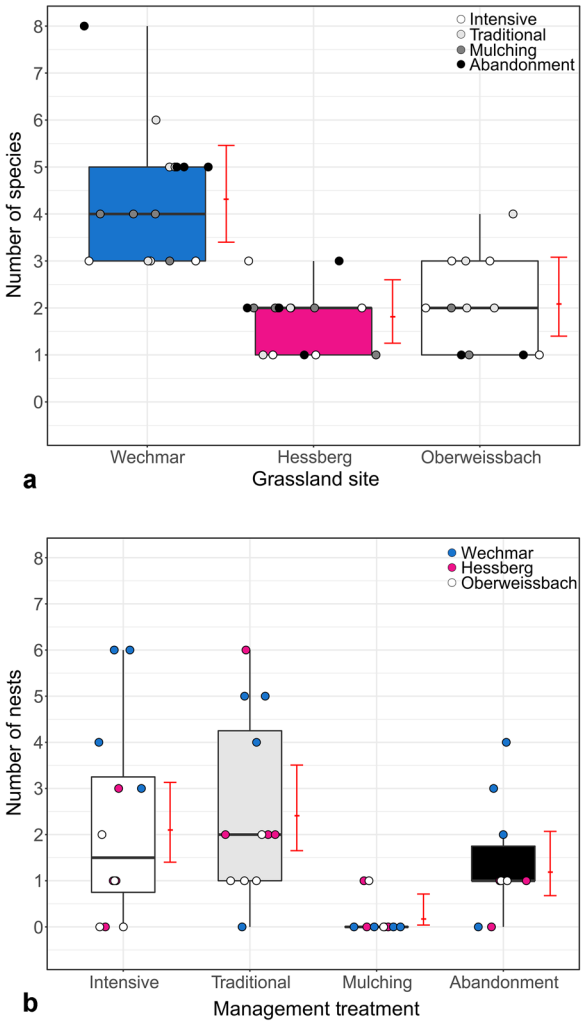

Fig. 1: Ant richness and nest abundance within grassland sites and management treatments. (a) Mean number of species per site, the jittered dots show the raw values per replicate (treatments color coded), and the error bars indicate the mean predicted values ± confidence intervals according with the candidate model 1 for ant richness (see Tab. 2). (b) Mean number of nests per treatment, the jittered dots show the raw data per replicated plot (sites color coded), and the error bars indicate the mean predicted values ± confidence intervals for the factor *Treat* according to the candidate model 1 for nest abundance (see Tab. 2).

The composition and structure of ant assemblages depended on the grassland site (Fig. 2). Wechmar experimental grassland had a diverse ant assemblage with high relative abundance of *Lasius niger* (Linnaeus, 1758) and the presence of exclusive elements of *Formica* and *Myrmica* species (Fig. 2a). At this site, the highest nest counts of *L. niger* and *L. flavus* (Fabricius, 1782) occurred at the intensive and traditional treatment, while *Myrmica sabuleti* Meinert, 1861 and *M. rubra* (Linnaeus, 1758) nests were detected only in the abandonment treatment (Fig. 2b). On the other hand, the Hessberg and Oberweissbach grasslands had less diverse ant assemblages with *M. scabrinodis* Nylander, 1846 as the most abundant species in all cases (Fig. 2a, b).

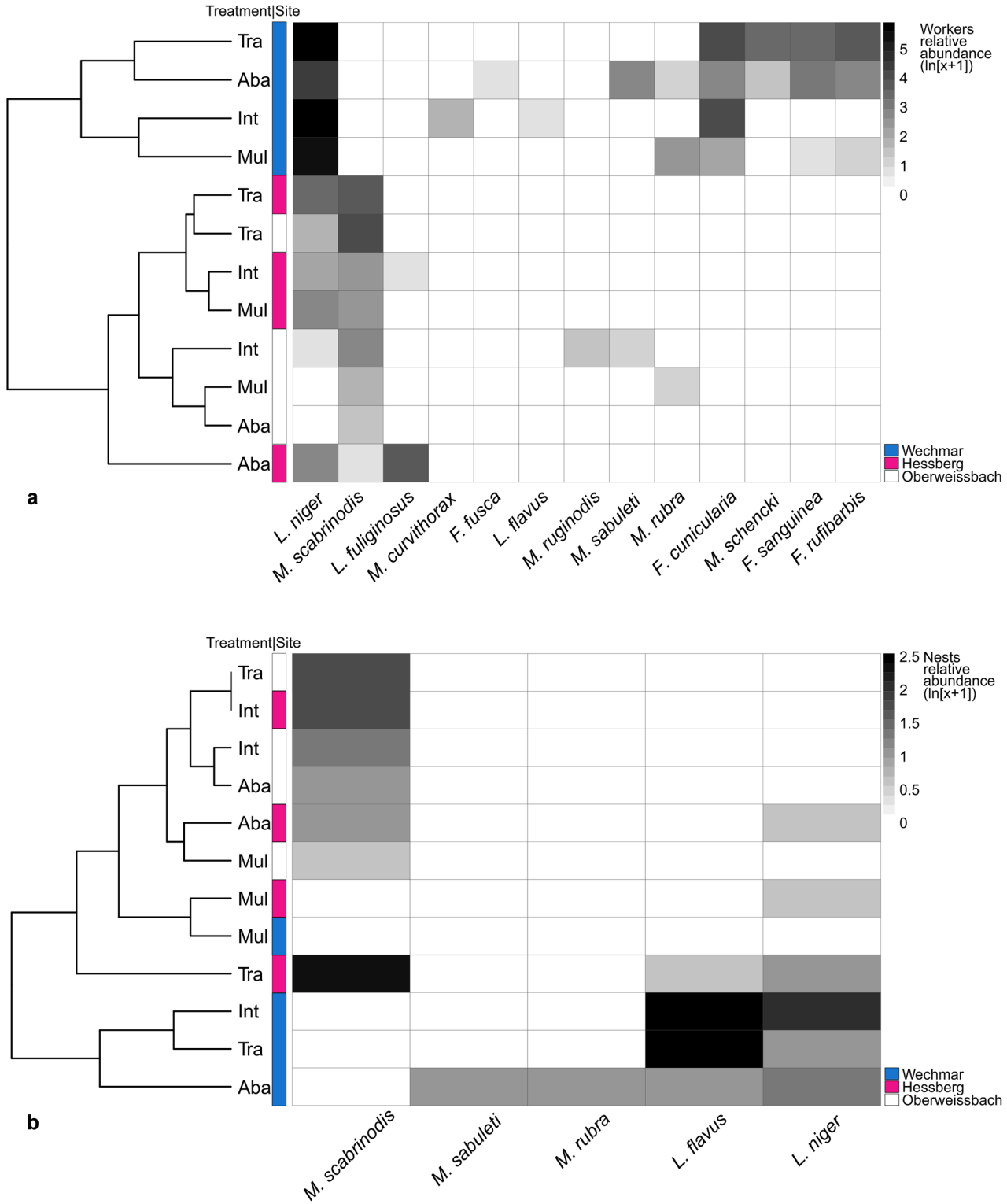

Fig. 2: Ant assemblage composition and structure based on (a) workers and (b) nest counts per species. In both cases the heat map plot shows the relative abundance of each species (ln [x + 1] transformed) at intensive (Int), traditional (Tra), mulching (Mul) and abandonment (Aba) treatment. The cluster heat map is based on similarity measures of the relative abundance of species per treatment.

Overall, cluster analyses showed high similarities on assemblage structure within the traditional, the abandonment and the intensive-mulching treatment (Fig. 2a), and confirmed the effect of the mulching treatment on nests counts in all sites (Fig. 2b).

Bait occupancy varied between grassland sites (Fig. 3). In Wechmar, more than 95% of the baits were occupied (Fig. 3a). Eight species were detected at this site, with *Lasius niger* occupying more than 80% of the baits (Fig. 3a). Baits at the intensive treatment of this site were mainly used by *L.*

niger, while the proportion of baits occupied by this species decreased in the abandonment treatment (Fig. 3a). Besides *L. niger*, the baits were partially used by *Formica sanguinea* Latreille, 1798 in the traditional and abandonment treatment at Wechmar. At this site, *F. cunicularia* Latreille, 1798 and *F. rufibarbis* Fabricius, 1793 occurred in low numbers in all treatments, while *Myrmica* species were restricted to the traditional and abandonment treatment (Tab. S1). The candidate model approach confirmed the effect of *Treat* on the bait occupancy by *L. niger*, and suggested that *%RH* explained the presence of this species on baits to a lesser extent (Tab. 3, Fig. 3a). On the other hand, three species were found on the baits in Hessberg, and four species in Oberweissbach (Tab. S1). At both sites, between 48 and 52% of the baits were occupied, with *M. scabrinodis* as the main species (Fig. 3b, c). In Hessberg, *M. scabrinodis* occupied a higher proportion of baits in the traditional and intensive treatment (Fig. 3b). In agreement with the model analysis, the incidence of *M. scabrinodis* on baits was affected by *Treat* and *%RH*, with both predictors having high relative importance within the subset (Tab. 3). Besides *M. scabrinodis*, *L. niger* was observed on baits in all treatments while *L. fuliginosus* (Latreille, 1798) was found only on baits in the abandonment treatment (Fig. 3b, Tab. S1). In Oberweissbach, a higher proportion of baits were occupied by *M. scabrinodis* in the traditional and abandonment treatment, while a lower proportion was detected in the intensive treatment (Fig. 3c). In this case, the model with the highest w_i value indicates that the presence of *M. scabrinodis* on the baits is mainly explained by *Treat* (Tab. 3). However, the models subset and the $w + (j)$ values also suggest an effect of *%RH* on the incidence of this species on the baits (Tab. 3). Additionally, *L. niger*, *M. lobicornis* Nylander, 1846 and *M. ruginodis* Nylander, 1846 were detected in low proportions on baits in the intensive and traditional treatment (Fig. 3c, Tab. S1).

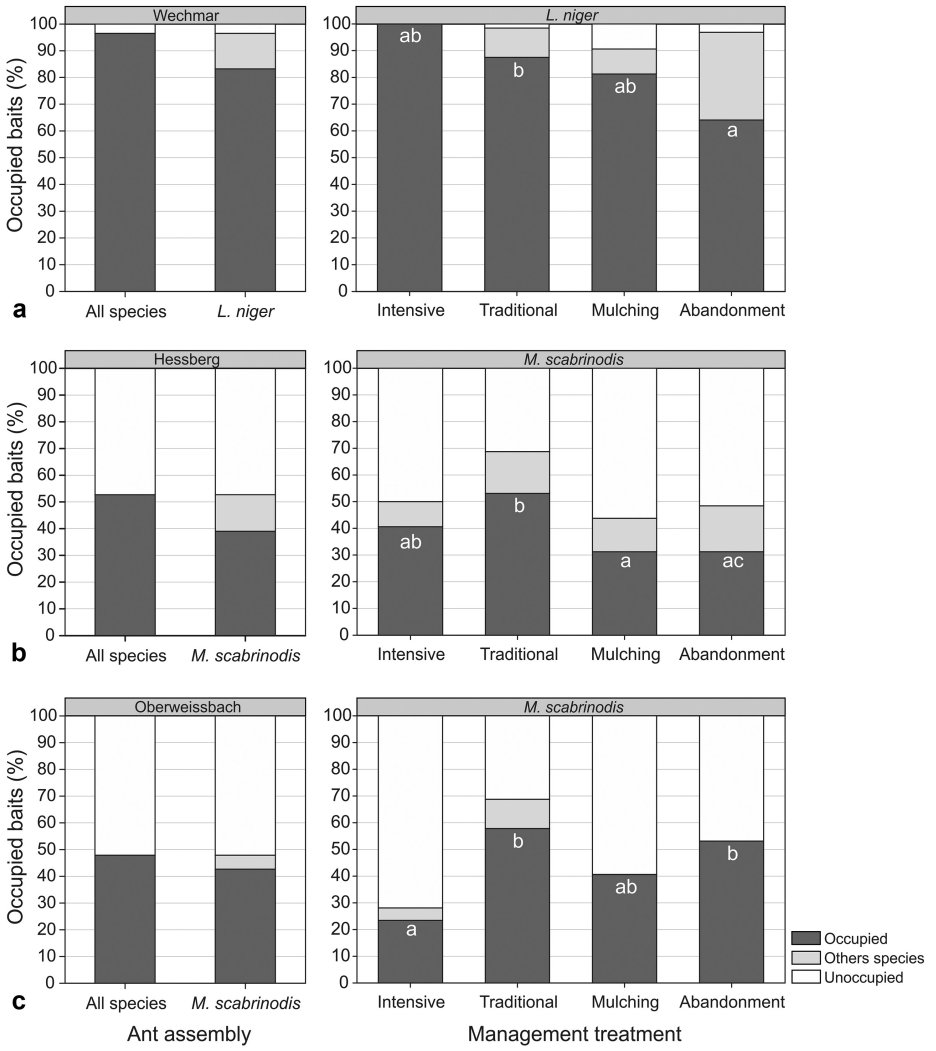

Fig. 3: Food resources occupancy by ants. An overview of the proportion of occupied baits by the assembly of species and most common species is provided for Wechmar (a), Hessberg (b), and Oberweissbach (c). Detail of the bait occupancy per treatment by the main species is also provided. [a, b, c] *post hoc* comparisons of each species incidence on baits among treatments (LSM Tukey, $P > 0.05$; Tab. 3).

Antagonistic or coexistence interactions among species were low, and the monopolization of baits was driven by numeral displacement. Baits shared by different species were scarcely recorded in Wechmar and Hessberg, and absent in Oberweissbach. Overall, Sh values were strikingly low among treatments and sites ($Sh \leq 0.1$). On the other hand,

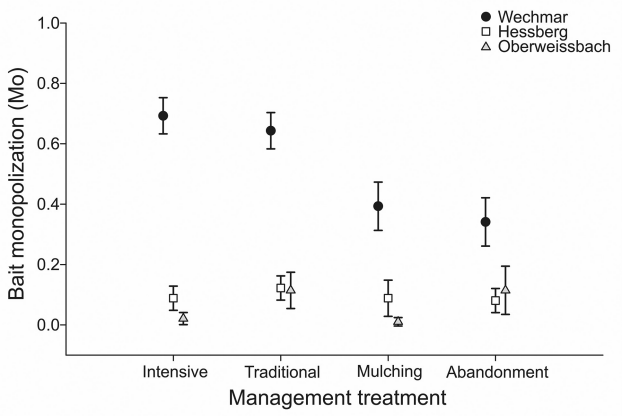

Fig. 4: Monopolization (Mo) of food resources by ants. Symbols show the Mo mean ± confidence intervals (2×SE) per treatment. Mo is the proportion of times (n = 6) at which a single species was dominant over a bait session.

Tab. 3: Subsets of candidate models, global and null model explaining the incidence of *Lasius niger* and *Myrmica scabrinodis* on food resources per grassland site. The table shows the Akaike's Information Criterion (AIC), the delta AIC values (ΔAIC), the Akaike's weight (w_i) and the relative importance of predictor variables ($w + (j)$) in the whole subset (Burnham & Anderson 2002). *Treat* = management treatment; *%RH* = percentage of relative humidity; "x" indicates interaction between two predictor factors, "X" indicates variable included in the model, and "-" parameter not included in the model. Only models with ΔAIC < 4 are considered as candidates.

Models	**Predictors**	**AIC**	**ΔAIC**	w_i	**Variables' relative importance**	
					Treat	***%RH***
Site Wechmar: *L. niger*						
Candidate 1	*Treat*	201.59	0.00	0.70	X	-
Global	*Treat* + *%RH*	203.25	1.66	0.30	X	X
Null	*1*	233.76	32.17	< 0.001	-	-
Distribution family: Binomial				$w + (j)$	**1**	**0.30**
Site Hessberg: *M. scabrinodis*						
Global	*Treat* + *%RH*	340.16	0	0.50	X	X
Candidate 1	*Treat*	341.11	0.95	0.31	X	-
Candidate 2	*%RH*	343.45	3.29	0.10	-	X
Null	*1*	343.64	3.48	0.09		
Distribution family: Binomial				$w + (j)$	**0.81**	**0.60**
Site Oberweissbach: *M. scabrinodis*						
Candidate 1	*Treat*	252.32	0	0.70	X	-
Global	*Treat* + *%RH*	254.25	1.93	0.27	X	X
Null	*1*	264.07	19.08	< 0.001		
Distribution family: Binomial				$w + (j)$	**0.97**	**0.27**

the dominance events were detected mainly in Wechmar (Fig. 4). At this site, the higher Mo values were observed in the intensive and traditional treatment compared to the mulching and abandonment treatment (Fig. 4). The model subset for this site indicates that monopolization of baits was affected mainly by *Treat* (AIC = 1121.82, ΔAIC = 0, w_i = 0.71). An additional effect of *%RH* was also detected (AIC = 1123.6, ΔAIC = 1.78, w_i = 0.29). Overall, *Treat* had higher relative importance ($w + (j) = 1$) in predicting the variation of Mo. The results of the Mo models at Hessberg and Oberweissbach are presented in the supplement (Tab. S3).

Discussion

We found that ant assemblages are sensitive to management within permanent grasslands of Thuringia, Central Germany. However, the responses of ant community composition and structure to long-term management regimes were affected by the grassland site. We detected diverse ant assemblages in Wechmar but impoverished assemblages in the experimental grasslands of Hessberg and Oberweissbach. This may be related to variations in climatic and soil conditions affecting the ant species pool of each site (Dauber & al. 2005, Dauber & Simmering 2006). It has been suggested that soil temperature and moisture along with their interplay with vegetation are important limiting factors for ant assemblages, where warm and dry conditions tend to be favorable for ants (Kaspari & al. 2000, Sanders & al. 2007, Seifert 2017). Likewise, ant species richness tends to be negatively associated with elevated soil moisture levels in temperate grasslands (Seifert 2017). In our case, Wechmar is characterized by semi-dry conditions with higher temperatures and drier soils than the other sites having more humid conditions (Tab. 1, Fig. S1). Such variation in soil conditions and microclimate may explain the ant diversity variation found among grassland sites. This is in accordance with the pattern described for Central European grasslands, where high ant diversity was found at hot and dry grasslands with well drained soils, while lower diversity was detected in more humid meadows (Seifert 2017). However, it is important to consider that our results are limited to the experimental plots and may not be representative for the overall regional diversity of each site. In any case, the patterns detected in the long-term and stable experimental systems of our study support the finding that the effects of land use on ants cannot be assessed independently of the site conditions (Dahms & al. 2005, Dauber & Simmering 2006, Wynhoff & al. 2011).

Along with the site effect mentioned above, ant assemblages vary between treatments but not consistently from management extremes to extensive practices as we had expected. Mean species richness did not vary significantly among treatments, which is in line with previous studies in Central European grasslands (Dahms & al. 2005, Pech & al. 2015, Korösi & al. 2014). However, the abandonment treatment at Wechmar harbored twice the number of species than the intensive treatment. In this particular situation, the abandonment of management practices at the scale of small plots could generate heterogeneous microhabitats with transitions from cooler conditions of tall vegetation in the plot center towards warmer microhabitats of short vegetation in the grassland matrix surrounding the plots

(Fig. S1). Such small-scale heterogeneity may provide wider resource availability for ant foraging and suitable nesting conditions for less thermophilic species (Dauber & Wolters 2004, Dauber & Simmering 2006). The fact that four species (*Myrmica sabuleti*, *M. rubra*, *Lasius niger*, *L. flavus*) with different nesting requirements were well established in this treatment supports this statement.

Nest abundance showed a differential response to the management regimes. The positive effects of the intensive and traditional treatment were site specific and related to the nesting preferences of the most abundant species: *Lasius niger* and *L. flavus* in Wechmar, and *Myrmica scabrinodis* in Hessberg. The first two species are well known for being resistant to and even favored by anthropogenic disturbance, while *M. scabrinodis* tends to be associated with less disturbed but warm and wet habitats, and is even favored by extensive management (Elmes & Wardlaw 1982, Grill & al. 2008, Wynhoff & al. 2011, Seifert 2017; but see Korösi & al. 2014). On the other hand, the total absence or low nest abundance within the mulching treatment at all sites might be attributed to changes in soil moisture and fertility. Doležal & al. (2011) showed that continuous mulching management over nine years increases water and nitrogen content within soils of upland meadows in Central Europe. This soil fertility improvement has also been reported in similar mulch-managed meadows of Thuringia (Hochberg & Zopf 2011). Although such changes in soil may benefit plant communities, high levels of moisture and nitrogen in soil have a negative impact on ant composition (Dahms & al. 2005), and long-term fertilization decreases species richness and nest density in managed grasslands (Petal 1976, Pihlgren & al. 2010). Therefore, we may expect that a continuous mulching management over 15 years alters the soil moisture and nitrogen levels within the studied plots, turning them into less suitable habitats for ant colonization and establishment. Although this explanation requires experimental verification, it may offer a starting point for formulating further research questions about the effect of mulching on ant diversity.

The effect of the management regimes at assemblage level was observed mainly in Wechmar, and to a lesser extent at the other sites. More complex ant assemblages were found in the traditional and abandonment treatment at Wechmar, which basically differ in the abundance of workers of either *Formica* or *Myrmica* species. The high T_{mean} and wide %RH in traditionally managed plots could benefit thermophilic species such as *F. cunicularia* and *F. rufibarbis* (Fig. S1). Both species tolerate high temperatures and are barely affected by extensive management like sheep grazing or traditional cutting (Seifert 2017). The heterogeneous vegetation structure of abandoned plots may favor species with different or wider requirements such as the thermophilic species *M. sabuleti* and *M. schencki*, or the phytodensity tolerant *M. rubra* (see Dahms & al. 2005, Seifert 2017). In contrast, less impoverished assemblages were associated with the intensive treatment in Hessberg and Oberweissbach. Considering the moist soil conditions at these sites, one could expect that the intensive mowing practices might improve the microclimate for ants by means of decreasing humidity and increasing temperature. This situation seems to be plausible in Oberweissbach, where the intensively managed plots showed the lowest levels of humidity but not important changes in temperature (Fig. S1).

Food resource usage between treatments was affected by local variations of ant assemblages, which corresponds to our previous results. However, these results highlighted the fact that almost all resources were exploited at Wechmar, while the bait use in the other grassland sites was two times lower. Although this situation might be a consequence of the differences in relative abundance between species at each site, this difference does not completely explain the pattern observed between sites. An alternative explanation may be related to the behavioral and colony features of the most abundant species at each site. In terms of behavioral interaction *Lasius niger* has been categorized as a subdominant species due to its non-territorial but aggressive behavior when defending or trying to take over resources (Cerdá & al. 2013). *Myrmica scabrinodis*, in contrast, has been considered as a subordinate species with a simple recruitment system that tends to avoid physical contact with workers of other colonies (Cerdá & al. 2013). The higher colony size and foraging distances of *L. niger* (≈ 7000 workers, ≈ 18 m; Elmes 1971, Seifert 2007) may allow them to cover and use a higher proportion of food resources when compared to *M. scabrinodis* which have smaller colonies and restricted foraging areas (600 workers, up to 2 m; Elmes & al. 1998). Some studies have reported relatively low frequencies (≈ 50%) of *Myrmica* species on baits, and it has been suggested that competition among this group is more related to nest site availability than food limitation (Elmes & al. 1998, Grill & al. 2008, Korösi & al. 2014). Hence, all these aspects could explain why *M. scabrinodis*, despite showing higher number of nests at Hessberg than *L. niger* at Wechmar, occurs in a lower proportion on baits.

It could be expected that changes in the habitat structure caused by management alter the biotic interactions within ant assemblages (Cerdá & al. 2013), promoting either the dominance of single species in intensively managed grasslands or the coexistence of several species in less disturbed habitats (Braschler & Baur 2005, Philpott & al. 2009). Contrary to such expectations, no significant results on species coexistence on baits were detected in this study. In Hessberg and Oberweissbach, this absence of coexistence events was not related to an increase of dominance events but to the low usage of the food resources by less diverse assemblages dominated by *Myrmica scabrinodis*. In contrast, the significant dominance events detected at intensively and traditionally managed plots in Wechmar might partially support our hypothesis about how a decrease of habitat structure promoted by management contributes to ant dominance. *Lasius niger* dominated all baits in the open and homogenous plots of the intensive treatment, and partially in the traditional managed plots where they share roles with *Formica sanguinea*. Both species were less frequent on baits in the abandonment treatment, which might allow other species to have access to the resources. This indicates that intensive mowing practices may not only promote the colonization by subdominant species as our nest results suggested (e.g., *L. niger*), but also contribute indirectly to a displacement of less competitive and subordinate species on food resources (e.g., *Myrmica* and *Formica* (*Serviformica*)). Similarly, Braschler & Baur (2005) found that changes of habitat structure caused by fragmentation can alter the competitive interaction within ant assemblages by promoting nest densities of the dominant species *L. paralienus* Seifert, 1992. The study by Braschler & Baur (2005), despite of being focused on fragmentation effects on ants, showed

a response of the ants to long-term and constant mowing management versus land abandonment (fragment vs control plots) within experimental grasslands.

Conclusions

Due to the enormous variety of plant community types, management regimes and local environmental conditions, it is difficult to elucidate the direct effect of management regimes on ant communities in European grasslands. Nonetheless, in this study we demonstrated that long-term grassland management affects ant communities in different ways, but those effects are strongly related to local climate conditions and soil attributes. We detected that in experimental plots under semi-dry conditions (Wechmar), traditional management and land abandonment can harbor diverse ant assemblages; while in mesic conditions (Hessberg and Oberweissbach) some ants can be favored by either intensive or traditional management practices. Additionally, despite of being considered an alternative grassland management for nature conservation, annual mulching affects ant nest abundance negatively. Habitat complexity mediated by management regimes also differently affected the success of ant species on baits, for example the intensive mowing management favored dominance within assemblages while abandonment enhanced more subordinate species. Overall, ant response to management regimes can be explained by how these practices (or absence of them) affected the microclimatic conditions under a local context. The interplay of those factors along with the ant species requirements are key elements for determining the impact of the land management. Against this background, new approaches that take into account the local climatic context and how this potentially affects ant species must be considered in order to evaluate the ant assemblage - management relationship and thus preserve this important group of insects in German and Central European grasslands.

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Invasive Northern Red Oaks benefit *Temnothorax crassispinus* (Hymenoptera: Formicidae) ant colonies

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Abstract

Non-native plant species can modify their environment, and their influence on food chains is well recognized. However, the phenomenon of non-nutrient dependent interaction between non-native plants and native animals has received little attention to date. The Northern Red Oak (*Quercus rubra*) is a non-native, invasive tree species in Europe, which strongly negatively influences co-occurring plants. However, a part of the native fauna is able to utilize the resources offered by this species in its non-native range. We studied a common species of wood ant, *Temnothorax crassispinus*, in forests under canopies of non-native Northern Red Oak and native oaks, Pedunculate Oak (*Q. robur*), and Sessile Oak (*Q. petraea*). These ants use acorns previously predated by insect larvae as nest cavities. We used the number of workers and number of larvae as a proxy for colony condition. *Temnothorax crassispinus* benefited from the occurrence of Northern Red Oak; their colonies were significantly more abundant and colony condition was significantly better than under canopies of native oaks. Laboratory experiments confirmed the significant preference of ants for Northern Red Oak acorns compared with native Pedunculate Oak acorns but only if ants had access to whole acorns. We found no significant preference when the choice was restricted to just the cotyledon material of the acorns. This suggests the ability of *T. crassispinus* to evaluate the solidity of a cavity based on the thicker pericarp of Northern Red Oak acorns. Overall, our data show that *T. crassispinus* ants benefit from the occurrence of Northern Red Oak in the environment.

Key words: Invasive plant species, cavity-nesting ant, seed damage, acorns.

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Introduction

The appearance of non-native plants can take the form of invasion and cause marked changes to ecosystems (Vilà & al. 2011). This invasion, in most cases, leads to negative effects manifested as decreased local plant species diversity (e.g., Gaertner & al. 2009, Hejda & al. 2009, Powell & al. 2011). However, these negative effects do not necessarily apply equally to all native plant species in the environment (Powell & al. 2013). Non-native plant species can also increase ecosystem productivity (Liao & al. 2008, Ehrenfeld 2010, Vilà & al. 2011), but, unfortunately, local herbivores are often unable to take advantage of this increased productivity. Therefore, non-native plants may be associated with significantly poorer communities of herbivores in their introduced range compared with their native range (Roques & al. 2006, Branco & al. 2015). This means that their resources are not fully utilized by locally occurring herbivores and may result in a reduction of biodiversity in the ecosystem and a simplification of trophic relationships. A very good example of such a non-native plant is the Northern Red Oak (*Quercus rubra*). This species does not have a positive effect on the occurrence of any vascular plants in the forests of Central Europe (Woziwoda & al. 2014). Additionally, Northern Red Oak regenerates naturally very well in Central Europe, much better than the native species of oak (Major & al. 2013). The acorns of Northern Red Oak are used by local herbivores much less than those of native species of oak (Myczko & al. 2014, 2017).

Ants of the genus *Temnothorax* form small colonies, composed of a few dozen to few hundred individuals. Depending on the species, they nest in, for example, fallen twigs, under rocks, or in the ground (Seifert 2007, Czechowski & al. 2012). *Temnothorax crassispinus* is among the most widely distributed and most common ant species, and it lives in light coniferous and mixed forests in Central Europe (Seifert 2007, Czechowski & al. 2012). Colonies of this ant are mainly found in cavities in acorns and sticks, situated in the litter layer (Białas & al. 2011, Czechowski & al. 2012). However, the acorns and sticks should have previously been bored by larvae of insects, whose foraging creates suitable ant cavities (Foitzik & al. 2004, Myczko & al. 2017). The

local density of *T. crassispinus* ant colonies can be higher than 2 nests / m^2 (Strätz & Heinze 2004, Białas & al. 2011), and the ant can disperse seeds of different herbaceous plant species (Fokuhl & al. 2012). Thus, the ants can significantly influence the distribution of myrmecophilic plants and, consequently, the forest ecosystem. The number of good quality nest sites for *Temnothorax* ant colonies is usually limited, and their availability changes seasonally (Herbers 1989, Foitzik & Heinze 1998, Herbers & Johnson 2007). Acorns and small sticks with cavities are typically ephemeral; they could be accidentally crushed or no longer habitable as a result of decaying processes (Herbers & Johnson 2007, Langridge & al. 2008). For this reason, *Temnothorax* ant colonies can be forced to find a new nest site and migrate to it frequently, even up to several times per year (Herbers 1989, Herbers & Johnson 2007).

We here report a study of the density and colony condition (using number of workers and larvae as a proxy for condition) of the ant *Temnothorax crassispinus* under canopies of native and non-native oak species in Central Europe. We also experimentally tested ant preferences for acorns of native and non-native oak species.

Methods

We conducted our study in the oak-pine forests of central Wielkopolska, Poland (52° 26' - 52° 36' N; 16° 48' - 17° 03' E), where mature trees of the non-native Northern Red Oak (*Quercus rubra*), native Pedunculate Oak (*Q. robur*) and some specimens of native Sessile Oak (*Q. petraea*) grow together. In total, we selected 50 Northern Red Oak trees paired with the nearest native oak species; Pedunculate Oak in 46 cases and Sessile Oak in the remaining 4 cases. We decided to include Sessile Oak because the morphological variability of acorns of the two native oaks and their chemical composition almost completely overlap (Shimada & Saitoh 2006, Łuczaj & al. 2014). Northern Red Oak trees within the forest stands were selected randomly, but were at least 15 m from each other. The nearest native oak tree of comparable age was selected as the second member of each pair.

Data were collected during October 2015. Under each selected oak tree we established a square study plot of size 2 × 2 m with one corner of the plot adjacent to the trunk and the opposite corner due south. For each plot we recorded: oak species (non-native or native), distance to the nearest mature Northern Red Oak (other than the target tree if appropriate), distance to the nearest mature native oak (other than the target tree if appropriate), diameter at breast height, percent of undergrowth cover, and percent of understory cover. Additionally we took a photo with the camera facing upwards at an angle of 45° to the south using a Sony Cyber-shot DSC-WX60 digital camera. The photographs were taken with 4.5 mm focal length. The digital photos were then converted into black-white mode in Image J software and the percentage of visible sky was used as an estimate of midday insolation of the plot. Then we searched the whole plot and collected all potential nest sites of *Temnothorax* ants into a container for further assessment in the laboratory. During the search for potential nest sites we checked carefully the whole litter layer and also the soil surface. Until assessed in the laboratory, all containers of potential nest site material were kept at a temperature of about 0 °C to avoid migration of ant colonies. In the laboratory we verified the presence of ant colonies and determined the numbers of workers and larvae in each colony and the presence of queens. Additionally we recorded for each study plot: the number of acorns with holes predated by insects which would be suitable for *Temnothorax* ant colonies, the number of this year's acorns fallen to the ground, the number of sticks collected, and the total length of all sticks.

To determine the ant species, we used Radchenko (2004). After confirmation of the ant species, the colonies were divided between two experiments. During the first experiment we used 16 colonies obtained from Northern Red Oak acorns and 16 colonies from acorns of native oak species. Additionally we used 5 colonies extracted from sticks. Because of the unequal number of suitable ant colonies originating from native and non-native acorns, for the second experiment we used 5 colonies from native oak acorns and 25 from Northern Red Oak acorns. For both of these experiments, we recognized the oak species as preferred if it hosted the queen and most of the workers. Each colony was used only once. We extracted the colonies by carefully breaking the acorn shell or the stick with a scalpel and then we excavated the large rests of the nest site using tweezers. In the first experiment we tested the preferences of *Temnothorax crassispinus* for acorns of either Northern Red Oak or Pedunculate Oak. In this experiment we used comparable size acorns of both species. We prepared artificial nests by cutting the acorns 5 mm from the basal edge and then drilling a 10 mm deep hole with a diameter of 8 mm towards the top of the acorn, resulting in the creation of a 0.5 cm^3 volume space within the cotyledons. Additionally we drilled a hole from the outside surface with a diameter of 2 mm as an entry for ants. This hole connected perpendicularly with the previously drilled hole. Then we re-secured the base of the acorn with two metal staples. We placed single acorns of both species together in Petri dishes (n = 37) and introduced a colony of ants. After 24 hours we recorded how many ants were in each acorn and which acorn species hosted the queen, which is a key factor during *Temnothorax* ant colony movement (Doering & Pratt 2016). In the second experiment we established if ants chose their nest site based on acorn cotyledon composition. We used 2 ml Eppendorf tubes lined with the cotyledon material of either Northern Red Oak or Pedunculate Oak. Firstly, in the tube wall we drilled a 2 mm diameter access hole at a 10 mm distance from the lid. Then, inside the tube we placed a cylinder cut from cotyledons of either Northern Red Oak or Pedunculate Oak sized to fit inside the tube (9 mm diameter 5 mm high) leaving a space above for the ant colony. The tubes were placed in pairs in Petri dishes (n = 30) and a colony of ants was introduced. After 24 hours we recorded how many ants were in each tube and which hosted the queen. For both these experiments, we recognized the acorn species as preferred if it hosted the queen and most of the workers. To test the difference in the thickness of the pericarp (an estimator of the strength of the acorn) we measured, using calipers, the pericarp thickness of randomly chosen Northern Red Oak and Pedunculate Oak acorns (n = 10 each).

We used model selection procedures based on information theory (Burnham & Anderson 2002) to identify factors affecting the numbers of *Temnothorax crassispinus* colonies in plots (Tab. S1, as digital supplementary material to this article, at the journal's web pages). We used a generalized linear mixed model (GLMM) with a Poisson distribution to determine factors affecting the number of *T. crassispinus* colonies in plots. Additionally, we used the same models to

determine factors affecting the number of ant colonies in acorns (both with (queenright) and without queens (queenless), and in sticks (both queenright and queenless)). We used plot pair as a random factor in all models. As environmental explanatory variables we included: non-native (coded as 1) or native (coded as 0) canopy oak species (oak.species), diameter at breast height (dbh.tree) as a proxy for the age of the tree, distance to the nearest native or non-native oak species as appropriate (dist.anoth.oak) as a proxy for the proportion of native or non native material in the litter layer, distance to the nearest same oak species (dist.oak) as a proxy measure of isolation from the same habitat, distance to the nearest Scots pine (*Pinus sylvestris*) (dist.pine) as a proxy distance to habitat where excavated acorns were not present (or rare), number of acorns with insect predation holes (no. acorn.hole) suitable for *T. crassispinus* colonies, number of this year's fallen acorns (no.acorn) indicating the fruit production of the tree, number of sticks (no.stick) suitable for *T. crassispinus* colonies, percent canopy openness (per. open.area) which was an indirect measure of the amount of sun from the south, percent of undergrowth cover (per. undergrowth) indicating reduced bare ground or leaf litter, percent of understory cover (per.understory) affecting soil insolation. To avoid multicollinearity, we excluded one variable (total length of sticks) from all models. Multicollinearity in the remaining explanatory variables in all models was not excessive (VIF < 2). All distances were measured from the middle of the study plots.

For model selection we used Akaike's Information Criterion adjusted for small sample sizes (AICc) to identify the most parsimonious model from each candidate set. We ranked all possible model combinations according to their ΔAICc values and used models with the lowest AICc together with associated weight values (the probability that a given model is the best) as those best describing the data. We considered candidate models differing by less than 2 AICc units (ΔAICc < 2.0) to be equally informative and subject to possible model averaging. For averaging, we used models with weights which had ΔAICc values lower than 4 (Burnham & Anderson 2002).

We used a Generalized Linear Model (GLM) with a binomial error structure and logit link function separately for non-native and native oak species to relate the probability of occurrence of ant colonies to acorn volume. The acorn volume was estimated from the formula: $4\pi / 3 \times \text{length} / 2 \times (\text{width} / 2)^2$. We used a log transformation of acorn volume to standardize the distribution and a cubic spline to visualize the probability of ant colony occurrence with respect to acorn volume, separately for non-native and native oak species. We used Kruskal – Wallis tests with a post hoc comparison to compare the number of ant workers and larvae between different nest site types (native oak acorn, non-native oak acorn, native oak stick and non-native oak stick). Chi-square independence tests were used to compare the numbers of all colonies and of the colonies with queens, between the non-native and native oak species, for acorns and sticks separately. We used a GLM with Poisson error to compare the number of acorns predated by insects but not used as ant nests between the non-native and native oak species plots. For the experiments we used binomial tests to determine the statistical significance of deviations from equality of observations in the two categories. In the first experiment the categories were artificial nests made from acorns of either Northern Red Oak or Pedunculate Oak. In the second experiment the categories were artificial nests made from Eppendorf tubes containing cores of cotyledons of each oak species. We used a simple 2 sample t test to compare thickness of acorn pericarp between Northern Red Oak and Pedunculate Oak acorns.

All analysis was carried out in R (R Core Developmental Team 2015). The model selection procedure was performed in the MuMIn library (Bartoń 2016), and the cubic spline visualization was performed in the mgcv package (Wood 2015). The threshold for significance was p = 0.05 throughout.

Results

We found 77 colonies of *Temnothorax crassispinus* ants in Northern Red Oak acorns, 33 in acorns of native oak species and 17 in sticks (seven from the Northern Red Oak plots and ten from the native oak plots). The number of acorns with colonies was significantly higher under non-native oak canopies than native oaks ($\chi^2 = 17.60$, $p < 0.001$). In contrast, the number of occupied sticks did not differ significantly between non-native and native oak species ($\chi^2 = 0.53$, $p = 0.467$) (Tab. 1). We also found three colonies in acorns of native oaks occurring under Northern Red Oak canopies; we excluded these from analysis.

Of the detected colonies, a significantly higher occurrence (57) in Northern Red Oak acorns was queenright than in native oak acorns (27) ($\chi^2 = 10.71$, $p = 0.001$). In contrast, the detected number of queenright colonies did not differ significantly between sticks under canopies of the non-native and native oak species (four under Northern Red Oak, nine under native oak; $\chi^2 = 1.92$ $p = 0.166$).

Based on Akaike's Information Criterion for model selection, seven models of the number of ant colonies were equally good and explained 41 - 54% of the variation. Fourteen models of the number of colonies in acorns were equally good and explained 26 - 56% of the variation. Four models of the number of queenright colonies in acorns were equally good and explained 49 - 55% of the variation. Fourteen models of numbers of colonies in sticks were classified as equally good and explained 6 - 28% of the variation.

The total number of colonies, number of colonies in acorns and number of queenright colonies in acorns was positively correlated with the number of acorns with holes, but numbers were lower in native oak species in comparison to Northern Red Oak (Tabs. 2 - 4). The number of colonies in sticks was significantly positively correlated with the number of sticks ($p = 0.02$, Tab. 5).

We use both type of colonies (queenright and queenless) to analyze differences in numbers of ant workers and larvae between types of nest sites (non-native oak acorns, native oak acorns, sticks under non-native oak canopies and sticks under native oak canopies) (Tab. 6). We found significant differences both in the number of workers and larvae ($\chi^2 = 18.18$, $p < 0.001$; $\chi^2 = 14.59$, $p = 0.002$ respectively). The number of workers was significantly higher in non-native oak acorns than native oak acorns ($p = 0.005$) and also in sticks under non-native oak canopies ($p = 0.005$). Additionally, the number of larvae was significantly higher in non-native oak acorns than native oak acorns ($p = 0.019$) and also in sticks under non-native oak canopies ($p = 0.007$).

The GLM for native oak species showed that the probability of colony occurrence was significantly related to acorn volume ($\beta = 2.052 \pm 0.004$, $Z = 530.1$, $p < 0.001$, Fig. 1), but the equivalent GLM for Northern Red Oak was not significant ($\beta = -1.706 \pm 1.620$, $Z = 1.05$, $p = 0.292$, Fig. 2).

Tab. 1: The mean density of *Temnothorax crassispinus* ant colonies per 1 m^2 on study plots associated with different oak species. SE = standard error.

Oak species	Colonies in acorns	Colonies with a queen in acorns	Colonies in sticks	All ant colonies
	Mean (SE)	Mean (SE)	Mean (SE)	Mean (SE)
Northern Red Oak	0.39 (0.07)	0.29 (0.05)	0.04 (0.01)	0.43 (0.07)
Native oaks	0.17 (0.03)	0.12 (0.03)	0.05 (0.02)	0.22 (0.04)

Tab. 2: Factors affecting the number of all *Temnothorax crassispinus* colonies in forest plots. Statistically significant values are emboldened. No.acorn.hole – number of acorns with insect predation holes, Oak.species – canopy oak species, Dist.oak – distance to the nearest same oak species, Dist.anoth.oak – distance to the nearest native or non-native oak species as appropriate, Per.undergrowth – percent of undergrowth cover, No.acorn – number of this year's fallen acorns, Dbh.tree – diameter at breast height, Per.open.area – percent canopy openness, Per.understory – percent of understory cover. SE = standard error; Z = Z value; P = probability.

Factors	Estimate	SE	Z	P
No.acorn.hole	**0.143**	**0.018**	**7.736**	**< 0.001**
Oak.species	**-0.866**	**0.216**	**3.960**	**< 0.001**
Dist.oak	-0.019	0.011	1.707	0.088
Dist.anoth.oak	-0.012	0.007	1.566	0.117
Per.undergrowth	-0.020	0.013	1.461	0.144
No.acorn	-0.005	0.003	1.399	0.162
Dbh.tree	-0.002	0.001	1.206	0.228
Per.open.area	-0.006	0.007	0.875	0.381
Per.understory	-0.003	0.005	0.617	0.538

Tab. 3: Factors affecting the number of *Temnothorax crassispinus* colonies in acorns in forest plots. Statistically significant values are emboldened. No.stick – number of sticks. For explanations of other variable codes: see Table 2.

Factors	Estimate	SE	Z	P
No.acorn.hole	**0.168**	**0.020**	**8.289**	**< 0.001**
Oak.species	**-1.148**	**0.254**	**4.468**	**< 0.001**
No.acorn	-0.006	0.004	1.800	0.072
Dist.oak	-0.017	0.012	1.417	0.156
Dist.anoth.oak	-0.012	0.008	1.465	0.143
Per.undergrowth	-0.018	0.015	1.194	0.233
Per.understory	-0.006	0.006	0.975	0.329
Per.open.area	-0.007	0.008	0.870	0.384
No.stick	-0.032	0.039	0.811	0.417

Tab. 4: Factors affecting the number of *Temnothorax crassispinus* colonies with queen in acorns in forest plots. Statistically significant values are emboldened. For explanations of variable codes: see Table 2.

Factors	Estimate	SE	Z	P
No.acorn.hole	**0.154**	**0.024**	**6.418**	**< 0.001**
Oak.species	**-1.179**	**0.279**	**4.174**	**< 0.001**
No.acorn	-0.007	0.004	1.631	0.103
Dist.anoth.oak	-0.017	0.010	1.616	0.106
Per.undergrowth	-0.014	0.014	0.956	0.339
Dist.oak	-0.010	0.011	0.880	0.379

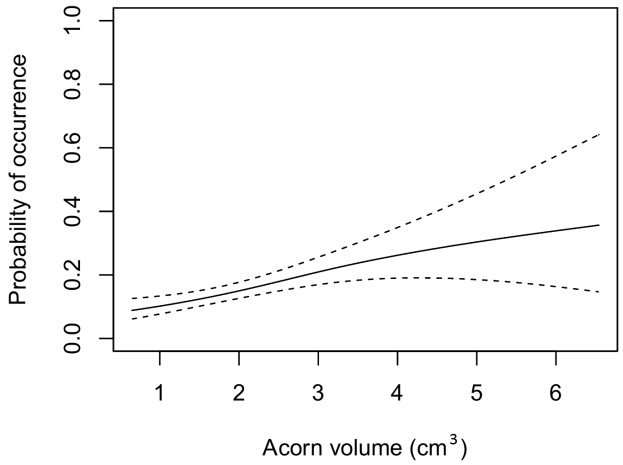

Fig. 1: The probability of occurrence of a *Temnothorax crassispinus* ant colony in relation to the volume of previously insect-bored native oak acorns. The solid curve is based on cubic splines and the dashed lines represent ± 1 standard error.

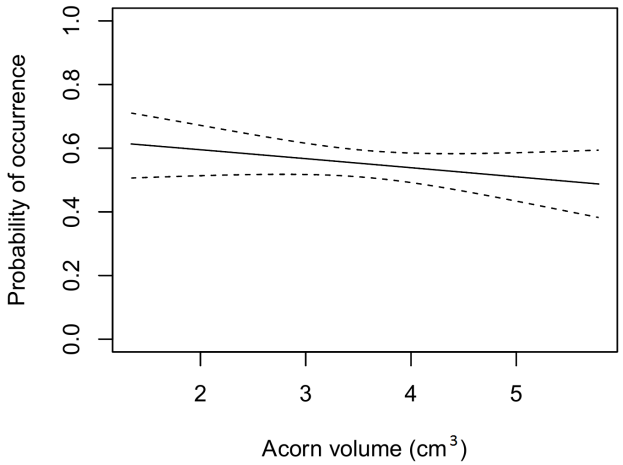

Fig. 2: The probability of occurrence of a *Temnothorax crassispinus* ant colony in relation to the volume of previously insect-bored Northern Red Oak acorns. The solid curve is based on cubic splines and the dashed lines represent ± 1 standard error.

Tab. 5: Factors affecting the number of *Temnothorax crassispinus* colonies in sticks in forest plots. Statistically significant value is emboldened. No.stick - number of sticks. For explanations of other variable codes: see Table 2.

Factors	Estimate	SE	Z	P
No.stick	**0.181**	**0.077**	**2.328**	**0.020**
Per.undergrowth	-0.039	0.041	0.954	0.340
Dbh.tree	-0.004	0.004	0.818	0.413
Oak.species	0.466	0.571	0.806	0.420
Per.open.area	0.015	0.020	0.728	0.467
Per.understory	0.009	0.013	0.724	0.469
Dist.oak	-0.040	0.056	0.716	0.474
Dist.anoth.oak	-0.010	0.015	0.660	0.510

Tab. 6: Mean, standard error and 95% confidence intervals (95% CI) of numbers of *Temnothorax crassispinus* workers and larvae in different colonies. SE = standard error.

Nest type	Workers		Larvae	
	Mean (SE)	95% CI	Mean (SE)	95% CI
Northern Red Oak acorns (n = 77)	125.60 (7.59)	110.49 - 140.71	139.57 (9.43)	120.79 - 158.35
Native oak species acorns (n = 33)	84.42 (9.26)	65.57 - 103.28	91.15 (11.64)	67.45 - 114.86
Sticks under Northern Red Oak canopies (n = 7)	43.43 (12.17)	13.66 - 73.20	58.43 (10.19)	33.49 - 83.37
Sticks under native oak species canopies (n = 10)	89.20 (17.96)	48.57 - 129.83	88.10 (22.64)	36.88 - 139.32

We also tested the availability of uncolonised acorn cavities. There were significantly fewer under Northern Red Oak canopies 61 acorns (Mean = 2.18 , SE = 0.462) than under canopies of native oak species 202 (Mean = 3.38, SE = 0.577) (Wald $\chi^2 = 12.74$, $p < 0.001$).

The binomial test in the first experiment showed that significantly more ($p = 0.008$) ant colonies chose nests in Northern Red Oak acorns (73%, n = 27) than in Pedunculate Oak acorns (27%, n = 10). In the second experiment we did not find significant differences ($p = 0.362$) in the choice of artificial nests based on fragments of cotyledons of Northern Red Oak (40% n = 12) or Pedunculate Oak (60%, n = 18). Northern Red Oak acorns had a thicker pericarp (Mean = 1.15 mm, SE = 0.03) than Pedunculate Oak acorns (Mean = 0.47 mm, SE = 0.02; t = 17.89, df = 18, $p < 0.001$).

Discussion

The occurrence in the environment of invasive Northern Red Oak positively affects the occurrence and colony condition (defined as the number of workers and larvae) of *Temnothorax crassispinus* ants. This is contrary to the most frequently described interactions between native fauna and invasive

plant species (see e.g., Shea & Chesson 2002, Roques & al. 2006, Branco & al. 2015). However, our described situation is not an herbivory interaction, or simple influence of plant cover, but a secondary interaction dependent on utilizing previously insect-infested acorns. This interaction has become possible only after the host range expansion by native insects whose larvae feed on cotyledons within developing acorns of Northern Red Oak (Myczko & al. 2017). The appearance of such interactions indicates the inclusion of this non-native species in the network of dependencies in the ecosystem and its "naturalization". Our results show that acorns of Northern Red Oak are significantly preferred as a colony cavity. Both in the forest and in the first laboratory experiment, colonies had access to whole acorns. The lack of preference for oak species in the second experiment where cotyledons were the only plant tissue provided suggests the ability of *T. crassispinus* ants to assess the durability of acorns. Acorns of native Pedunculate Oak had significantly thinner pericarps compared to Northern Red Oak acorns. Furthermore, Łuczaj & al. (2014) reported a significantly lower proportion of seed material within Northern Red Oak acorns indicating a greater share of pericarp tissue in the total weight of the acorn.

In addition to the robustness of the acorn for *Temnothorax crassispinus* ants, cavity volume also seems to be important. Mitrus (2015) has shown that ants prefer larger artificial nests. However, the volumes tested (up to 1.76 cm^3) were smaller than the volume of most insect-infested acorns found in our study plots. In the current study, a positive relationship between acorn occupation and acorn volume occurred only in native oaks (Fig. 1). The increased occurrence of ant colonies in larger native acorns may be associated with an increase in "solidity" in larger acorns. However, in the current study we did not collect data on pericarp thickness of all acorns to confirm this statement. In contrast, Northern Red Oak acorn volume did not significantly influence occupation by *T. crassispinus* ants (Fig. 2). This suggests that a factor other than the simple volume difference affects the decision on occupation of an acorn.

We found two key factors affecting the number of ant colonies on our plots, irrespective of whether total number of colonies, number of acorn colonies or number of queen-right acorn colonies was considered. These were the oak species and the number of acorns with holes. Numbers of all types of colony were higher in Northern Red Oak plots. Northern Red Oak acorns were also characterized by significantly higher numbers of both workers and larvae in colonies (proxy for colony condition). This suggests better food resources under Northern Red Oak canopies. This finding is consistent with McCary & al. (2016) who showed increased abundance of secondary consumers in woodland brown food webs based on invasive plants. Also Kjar & Park (2016) showed that the ant abundance and species richness can be positively associated with alien plant cover. The second factor significantly affecting the number of all types of ant colony was the number of acorns previously predated by insects. This factor indicates the ability of colonies to more frequently change nest location which should allow avoidance of social parasites, for which the *Temnothorax* ants are particularly vulnerable (Brandt & Foitzik 2004, Foitzik & al. 2004). However, it should be kept in mind that under Northern Red Oak canopies there were significantly fewer potential acorn cavities than under native oaks. This suggests two possibilities or a combination of them; firstly that the better quality of Northern Red Oak acorns compensated for their smaller number or, secondly, that compensation was via a higher quality of habitat under Red Oak canopies in comparison to habitat under native Oak species.

The colonies located in acorns coexisted on the same plots with colonies located in sticks previously bored by insects. If we analyze only the differences between types of nest choice, the ant colonies located in sticks under Northern Red Oak canopies contained fewer workers and fewer larvae (proxy for colony condition) than colonies in acorns of Northern Red Oak, but these numbers were comparable to those in native oak acorns. Additionally, only the number of sticks significantly explained the number of ant colonies in sticks. This type of cavity may by a refuge for local ant colonies during subsequent non-mast years when acorns may be scarce. The differences in the quality of colonies between Northern Red Oak acorns and native oak acorns should influence sex ratio decisions during production of sexual specimens. Strätz & Heinze (2004) showed that well-supplied *Temnothorax crassispinus* colonies reared more numerous female sexuals in comparison to weaker colonies which preferably invested in the production of male sexual specimens. This means we can anticipate differences between sex ratio decisions from colonies living under Northern Red Oak canopies and those under native oak canopies, but this question needs future investigation.

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Reconstructing the relatedness of cooperatively breeding queens in the Panamanian leaf-cutting ant *Acromyrmex echinatior* (Hymenoptera: Formicidae)

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Abstract

The evolution of permanent inquiline social parasites in ants has been conjectured to be facilitated by secondary polygyny, that is, the re-adoption of new queens into existing mature colonies. This idea was first formulated by Wasmann, Wheeler, and Emery more than a century ago. Emery predicted that inquilines should be the sister-lineages of their hosts, which prompted Alfred Buschinger to propose that they evolve by sympatric speciation. However, these scenarios hinge on two vital conditions that have not been quantitatively documented: 1. That host sister species are secondarily polygynous and primarily recruit close kin, and 2. That such adoptions are prone to occasional mistakes that would select for the condition-dependent expression of exploitative traits and reproductive isolation by disruptive selection. Here, we use a long-term data set on the leaf-cutting ant *Acromyrmex echinatior* (Forel, 1899), known to have a closely related inquiline social parasite *A. insinuator* Schultz, Bekkevold & Boomsma, 1998, to address the first of these conditions. We estimate the frequency of secondary polygyny and the degree to which cooperatively breeding queens are related. We find that the overall frequency of polygynous colonies is ca. 8% and that polygynous colonies typically have two queens. Most queen pairs are first-degree relatives, consistent with colonies adopting one or two daughters either before or just after becoming orphaned. However, we also document a few pairs of cooperatively breeding queens that are unrelated and estimate that this social structure may apply to ca. 20% of the polygynous colonies, and thus ca. 1% of all colonies. Our findings show that the breeding system of *A. echinatior* matches the polygyny characteristics that are believed to facilitate the emergence of socially parasitic queen morphs.

Key words: Polygyny, social parasitism, inquilines, leaf-cutting ants.

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Introduction

The evolutionary origin of inquiline ant social parasites ranks among the best documented cases of adaptive (sympatric) speciation (Boomsma & Nash 2014). The rationale is that such inquilines, which have convergently evolved in many ant genera (Huang & Dornhaus 2008), emanate from disruptive selection on alternative queen morphs that coexist in the same colonies (Emery 1909, Heinze & Keller 2000, Savolainen & Vepsäläinen 2003, Buschinger 2009, Rabeling & Bacci 2010, Boomsma & al. 2014). This implies that cooperative breeding of multiple queens in the same perennial colony is a prerequisite for the evolution of permanent inquilines. Lasting polygyny is essentially absent in most social insect lineages with permanent caste differentiation (corbiculate bees, vespine wasps, and higher termites) but common in the ants (Boomsma & al. 2014), consistent with permanent inquilinism appearing to be restricted to the ants. Cooperative breeding of multiple ant queens in the same colony is almost always a consequence of monogynous colonies re-adopting offspring queens back into the colony after they have been inseminated by unrelated males just outside their natal nest (Hölldobler & Wilson 1977, Elmes & Keller 1993, Boomsma & al. 2014). In such cases, kinship ties via the maternal line likely explain how cooperative queen breeding can be evolutionarily stable (Hölldobler & Wilson 1977, Nonacs 1988, Keller 1993, 1995, Boomsma & al. 2014). Selection for polygynous cooperative breeding

would be stronger when the alternative strategy, dispersal and de novo solitary colony founding, carry high mortality costs and are thus unlikely to be successful (Bourke & Heinze 1994). However, these dispersal constraints are counterbalanced by increasing sib-competition (West & al. 2002, Boomsma & al. 2014), so it is impossible for natural selection to remove all tendencies to disperse (Hamilton & May 1977).

It has been proposed that ant polygyny arises from exclusive single-queen ancestry (Boomsma 2007, Hughes & al. 2008) when some newly-inseminated queens occasionally succeed in re-entering their natal colonies. Any traits that favour this form of social promiscuity are also expected to select for some queens entering unrelated colonies, where they may be admitted in spite of the absence of maternal kinship (Boomsma & al. 2014). This produces a classic dichotomy between vertical transmission selecting for loyalty to kin and horizontal transmission selecting for parasitic exploitation of unrelated conspecifics (May & Anderson 1979, Frank 1998). Disruptive selection is likely to accumulate behavioural and morphological adaptations for a more efficient parasitic life-style in one of the queen morphs (Rabeling & Bacci 2010, Boomsma & Nash 2014, Rabeling & al. 2014). These adaptations normally include smaller body size, cryptic nest-intruding behaviours, cuticular chemistry that precludes easy recognition by non-kin. Ultimately, assortative mating could then arise and gradually sever gene-flow, so that inquiline populations become irreversibly reproductively isolated (Buschinger 2009, Rabeling & Bacci 2010). Over the years, substantial genetic evidence for adaptive sympatric origins of ant inquilines from their directly ancestral host lineages has accumulated (Huang & Dornhaus 2008, Buschinger 2009), and some recent studies have reconstructed this process in considerable detail (Savolainen & Vepsäläinen 2003, Rabeling & al. 2014, Schär & Nash 2014).

An often-implicit assumption for sympatric speciation of ant inquilines is that evolutionary derived, polygynous species are more vulnerable to the emergence of inquiline social parasites over evolutionary time than monogynous sister lineages. This is because regular cooperative breeding of queens may lead to worker-controlled reproductive skew based on queen age (Ozan & al. 2013), i.e., differential contributions to the colony's brood of reproductives rather than sterile workers (Heinze & Keller 2000, Boomsma & al. 2014). However, no permanent inquiline species are known to have evolved in the polygynous *Formica fusca* Linnaeus, 1758 (*Serviformica*) ants studied by Ozan & al. (2013), in contrast to other genera such as *Myrmica* and *Acromyrmex* in which such social parasites have originated multiple times.

The secondary nature of polygyny is important because forms of primary polygyny (pleometrosis), i.e., cooperative colony foundation, are also relatively common in ants, and have led to other forms of social parasitism involving aggressive colony usurpation. This type of social parasitism has evolved in all lineages of social Hymenoptera, both basal ones with merely behavioural reproductive division of labour, and evolutionary derived ones in which all colony members belong to a single morphologically differentiated caste phenotype for life (Boomsma & Gawne 2018). Early students of inquiline and slave-making ants did not always clearly separate between usurpation of incipient colonies by competing foundress queens and parasitism by dulosis (slave-making), but appear to have agreed that parasitism is derived from intraspecific queen adoption (Emery 1909, Wasmann 1909, Wheeler 1910). Their main conceptual struggle was to explain dulosis due to the many secondary host switches in this type of social parasitism. However, while secondary polygyny might be the logical precursor state for the sympatric evolution of permanent inquilines (cf. Boomsma & Nash, 2014), there is no reason to predict that aggressive usurpation during colony founding should necessarily evolve directly from within host populations, i.e., according to the strict version of what came to be known as Emery's rule (Emery 1909, Huang & Dornhaus 2008, Lopez-Osorio & al. 2011).

The conditions that might allow the emergence of early (intraspecific) inquiline traits and the final adaptations of established inquiline species have been reasonably well studied (Heinze & Keller 2000, Huang & Dornhaus 2008, Buschinger 2009, Boomsma & al. 2014, Rabeling & al. 2014), but evidence for the intermediate steps in this process remains rare. The most critical transition appears to be the switch from the routine adoption of newly inseminated colony daughters to the derived establishment of a parasite lineage that relies completely on the exploitation of non-related colonies. In the present study we provide to our knowledge the first case study documenting secondary polygyny of a kind that is consistent with the putative necessary condition for making the transition to inquilinism in sympatry. Our study draws on almost 20 years of field surveys in Gamboa, Panama collecting hundreds of colonies of *Acromyrmex echinatior* (Forel, 1899), which is host to a rare sister-species inquiline *A. insinuator* Schultz, Bekkevold & Boomsma, 1998 (Schultz & al. 1998, Sumner & al. 2004a) and is known to have a low frequency of polygynous colonies (Bekkevold & al. 1999).

The leaf-cutting ant genus *Acromyrmex* is notorious for the convergent evolution of inquiline social parasites (Schultz & al. 1998, de Souza & al. 2007, Rabeling & al. 2015), in spite of monogyny remaining the default colony structure. This contrasts with many other ant lineages (e.g., *Temnothorax*, *Myrmica*, *Crematogaster*) in which polygynous colonies have become the norm. When inquiline queens enter colonies that already have multiple host queens, overall relatedness among workers is low and kin-recognition efficiency possibly compromised (Heinze & Keller 2000, Foitzik & Heinze 2001). *Acromyrmex* leaf-cutting ants thus provide an interesting system to evaluate whether and to what extent colonies preferentially accept close kin as new reproductives, and whether occasional exceptions can also be found. We use multiple types of genetic marker analyses to reconstruct the most likely types of facultative polygyny that occur in *A. echinatior*. The present study greatly expands a previous one by Bekkevold & al. (1999) which documented facultative polygyny in *A. echinatior* for the first time.

Material and methods

Sampling of polygynous colonies: During 15 field trips to Gamboa, Panama in the period 1993 - 2010, 197 colonies of *Acromyrmex echinatior* (identified according to Schultz, Bekkevold & Boomsma, 1998) were completely excavated so that at least one queen could be collected. For the purpose of the present study, we screened the field records for colonies in which more than a single potential mother-queen, characterized by a relatively dull and dark

Tab. 1: Details for seventeen colonies of *Acromyrmex echinatior* (Ae) and two of *A. octospinosus* (Ao), which either had two or more observed queens that were mostly but not always available for genetic analysis, or produced workers whose genotypes we could only explain by deducing more than a single mother queen. For colonies where the genotypes of two queens were known, we highlighted the likelihood of them being related by: 1) listing the number of marker-loci for which they shared alleles (out of the maximum of four loci genotyped; 3 in one case), 2) estimating the pairwise relatedness ± SE via jackknifing over all loci (Goodnight & Queller 1999), and 3) providing the relationship with the highest likelihood in a formal likelihood-analysis (Konovalov 2004). For some colonies, additional queens were observed once the colonies were established in the laboratory. Relatedness and formal likelihood analyses require population-wide background allele frequencies, which were not available for *A. octospinosus* (*). Some deduced offspring numbers are based on published results ([1] Sumner & al. 2004b; [2] Dijkstra & Boomsma 2007).

Colony	Queen number			Number of offspring genotyped	Number of loci with shared alleles	Queen-queen Relatedness coefficient (SE)	Maximum likelihood relationship
	Observed in field (lab)	Available for genetic analysis	Deduced from offspring genotypes				
Ae012	2	2	2	66	4/4	0.26 (0.07)	mother-daughter
Ae020	2	2	2	151	3/4	-0.08 (0.35)	half-sisters
Ae022	4	0	-	0	-	-	-
Ae028	0 (1)	1	2	40	4/4	0.57 (0.22)	full-sisters
Ae043	2	0	1	32	-	-	-
Ae047	2	2	2	145	4/4	0.15 (0.13)	mother-daughter
Ae109	2	1	2	79	2/3	0.23 (0.25)	half-sisters
Ae134	2	0	-	0	-	-	-
Ae141	2	0	1[1]	0	-	-	-
Ae144	1 (3)	3	2[2]	0	4/4	0.23 (0.17)	mother-daughter
Ae154	1 (2)	0	2	47	4/4	0.73 (0.13)	mother-daughter
Ae168	2	0	1	32	-	-	-
Ae263	2	0	-	0	-	-	-
Ae266	1	0	2	20	4/4	0.52 (0.26)	full-sisters
Ae394	2	2	2	40	3/4	0.09 (0.31)	unrelated
Ae406	2	2	2	63	3/4	-0.07 (0.32)	unrelated
Ae480	2	2	2	40	4/4	0.34 (0.14)	mother-daughter
Ao044	3	0	1	44	-	-*	-*
Ao273	1 (2)	0	2	29	2/4	-*	-*

cuticle and acquiescent behaviour, had been observed. In 13 of the colonies two mother-queens were recorded during collection, and in two further colonies two or three potential mother-queens were discovered after the colonies had been set up in the lab (for details see Tab. 1 & Appendix S1, available as digital supplementary material to this article, at the journal's web pages). Of these 15 observationally polygynous colonies, we genotyped all queens in seven cases and one of two queens in an eighth colony, using four highly polymorphic microsatellite loci *Ech1390*, *Ech3385*, *Ech4126*, and *Ech4225* (Ortius-Lechner & al. 2000). A single colony (Ae144) contained a third queen, which was homozygous at all loci, so we inferred she was a gynandromorph, which are known to occur in *Acromyrmex* leaf-cutting ants (Wheeler, 1931), and we omitted her from all further analyses. We also genotyped workers and in some cases males and/or gynes in 12 of the observationally polygynous colonies (for the remaining colonies no offspring samples had been kept; see Tab. 1 and Appendix S1 for details).

Detecting polygyny through offspring genotypes: For other projects (Appendix S2), we genotyped 27 colonies with one observed queen each and, in addition to the 197 queenright colonies already mentioned, another 6 colonies that had been collected without a queen. Among the 33 observationally not polygynous queens with genotyped offspring, we found two colonies (Ae028, Ae266) with genotypes that could not be explained by a single multiply mated mother queen (Bekkevold & al. 1999), suggesting that these colonies had contained another fecund mother-queen as well (Tab. 1). We realize that the detection of

second queens based on offspring genotypes is prone to false positives when these genotypes could represent unrelated stray workers from neighbouring colonies. Stray workers would seem most likely for samples from lab colonies because nest boxes may be close to neighbouring colonies on the same shelf. However, any such stray workers would be rare and unlikely to be related to each other, and their mothers should be unrelated to the queen(s) of the colony they were found in. These expectations were not met in the two colonies that had some workers whose genotypes could not be explained by a single queen despite there being no observation of a second queen (Ae028, Ae266). In both colonies, we found many offspring workers were incompatible with a single queen (18 / 40 workers in Ae028, 7 / 15 males and 5 / 20 gynes in Ae266), and in both cases, these workers were related among each other, suggesting they were likely offspring of the same mother who was in fact related to the single queen found in the colony. This would be an extremely unlikely coincidence for occasional stray workers and an impossible match for the sexuals, hence we explained these two cases as being consistent with a cryptic second mother queen.

Because the genotyped individuals of colonies Ae028 and Ae266 were collected well after excavation in the field, it is possible that these second queens were adopted after the colonies had become established in the laboratory (Tab. 1). Winged gynes and males sometimes start mating flights in the laboratory rearing rooms so we cannot exclude that some of these gynes can be adopted by their sister workers after insemination (Nehring & al. 2015). However, our laboratory note-books indicate that only colony Ae266 had produced gynes in previous years in the laboratory, so the alternative explanation that a second queen was present in the field colony but missed upon collection seems more likely.

After dismissing that these two additional cases of polygyny were false positives, we concentrated our further analyses on estimating the possible non-detection error, i.e., the likelihood of having obtained false negatives because additional queens were overlooked. Such polygynous colonies that were assessed as monogynous are most likely when we had to base our estimate of queen number on offspring genotypes only, or when we observed two queens but the offspring genotypes only supported one mother (Ae043, Ae141, Ae168). Support for a second queen producing offspring is only obtainable when there is at least one marker locus for which the total set of offspring genotypes has at least three different maternal alleles, after adjusting for multiple paternity because *Acromyrmex echinatior* queens are always inseminated by multiple males (see below). Colonies for which we only had offspring genotypes are therefore challenging even with highly variable co-dominant marker loci, particularly when the two putative mother-queens are full-sisters related by 75%.

To approximately quantify the likelihood of non-detection errors, we simulated 1000 pairs each of full-sister queens, half-sister queens, mother and daughter, and unrelated queens, using the Kingroup software (Konovalov 2004), based on the observed population-wide allele frequencies that were deduced from the genotypes of 24 queens from monogynous colonies that had been genotyped over the years (Appendix S2). We then counted how many of the queen-pairs had a maximum of two alleles in common for each of the four marker loci, so their proportional presence could be used to adjust our overall likelihood estimates of the three possible scenarios of positive queen relatedness (full-sisters, half-sisters, mother and daughter) against a null hypothesis of no relatedness. We did not evaluate non-sampling error (Boomsma & Ratnieks 1996) i.e., the probability of not detecting a second queen due to low offspring sample size, because the number of offspring genotyped was at least 32 in colonies deduced to be monogynous (Tab. 1).

Inferring genetic colony structures: Genotypes of queens that had not been directly genotyped were deduced from worker offspring both manually and by using the program COLONY (Jones & Wang 2009), the latter to check our manual inferences and to quantitatively assign maternity because COLONY allows for likelihood-based deduction of ambiguous alleles before assigning parentage. The results obtained were generally congruent across methods, with only a few exceptions. In two colonies for which we could document that two queens had contributed to the worker castes, we also found some offspring among the laboratory samples that could not be daughters of either of these queens and inferred they must have been rare escape workers from neighbouring colonies, i.e., false positives erroneously suggesting more than two queens (colony Ae020: 6 / 151; Ae047: 1 / 145). To reduce the likelihood of mistakenly diagnosing colonies as being polygynous, we always analysed maternity shares and included only workers with likelihoods of correct maternity assignment of at least 90% into the COLONY analyses. This also allowed us to evaluate the significance of reproductive skew with a binomial test. Resampling procedures suggested that, depending on the number of offspring workers genotyped (n), we would detect reproductive skew > 80% (for n = 30), > 74% (n = 60), > 68% (n = 90) and > 67% (n = 120). Finally, we analysed inbreeding (F_{IS}) as the proportion of observed heterozygous workers and gynes relative to the expected heterozygosity based on each colony's allele frequencies across the four marker loci. There is some evidence that queens of *Acromyrmex insinuator* do occasionally mate with brothers (Bekkevold & Boomsma 2000), so checking whether the host might have such tendencies is relevant.

Reconstructing queen relatedness: We analysed genetic relatedness (R) among queens with the program Relatedness (Queller & Goodnight 1989, Goodnight & Queller 1999) against the background of the population's allele frequencies (see above), focusing on the estimation of relatedness between pairs of queens in the same colony. We derived standard errors for relatedness by jackknifing over loci for the estimates of individual queen pairs, and by jackknifing across queen-pairs for the estimation of the average relatedness between cooperatively breeding queens. Based on the 1,000 simulated queen pairs (see above) of full-sisters, half-sisters, mother and daughter, and unrelated individuals, we inferred how much the values of R overlapped between these four hypothetical queen-relatedness scenarios to estimate the power of our R-estimates in discriminating among the specific scenarios of queen relatedness.

The power of relatedness estimates for discriminating between different kinship scenarios can vary widely and maximum likelihood approaches have proven to be the most appropriate tools to achieve inferences of this kind without bias (Arévalo & al. 1998, Blouin 2003). We therefore used Kingroup (Konovalov 2004) for a direct analysis of the likelihood that two cooperatively breeding queens were unrelated, a mother and daughter, half-sisters, or full-sisters, based on the observed allele frequencies in the genotypes of

Tab. 2: Estimates of the prevalence of functional polygyny among the 203 analysed colonies of *Acromyrmex echinatior* collected in Gamboa, Panama (158 collected with at least one queen but not genotyped; 39 collected with one or more queens and genotyped; 6 collected without a queen and genotyped). Estimates are ranked from top to bottom starting with the most reliable estimation method (only counting colonies with both observational and molecular evidence for polygyny) and stepwise including less rigorous evidence.

Estimate	Number	% of total	Cumulative number	Cumulative %
Total colonies with and without queen observation	203			
Colonies in which multiple queens were both observed and confirmed by microsatellite analysis	9	4.4%	9	4.4%
Colonies in which only a single queen was observed, but where genetic marker data provided evidence for a 2nd queen	2	1.0%	11	5.4%
Adjustment for the previous row, based on the distribution of false-negatives in queen pairs of different theoretical relatedness	0.4	0.2%	11.4	5.6%
Adjustment for the likelihood (7.31%) of not detecting a second queen in the monogynous colonies where neither queens nor workers were genotyped	11.3	5.6%	22.7	11.2%
Colonies in which a second queen was observed, but without any genetic data being available to validate multiple maternity	3	1.6%	25.7	12.7%

the queen-pairs. For each of the four alternative hypotheses, we also calculated the overall likelihood that all queen-pairs in our sample represented the same kinship scenario, by multiplying the likelihoods of all individual queen pairs. Some queen-pairs had at least one locus where they did not share at least one allele, which excluded the possibility of a full-sisters or mother-daughter relationship, so we inevitably obtained an overall likelihood of zero for these two specific scenarios. We resolved this by excluding these zero cases and re-calculating the overall relative likelihoods of being sister or mother-daughter pairs for all other queen-pairs.

To generate population-wide estimates, we also calculated likelihoods of the sister-queen scenarios at a finer scale, to account for the fact that *Acromyrmex echinatior* is polyandrous and a colony's offspring are thus a mix of full-sisters and half-sisters. If cooperatively breeding queens were randomly drawn sisters from a colony's offspring, the expected relatedness would thus be expected to lie somewhere between full-sister (R = 0.75) and half-sisters (R = 0.25).

Results

Relative abundance of polygynous colonies: We found more than a single queen upon collection in 13 of the 197 queenright *Acromyrmex echinatior* colonies collected in Gamboa, Panama (Ae012, Ae020, Ae022, Ae043, Ae047, Ae109, Ae134, Ae141, Ae168, Ae263, Ae394, Ae406, Ae480). For ten of these colonies we had offspring genotypes, which supported two fertile queens in seven but not in the three remaining colonies (Ae043, Ae141, and Ae168; Sumner & al. 2004b), in which all workers could be explained as offspring of a single queen (Tab. 1). We further found a second queen in two colonies after they had been established in the laboratory (Ae144 and Ae154). Molecular offspring data confirmed maternity by at least two fecund queens in both colonies (Tab. 1; Dijkstra & Boomsma 2007). The second queen could have been present in the field colony but have been overlooked upon collection, because collectors might not have scrutinized all fungus gardens by thorough fragmentation after the colony was confirmed to have a queen. Later queen discoveries also happened sometimes when a colony was registered as queenless in the field but later turned out to have a queen as established lab colony (Dijkstra & Boomsma 2008). Estimates of the frequency of polygyny purely from field observations about observed queen numbers are thus likely to underestimate the true number of polygynous colonies. Our subsample of 12 colonies with observed polygyny and genotyped offspring produced nine positively confirmed cases of functional polygyny and three ambiguous cases, yielding a minimal population-wide prevalence of polygyny of 9/197 = 4.6%.

As mentioned in the Materials and methods section, two further polygynous colonies (Ae028 and Ae266) were detected by offspring genotyping (Tab. 2; Appendix S2). If we assume that our sample of 33 genotyped but observationally non-polygynous colonies across the years is representative of all field colonies and that both colonies Ae028 and Ae266 were functionally polygynous in the field, this would imply that only counting queens upon field excavation would underestimate the prevalence of polygyny by 2 / 33 = 6.1%.

An additional risk of obtaining false negatives arises when genetic markers are not variable enough to discover a second queen, in particular when queens are related. In our simulations, 556 of the 1000 full-sister pairs, 926 of the 1000 half-sister pairs, 842 of the 1000 mother-daughter pairs, and 975 of the 1000 unrelated queen-pairs had between them at least three different alleles for at least one of the four marker loci. This implies that 43% of the full-sister queen pairs would have all alleles in common, so they would be scored as a single queen when only offspring genotypes are available. In contrast, our set of genetic markers would only have missed 2.5% of any unrelated queen pairs, 7.4% of the half-sister queen-pairs, and 15.8% of the mother-daughter queen-pairs.

Relatedness estimates of queen pairs: To assess whether second queens are kin or whether colonies adopt strangers, we directly estimated the relatedness among queen pairs. In eleven polygynous *Acromyrmex echinatior* colonies we could either genotype all queens (Ae144), or infer the genotypes of two queens from offspring (Ae28, Ae109, Ae154,

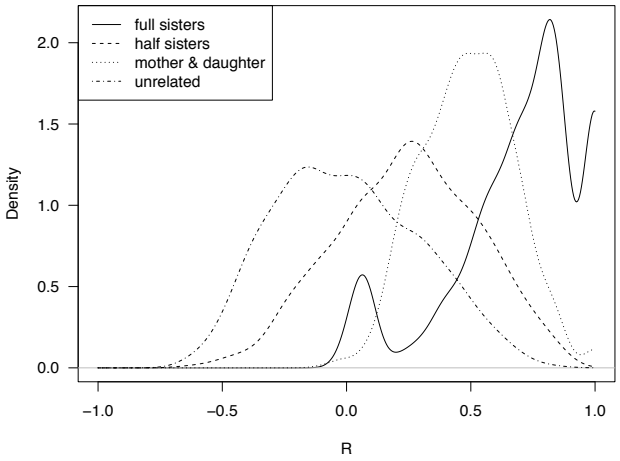

Fig. 1: Density functions illustrating the relatedness (R) values obtained in 2000 simulations of sampling full-sisters, half-sisters, mother-daughter combinations, and unrelated (random) queen-pairs, based on the observed population-wide allele frequencies for the four marker loci used. The peaks of the first three curves coincide with the expected values of 0, 0.25 and 0.5, but the full-sister curve is more erratic, consistent with the available diversity at marker loci not being quite high enough to resolve these cases. The upper and lower values of each of the curves further illustrate that there remains a low, but non-zero, likelihood of full-sister pairs producing a relatedness estimate of zero by chance and of mother-daughter pairs producing a relatedness estimate of one by chance.

Ae266), or do both (Ae012, Ae020, Ae047, Ae394, Ae406, Ae480). In seven of these cases (Ae012, Ae028, Ae047, Ae144, Ae154, Ae266, Ae480) the two queens shared at least one allele for each marker locus (Tab. 1). This implies they could be either mother-daughter or full-sister pairs. We could exclude such close relatedness for the queen pairs in the other four colonies (Ae020, Ae109, Ae394, Ae406), which must therefore have been half-sisters or unrelated.

The cross-colony average coefficient of relatedness between queen-pairs was R = 0.29 (SE = 0.09), which is significantly greater than zero (one-sample t = 3.42, n = 11, two-tailed $p < 0.01$). The pairwise relatednesses across these eleven queen-pairs ranged from -0.08 to 0.73, spanning the entire range from unrelated (R = 0) to full-sister relatedness (0.75; Tab. 1). Our simulation of queen pairs showed that the four alternative kinship scenarios are difficult to disentangle based on pairwise relatedness estimates alone, because R can vary substantially within each kinship scenario so that estimates of alternative scenarios overlap (Fig. 1). However, unrelated queens had a median R = -0.01 (interquartile range - 0.22 - 0.22), half-sister queens a median R = 0.25 (0.05 - 0.45), full-sister queens a median R = 0.76 (0.57 - 0.86), and mother-daughter queens a median R = 0.49 (0.36 - 0.62), suggesting that our simulation approach produced unbiased results with correct averages.

All relationships mentioned above are possible in principle because most colonies in the analysis were large enough to have produced gyne offspring before we observed the second queen or took the offspring samples from which we deduced the presence of a second queen. For nine of the 17 suspected or confirmed polygynous colonies we had directly observed sexual offspring from the field (Appendix S1). In two of the remaining colonies (Ae043, Ae047), we observed two queens during the field collections and both of these colonies were large enough at the time of collection to have produced winged reproductives in earlier years (i.e., having > 1 L fungus garden volume; Bekkevold & Boomsma 2000). Four out of the 17 colonies did not contain alates (winged reproductives) and may have been too small to produce any (Ae012, Ae020, Ae141, Ae266). In these cases the additional queens may either have come from outside (unlikely given the relatedness estimates) or they may have been adopted years before we collected the colony, if colony size would have been declining since.

Tab. 3: The majority contribution of one of the paired queens to the offspring workers and gynes in polygynous *Acromyrmex echinatior* colonies based on genotypes for four microsatellite loci (see Tab. 1). The percentage of offspring produced by the focal queen, the total number of offspring analysed (n), and the p-values from binomial tests of whether deviation from 50 / 50 was significant are given. We also tested whether skew in gyne production differed from skew in worker production, using χ^2 tests (final column). Offspring that could not be unambiguously attributed to either of the two queens were omitted. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$.

Colony	Workers			Gynes			Worker skew vs. gyne skew
	Focal queen	n	p	Focal queen	n	p	p
Ae012	55%	29	0.71	48%	25	0.99	0.80
Ae020	50%	109	0.99	33%	36	0.07	0.13
Ae028	52%	40	0.87	-	-	-	-
Ae047	64%	141	0.001***	-	-	-	-
Ae109	58%	69	0.23	10%	10	0.02*	0.01**
Ae154	60%	15	0.61	50%	32	0.99	0.74
Ae266	-	-	-	76%	21	0.03*	-
Ae394	57%	40	0.43	-	-	-	-
Ae406	56%	32	0.60	56%	32	0.60	0.99
Ae480	63%	40	0.15	-	-	-	-

Since relatednesses of paired queens could not be unequivocally derived from the overall estimates of R (see also Blouin 2003), we also used a likelihood approach to find the queen relationship that was most likely for each colony. This indicated that queen-pairs were most likely to be mothers and daughters (5 pairs) or sisters (4 pairs) (Tab. 1, Fig 2), but that two of the pairs were unlikely to be related at all. Finally, we reconstructed the best overall maternity matches across all colonies by combining the colony-specific likelihoods that we obtained. The assumption that all queen-pairs are sisters (either full-sisters or half-sisters) explained the observed genetic marker data very well, whereas the likelihoods of all queens being mother-daughter pairs or unrelated pairs were zero or very low (Fig. 3). The likelihood curve peaked at an average offspring relatedness value of R = 0.285, i.e., close to the expected value for queen-pairs being half-sisters (0.25) and suggesting that only ca. 7% of the queen pairs were in fact full-sisters.

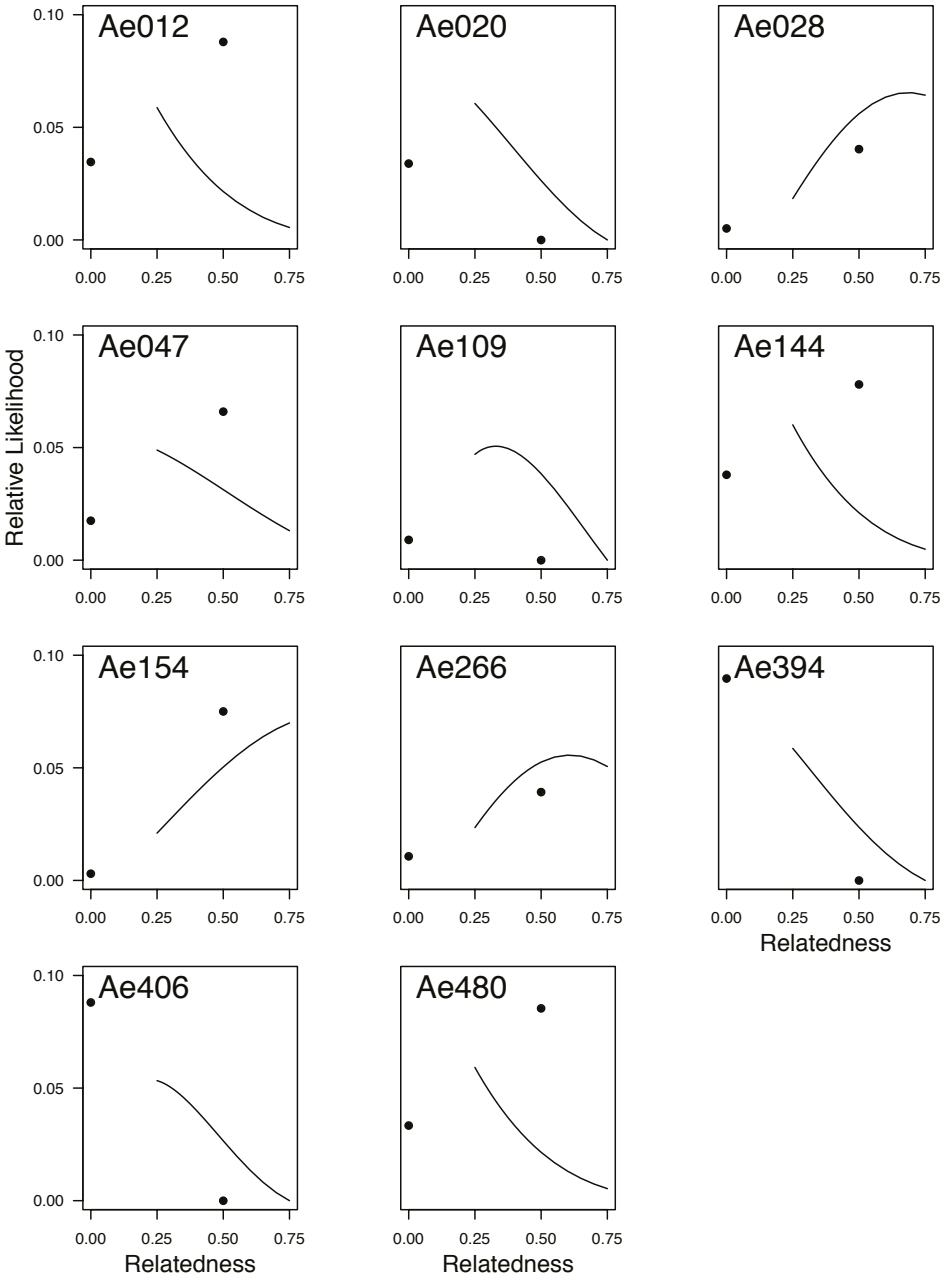

Fig. 2: Likelihoods of the four alternative types of relatedness relationship between paired queens for the 11 separate *Acromyrmex echinatior* queen pairs. Pairs could either be full-sisters or half-sisters (curves between R = 0.25 and R = 0.75), mother and daughter (black dot at R = 0.5), or unrelated (black dot at R = 0). See Table 1 for comparisons between these likelihood estimates and direct relatedness estimates.

As *Acromyrmex* queens are obligatorily polyandrous, daughters of the same queen are known to be either full-sisters or half-sisters. The likelihood of being full-sisters is then $R_p = 0.19$ (the inverse of the genetically effective paternity $R_e = 5.3$ (Nielsen & al. 2003, Sumner & al. 2004b). When queen pairs are assumed to be daughters of the same mother, an estimate of average relatedness of *Acromyrmex echinatior* queen pairs based on this likelihood comes out at R = 0.345 ($0.81 \times 0.25 + 0.19 \times 0.75$). This is somewhat higher than the R = 0.285 that we found for our dataset, which might be consistent with a few queen-pairs being in fact unrelated or with the analysis failing to recognize a significant number of the full-sister queen-pairs (see discussion of false-negatives for full-sister detection). In this population-wide estimate, the relative effect of less related or unrelated queen-pairs on the overall average is strong because even a single queen-pair not sharing at least one allele per locus will render the likelihood of the average queen-pair being full-sisters or mother and daughters zero, because such queen-pairs would always share alleles. When we excluded the queen-pairs that did not share alleles at all four marker loci, the maximum likelihood peaked at ca. $R_p = 0.15$, which is rather close to the expected value of $R_p = 0.19$. However, this analysis considered mother-daughter relationships to be the most prevalent kin structure among cooperatively breeding queens (Fig 3).

Possible effects of reproductive skew and sib-mating in queen pairs: Both queens contributed to the offspring in ten out of twelve colonies where offspring workers or gynes were genotyped. We tested for maternity skew in workers and gynes separately and found evidence for unequal contributions in three of the colonies (Tab. 3). We also tested for a difference in skew between worker and gyne

Fig. 3: Results of maximum likelihood analyses to evaluate the overall probability of all queen-pairs of *Acromyrmex echinatior* to be full-sisters, half-sisters, mother-daughter, or unrelated. **A.** The likelihood that either of the four relatedness scenarios between paired queens were universally true for all eleven pairs for which queen genotypes were available, calculated as the product of the likelihoods for individual queen pairs (see Fig. 2). The filled circles are the probabilities of all queen pairs being unrelated (at R = 0; Relative Likelihood RL = 0.00006) and being mother and daughter (R = 0; RL = 0 since some queen pairs could definitely not be mother and daughter). For sister scenarios, likelihoods were simulated for a range of combinations with varying probabilities of two queens being half-sisters or full-sisters (i.e., having relatedness of one or zero through the father R_p), which produced a dome-shaped likelihood curve of open circles each representing an individual simulation and gave a maximal RL of 0.176 when $R_p = 0.05$ and almost the same maximal RL of 0.173 for $R_p = 0.10$. These values are lower than those predicted from the earlier observed effective queen mating frequency of 5.3 (Sumner & al. 2004b) and suggest that ca. 20% of all queen-pairs would be expected to be full-sisters ($R_p = 0.19$; dotted vertical line). In these simulations, the likelihood of all queen-pairs being full-sisters (R = 0.75) was zero (RL = 0) because this scenario was definitely impossible for some queen-pairs. **B.** The same results plotted with a logarithmic vertical axis to illustrate that likelihood values differ only slightly towards the left of the dotted vertical line and to show that there is still a positive, but very low, likelihood of queens being unrelated, whereas the likelihood of all queen-pairs being full-sisters or mother-daughter combinations is truly zero, so they could not be plotted. **C**. A log likelihood plot similar to the B-panel, but only including the seven colonies where queen-pairs shared at least one allele at each of the four marker loci, so they could in theory be full-sisters or mother-daughter combinations, whereas being unrelated or half-sisters seemed unlikely but not impossible because shared alleles may also be due to chance. Here the likelihood curve (open circles) peaks closer to the expected value based on $R_p = 0.19$, and mother-daughter combinations became more likely while being unrelated remains possible but with very low likelihood.

production within the five colonies for which both worker and gyne genotypes were available (Tab. 3). In one of these (colony Ae109), one of the queens contributed relatively more gynes than workers, but only ten gynes could be unequivocally attributed to either of the queens. Overall, these results appear to imply that reproductive skew is relatively minor, consistent with queen-pairs not forming direct dominance hierarchies. This implies that reproductive skew does not provide a significant overall complication for estimating the overall prevalence of functional polygyny in *Acromyrmex echinatior*.

We found no indication for sib-mating because the colony-level inbreeding coefficients ranged from $F_{IS} = -0.27$ to $F_{IS} = 0.17$ (median -0.07). The only reasonable scenario

for sib-mating to occur would require that gynes staying in their maternal colony at least sometimes refrain from a mating flight and mate with a brother inside the colony. Estimates of worker inbreeding coefficients did not covary with queen-queen relatedness, which is consistent with all queens mating outside the nest (Appendix S3). Also a high number of genetically distinct reconstructed mates for each queen (median 9.5, ranging from 4 - 18) seems impossible to match with a sib-mating scenario.

Combining alternative estimates for the prevalence of polygyny: Reviewing our combined analyses it seems clear that our first estimate of 4.6% polygyny across the population and sampling years, based on colonies for which both observational and molecular data supported two queens, is likely to be an underestimate. Considering colonies for which only observational or molecular data are available, and taking the estimated false-negatives specific to our methods into account, makes it possible to adjust this estimate, although at some danger of overestimating the true percentage.

Our best possible adjustments are as follows. We add two colonies, for which evidence for polygyny came only from offspring genotypes and a second queen might thus have been overlooked (6.1% of the genotyped observationally not polygynous colonies; Table 2). To further adjust this estimate for the detection efficiencies specific to the four alternative queen-relatedness scenarios, we multiplied the observed queen-queen relatedness-prevalences with their specific likelihood of overlooking a second queen when only offspring molecular data are available. The sum of these products ($0.025 \times 0.18 + 0.074 \times 0.18 + 0.44 \times 0.18 + 0.16 \times 0.45$) then produces the likelihood of a polygynous colony having been overlooked based on molecular data. This estimate (17%) implies an overall detection efficiency of 83% (100% - 17%), which then produces a best estimate of $2 / 0.83 = 2.4$ polygynous colonies among the 33 genotyped colonies that were observationally monogynous, which would imply that 7.3% of all observationally monogynous colonies are in fact polygynous (Tab. 2). Applying this proportion to the remaining 155 observationally monogynous colonies that were not genotyped would add thus another 11.3 polygynous colonies, so that a revised overall prevalence estimate accounting for both, average molecular non-detection error and false-negatives owing to related queens, is 11.2%, i.e., an estimated 22.7 out of the 203 excavated colonies being polygynous.

Finally, there were three colonies (Ae022, Ae134, Ae263, see Tab. 1) in which two queens were observed but no molecular data were available. Adding these would increase the overall polygyny prevalence estimate for the Gamboa population of *Acromyrmex echinatior* to 12.7% (25.7 out of 203 colonies, Tab. 2), but some of these queens might not have had offspring among the workers. Overall, therefore, we inferred that our combined data suggest that the frequency of colonies with two cooperatively breeding queens was probably intermediate between the scenarios that we explored. To facilitate discussion in round figures we will use a polygyny prevalence of 8%, which, combined with the breakdown of types of polygyny (Tab. 1), implies that ca. 92% of the *A. echinatior* colonies in Gamboa, Panama were monogynous, 3.5% were polygynous with a mother-offspring pair of queens, another 3% with a sister pair of queens, leaving 1.5% being polygynous without any relatedness between the pair of queens.

Discussion

Our analysis encompassed a total of 203 excavated colonies of *Acromyrmex echinatior*, of which 45 colonies were genotyped with microsatellite markers. It confirms and adds considerable detail to an earlier study (Bekkevold & al. 1999) showing that polygyny in this leaf-cutting ant is a real but rather rare phenomenon (ca. 8% prevalence). It also became clear that colonies do not typically have more than two co-breeding queens, which are first degree relatives in the large majority of cases. This implies that workers may adopt a single reserve queen even though their mother queen is still fully functional, or two sister queens before or just after they become orphans. However, we also provide evidence that it is in fact possible that ca. 20% of the co-breeding queens are unrelated, suggesting that mistakes in the adoption of inseminated daughter queens can occur. To our knowledge this is the first study to provide robust estimates of relatedness structures among queens in an ant species where the prevalence of polygyny is low, which is difficult to achieve due to the large number of colonies that need to be sampled and genotyped.

Co-breeding queens in polygynous colonies are related: Our two independent types of analyses both showed that queen-pairs are likely to be first degree relatives, but emphasized different aspects. Direct relatedness analyses showed that queen-pairs heading polygynous colonies have an average relatedness of $R = 0.29$, suggesting mostly half-sister ($R = 0.25$) and occasional full-sister ($R = 0.75$) relationships, but not excluding mother-daughter ($R = 0.5$) combinations or occasional unrelated queen-pairs ($R = 0$). Alternative maximum likelihood analyses also identified a combination of full-sister and half-sister queen pairs as the most likely scenario for explaining the overall genetic marker patterns across colonies, but identified mother-daughter combinations as the most likely scenario after excluding colonies whose queens could not possibly be closely related.

In spite of these relatively consistent first-degree relative patterns, it is not straightforward how newly inseminated queens occasionally end up being adopted in their natal colonies. Mother queens from established colonies of the related *Acromyrmex subterraneus molestans* Santschi, 1925 have a high chance of being accepted into conspecific non-nestmate colonies, which is likely the reason for the substantial prevalence of polygyny in this species (Souza & al. 2005). Although it is not known to what extent these cooperatively breeding *A. subterraneus molestans* queens are related, it seems of interest that other subspecies of *A. subterraneus* are also polygynous (*A. subterraneus subterraneus* (Forel, 1893), see de Souza & al. 2004; *A. subterraneus brunneus* (Forel, 1912), see Delabie 1989) and harbour an inquiline social parasite, *A. ameliae* de Souza, Soares & Della Lucia, 2007 that convergently evolved many similar traits as the *A. insinuator* inquiline of *A. echinatior* in Panama (de Souza & al. 2007).

Rare field observations during the 15 years of field collections in the period 1993 - 2010 reported here have suggested that virgin reproductives of *Acromyrmex echinatior* aggregate and mate at landmarks such as big trees, and that inseminated queens may fly away from these trees before shedding their wings and excavating a shallow cavity to deposit the fungus-garden fragment that they transported in their infrabuccal pocket (Weber 1972, Fernández-Marín & al. 2004, Poulsen & al. 2009). As long-distance dispersal

seems incompatible with finding the natal nest, it might be that colonies are more likely to become polygynous when they happen to be located near such mating landmarks, also because *A. echinatior* colonies typically kill non-nestmate queens (Nehring & al. 2015), in contrast to *A. subterraneus molestans* colonies. The ants' nestmate recognition system would then act as a natural filter so that normally only a former nestmate gyne would be allowed to re-enter the colony. However, rare recognition errors can happen (Nehring & al. 2013, Larsen & al. 2014, Nehring & al. 2015), consistent with few colonies adopting a non-related additional queen. An alternative hypothesis would be that adopted daughter queens never leave their natal colonies and mate in or very close to their nest. However, that hypothesis seems unlikely as it should imply that such queens mate with fewer males, likely brothers, so that their offspring would be inbred. These implications were incompatible with our data sets when we manually checked for paternity nested within maternity and offspring homozygosity. The absence of sparsely inseminated queens also makes the scenario of some colonies acquiring a second queen after insemination of gynes in the lab a rather academic possibility.

If daughter queens are occasionally re-adopted, they would have some likelihood of breeding alongside their mother, which would explain that the mother-daughter scenario appeared to be most likely in a number of polygynous colonies. Once the old mother queen dies, such colonies would then either return to the monogynous state or remain polygynous when two newly mated daughter queens were adopted. Sister queen pairs might also arise in recently orphaned *Acromyrmex* colonies that raise "emergency gynes", i.e., turning all female brood into gynes before starting to produce worker sons from unfertilized eggs (Dijkstra & Boomsma 2007). As gynes are related to half-sister sons by only 0.125, inbreeding would be less detectable than in the brother-sister mating (R = 0.25) scenario rejected above. However, the explicitly outbred scenario of landmark-related adoption seems to remain the most parsimonious hypothesis.

While landmark-related adoption of daughter queens might occur due to an almost "passive" filtering process, it might well be adaptive since it allows versatile colonies at high quality nest sites to considerably extend their life-span. *Acromyrmex echinatior* colonies might thus sometimes resemble termite colonies in which dying queens or kings can be replaced by their own offspring without much cost to colony efficiency (Hartke & Baer 2011). However, in contrast to the termites producing replacement reproductives to mate with siblings, newly adopted *Acromyrmex* queens introduce new alleles into the colony when they are inseminated by unrelated males. This has the potential to introduce reproductive conflict between the worker offspring of the old and the new queen, similar to what happens during queen replacement in honeybees (Woyciechowski & Kuszewska 2012). While this conflict is expressed in the honeybee, in which queens never co-breed, it may never become an actual conflict in *Acromyrmex* colonies unless the required kin-discrimination mechanisms evolved to be sufficiently efficient (Nehring & al. 2011).

Pleometrosis appears to be unlikely in *A. echinatior*: An alternative route to polygyny would be cooperative colony founding by unrelated queens. This has been observed in several ant lineages, but usually results in the elimination of all but one queen when the first workers hatch (Strassmann 1989, Bernasconi & Strassmann 1999). Pleometrosis has been documented in a relative of *Acromyrmex echinatior*, the desert-living species *Acromyrmex versicolor* (Pergande, 1894), which often founds colonies by groups of unrelated queens (Hagen & al. 1988, Rissing & al. 1989). Co-founding *A. versicolor* queens appear to survive for at least half a year, i.e., well into the stage when they already have a considerable workforce (Clark & Fewell 2014), but it remains unclear whether this ever results in lasting polygyny of mature colonies. As in *Lasius* ants in which pleometrosis enhances early colony survival (Sommer & Hölldobler 1995, Holman & al. 2010), cooperative colony founding in *A. versicolor* improves the chance of successful fungus garden initiation, which may be beneficial in a desert environment where many foundresses compete for rare habitat patches with suitable resources (Rissing & al. 1986, Rissing & Pollock 1987, Cahan 1999). However, incipient colonies with two or more queens are almost never observed in the Gamboa population of *A. echinatior* (see Fernández-Marín & al. 2004; we only know of a single case among over two hundred founding colonies that were collected; J. Howe, unpublished observations), likely because founding queens are normally aggressive against intruders (Howe & al. 2016). It thus appears that queen adoption in mature colonies is the prime viable route towards polygyny in *A. echinatior*, although the mating at landmark scenario does not completely exclude that two sister queens might find each other and initiate a colony together.

Adoption of unrelated queens and the evolution of social parasitism: While the majority of cohabiting queens had been adopted by their own mother colonies, two out of eleven polygynous colonies likely contained unrelated queens. In these instances, new queens apparently succeeded in becoming adopted in an alien mature colony despite the typically efficient recognition and expulsion of non-nestmate queens (Nehring & al. 2015). Such adoption of an unrelated queen may double the productivity of the joint colony and create a cooperative win-win situation. However, when joint productivity is constrained, the immigrating unrelated queen may be under selection to cheat by investing less in somatic colony growth (i.e., producing worker brood) and more in colony reproduction. This is because an unrelated immigrant queen gains no indirect fitness benefits from the offspring of the resident queen. She would benefit disproportionally from diverting resources towards the production of her own reproductive offspring. This scenario reflects what has been hypothesized for the sympatric evolution of permanent inquiline social parasites from within their host species, with facultative polygyny as necessary condition for the early evolution of intraspecific social parasitism (Savolainen & Vepsäläinen 2003, Buschinger 2009, Rabeling & Bacci 2010, Boomsma & Nash 2014). The results of our analyses are consistent with outbreeding and not with any queens being inseminated in the nest by brothers or more distantly related males. This supports Buschinger's (2009) conjecture that sib-mating of inquiline species evolves as a derived trait and is not part of any pre-adaptive syndrome in the host ants from which inquiline evolution may start.

Our sample of merely two cases of a putative unrelated queen-pair was insufficient to detect forms of cheating because we could not tell which of the queens was the original resident. Detecting intraspecific cheating in the form of overproduction of reproductives may be subtle and would require large numbers of gynes and workers to be genotyped, as in an analysis of cheating patrilines

in *Acromyrmex echinatior* (Hughes & Boomsma 2008). However, if exploiting the joint public good pays off, queens entering non-related colonies may come under selection to start expressing incipient inquiline traits such as body size reduction, loss of the worker caste, and chemical adaptations to avoid being recognized as a non-nestmate (Heinze & Keller 2000, Buschinger 2009, Rabeling & Bacci 2010, Boomsma & Nash 2014, Schär & Nash 2014). If assortative mating arises during this process, e.g., through a shift in mating locality or mating time, reproductive isolation from the host may follow and a new social parasite species would evolve. This scenario matches what is so far known from the biology of the *A. echinatior* inquiline *A. insinuator* (see Schultz & al. 1998, Bekkevold & Boomsma 2000, Sumner & al. 2004a, Lambardi & al. 2007, Nehring & al. 2015). However, much of this remains conjecture, because of the low number of polygynous colonies that were available.

During the entire sampling period (1993 - 2010), sympatric colonies of the related species *Acromyrmex octospinosus* (Reich, 1793) were also routinely collected and transported to rearing rooms in Copenhagen, amounting to a total sample of 166 colonies. One of these colonies (Ao044) contained three potential mother-queens upon collection, but a single queen was sufficient to explain all offspring, suggesting that the additional queens were in fact unmated soldier-like helpers (Nehring & al. 2012). In only one other colony (Ao273) did we find a second queen after it had been set up in a Copenhagen rearing room. In this case, the queen pair shared alleles in two out of the four marker loci, suggesting that the queens were half-sisters, and not full-sisters or mother and daughter, consistent with multiple insemination and effective paternity ($Ne = 5.3 - 6.8$) being of similar magnitude as in *A. echinatior* (see Ortius-Lechner & al. 2003). These data suggest that functional polygyny in Panamanian *A. octospinosus* is possible but truly exceptional and perhaps an order of magnitude less common than in *A. echinatior*.

Queens of the inquiline *Acromyrmex insinuator* manage to become adopted in colonies of *A. octospinosus* with about equal efficiency as in *A. echinatior* colonies, but they almost never suppress the host queen and realize their typical semelparous burst of reproduction that normally kills *A. echinatior* host colonies (Schultz & al. 1998, Bekkevold & Boomsma 2000, Sumner & al. 2003, Nehring & al. 2015). This pattern is consistent with Emery's rule for the evolution of inquiline social parasitism in ants (Sumner & al. 2004a, Huang & Dornhaus 2008, Buschinger 2009, Rabeling & al. 2014), predicting that new inquiline species initially evolve as sister species of their host (the strict version). Once they have become fully specialized on a parasitic life history, they may later colonize additional host species of the same genus, which can lead to further adaptive radiation in the inquiline lineage (captured by the loose version of Emery's rule). The barely successful colonization of *A. octospinosus* as a secondary host may thus represent the incipient stage of this further speciation process.

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Author contributions

JJB, MBD, VN, and WOHH developed the rationale of this study over the last decade; MBD, SRS, VN, and WOHH obtained the genotyping data; VN did most of the analyses after MBD and WOHH had initiated some of them; JJB and VN wrote the paper with input from WOHH. All authors approved the final version of the manuscript.

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Diversity and distribution of *Solenopsis* (Hymenoptera: Formicidae) thief ants belowground

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Abstract

Subterranean ant communities are vastly understudied relative to aboveground ant communities. The thief ants of the genus *Solenopsis* are a globally abundant and widespread group that is a conspicuous and important part of the belowground ant community. Thief ant ecology, including their distribution and diversity at local scales, has also rarely been documented. In this study, we sampled the subterranean ant community of central Florida, a region with conspicuously high subterranean thief ant abundance. We used a stratified-random sampling protocol and collected soil environmental variables at each sampling plot to model subterranean ant diversity in relation to abiotic conditions in the soil environment. Furthermore, we utilized non-parametric ordination methods and permutation-based analyses of variance to visualize and quantify associations of species based on habitats and soil strata. Our study yielded 15 species from six genera of which five were thief ant species. These five *Solenopsis* species represented 64% of all ant individuals found. We also identified distinct differences in species composition between two habitat types (pine flatwoods and high pine sandhills) and significant associations of soil abiotic conditions with the diversity of the subterranean community. This study finds that thief ants dominate belowground and respond predictably to soil habitat conditions. Biotic effects among ant species may be important given their purported lestobiotic behaviors.

Key words: Cryptic, fire ants, hypogaeic, lestobiosis, ant sampling, soil temperature, soil moisture, subterranean.

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Introduction

Subterranean ants nest and forage almost entirely belowground. They are a group that may represent the final unexplored frontier for global ant biodiversity (Wilkie & al. 2007). In general, these ants are usually small-bodied and cryptic in their morphology, most likely a result of a hypogaeic life history (Wong & Guénard 2017). Despite recent evidence of the diversity represented in subterranean communities as well as their potential impacts on soil ecosystems, little information exists on their basic biology and ecology (Wilkie & al. 2007, Schmidt & Diehl 2008, Andersen & Brault 2010, Wilkie & al. 2010, Ribas & al. 2012, Wong & Guénard 2017). This also extends to what little is known about the ecology and belowground activities of most epigaeic ants. Subterranean sampling has not been integral to ant diversity assessments, and its practice has only recently become more widespread (Schmidt & Solar 2010). Most sampling of subterranean ant communities has only been done in the Neotropics (Wong & Guénard 2017). Thus, subterranean ant distributions and interactions with other soil invertebrates are scarcely known. This dearth of information is because of the difficulties associated with sampling belowground where traps and direct soil sampling are usually the only logistically feasible approaches.

Given this sparse background, an important question is: What are the potential drivers of subterranean ant species distributions at local scales? At broader scales, soils (type, compression, temperature) and elevation have been shown to affect subterranean ant diversity (Lynch & al. 1988, Wilkie & al. 2010, Berman & Andersen 2012, Canedo-Júnior 2015). However, substantial variation exists among local sample sites in the above studies. For example, subterranean ant abundances in Ecuador are not predicted by some soil conditions, such as soil pH or mineral content (Jacquemin & al. 2012). However, another study in the Brazilian savannah showed significant effects of soil temperature and compression in association with changing subterranean ant species compositions (Canedo-Júnior 2015). Collectively, these few studies represent most of what is known about environmental factors affecting subterranean ant diversity and distributions (Wong & Guénard 2017). These studies suggest that the relationship between the diversity and distributions of subterranean ants and soil conditions may jointly depend on broad-scale geography and the local composition of the local subterranean ant community.

Subterranean ant communities, especially in the tropics, contain a variety of genera. However, the genus *Solenopsis* is found globally in belowground communities and is among the most abundant group of species in these communities and thus warrants special attention (Wilkie & al. 2007, Berman & Andersen 2012, Pacheco & Mackay 2013). Thief ants in the genus *Solenopsis* are a group of relatively small-bodied, largely subterranean or litter-dwelling species (although there are even some arboreal species) that are abundant in communities from the warm temperate to the

tropical zones (Pacheco & al. 2007, Andersen & Brault 2010, Hernández 2010, Pacheco & Mackay 2013). About 86 described thief ant species occur across the globe as a common and conspicuous group in most ant communities (Mackay & Mackay 2002, Pacheco & Mackay 2013). In Florida, the thief ant species considered to be completely subterranean are *Solenopsis tonsa* Thompson, 1989, *Solenopsis pergandei* Forel, 1901, and quite possibly *Solenopsis tennesseensis* Smith, M.R., 1951. Some (or perhaps most) thief ant species are purported to be "lestobiotic", nesting near the nests of host ant colonies, tunneling belowground into their nests, and stealing their brood (Hölldobler 1973, Hölldobler & Wilson 1990, Tschinkel 2006, Deyrup 2016). Thief ants are assumed to practice lestobiosis upon a wide range of ant species that are often much larger in size, as this interaction often emphasizes the interaction between small and large-bodied ants (Hölldobler & Wilson 1990). Although thief ants may also be dietary generalists and even predators of other ants when not stealing brood as they have also been observed actively preying on founding queens (Wheeler 1901, Blum & al. 1980, Thompson 1980, Buren 1983, Lammers 1987, Nichols & Sites 1991, Vinson & Rao 2004, Deyrup 2016). The small body size of thief ants (which includes some of the smallest workers among all ants) may also allow them to move through soil and escape via pathways not accessible to their larger-bodied prey (Kaspari & Weiser 1999). This potential behavior coupled with their high abundance and broad, global distribution suggests that lestobiosis by thief ants, and preying directly on brood and, especially, founding queens (Lammers 1987, Nichols & Sites 1991, Vinson & Rao 2004), may be an important regulator of both subterranean and aboveground ant communities.

What is actually known about subterranean ant interactions with other ants is largely based on a few descriptions (Wheeler 1901, Schneirla & al. 1944, Deyrup 2016). This gap in knowledge is all the more important in regions such as the southeastern US, and especially upland habitats in Florida, where thief ants dominate subterranean ant diversity and abundance (Lubertazzi & Tschinkel 2003, King & Porter 2007, Deyrup 2016). Furthermore, the subterranean thief ant complex from these localities has been taxonomically well described for many years, meaning that community diversity analyses may be confidently conducted (Thompson 1980, 1989, Moreno Gonzalez 2001).

In the most comprehensive treatment of thief ant ecology to date, Thompson (1980) found that thief ant species composition differed between shrubby and grassy habitat types. Otherwise, only unpublished observations inform the ecology of thief ant distributions. Depth to water table or soil moisture content may be the main environmental drivers of thief ant distribution and diversity as long-term soil moisture dynamics may limit the foraging capabilities of these ants (Lammers 1987). It is also known that thief ants are sensitive to low humidity when being raised in a laboratory setting but in the wild are incapable of building mounds like the fire ant (*Solenopsis invicta* Buren, 1972) to escape inundation (Thompson 1980, Tschinkel 2006). Therefore, well-drained soils in otherwise mesic regions likely maintain conditions ideal for thief ant populations. In Florida, upland habitats such as drier pine flatwoods and especially high pine sandhills (Myers & Ewel 1990) appear to support robust populations of a number of thief ant species (Thompson 1980). Nearby habitats (e.g., more mesic flatwoods and dry prairies) are more prone to flooding (Myers & Ewel 1990) and appear to have reduced subterranean ant diversity and abundances (Deyrup 2016). We therefore conducted this study in upland sandhill and flatwood habitats to determine if there are differences in thief ant communities associated with these common habitat types in this region.

To better understand the factors affecting ant distribution and activity belowground, we sampled belowground foraging ants in the two habitat types (sandhill and flatwoods) using baits and collected associated soil environmental variables to identify relationships between the subterranean ant community and local habitat conditions. Considering that many subterranean ants are known for their small-bodied form and cryptic morphology we specifically targeted small-bodied ants in our sampling. We understand that not all subterranean ants are small-bodied as seen in Wong & Guénard (2017) but based on previous surveys and studies in central Florida and in other parts of the state we have evidence that subterranean ants in our locality were small-bodied (Thompson 1980, Prusak 1997, Lubertazzi & Tschinkel 2003, King & Porter 2007, King 2010). Furthermore, our primary focus, the *Solenopsis* thief ants, are all small-bodied (Thompson 1989, Deyrup 2016). However, not all the ants that were baited truly practice a subterranean life history, that is, nesting and foraging entirely belowground but they were still classified as part of the subterranean ant community for the purpose of this study. Therefore, we defined the "subterranean ant community" to be composed of ants with a hypogaeic life history as well as the ants that were found to co-occur with them in our subterranean sampling. These co-occurring species may forage or nest aboveground but may be opportunistically foraging belowground as well. We later differentiate subterranean versus other ant species, based on what is known of their natural history. Nevertheless, even small-bodied aboveground foraging or nesting ants that forage opportunistically belowground likely play a role in the subterranean ant community.

We asked: (1) Do subterranean ant communities (with an emphasis on thief ants) differ in composition and abundance between flatwood and sandhill habitats? (2) Do soil environmental gradients predict the species diversity of this subterranean ant community? (3) Do these gradients also predict the occurrence of thief ant species?

We also compared those data to the only two other subterranean sampling studies conducted in Florida (Thompson 1980, Lubertazzi & Tschinkel 2003). Lubertazzi & Tschinkel (2003) carried out their subterranean assessment in the longleaf pine forest of the Apalachicola National Forest outside of Tallahassee, Florida. Thompson (1980) conducted a sampling survey comprised of two total plots, one in turkey oak woods and the other in an open field outside of Gainesville, Florida. Comparisons to aboveground ant diversity and relative abundance in our study site were also made possible using aboveground pitfall sampling data (from 2012) collected from the same areas as our subterranean sampling.

Materials and methods

Study site: Sampling was conducted during the months of July and August, 2017, at Wekiva Springs State Park (2,750 hectares) situated in Orange County, Florida at 28.7118° N, 81.4628° W. Average annual rainfall in the area is approximately 1350 mm. The general seasonality of the site

Tab. 1: A table showing the top five performing simple linear regression models under AICc (Akaike Information Criterion with correction for small sample sizes) rankings. Predictor variables for each model are shown along with each model's AICc score, the change in AICc for every lower ranked model, AICc weights, and the adjusted-R^2.

Model	AICc	ΔAICc	Weight (w_i)	Adjusted-R^2
D ~ Habitat * Avg. Change in Daily Soil Temp.	94.4	0.0	0.34	0.60
D ~ Habitat * Avg. Soil Maximum Temp.	96.2	1.8	0.14	0.57
D ~ Habitat + Avg. Soil Moisture * Avg. Soil Temp.	97.2	2.9	0.08	0.58
D ~ Habitat + Avg. Soil Minimum Temp.	97.4	3.0	0.08	0.53
D ~ Habitat + Avg. Soil Moisture	97.6	3.3	0.07	0.53

involves a cycle of wet and dry seasons with the wet season beginning around May and ending in November and the dry season occurring December-April. We distinguished two main habitat types within this park to conduct our survey, high pine sandhills and mesic pine flatwoods.

High pine sandhill is a pyrogenic habitat characterized by well-drained sandy soils, an overstory of longleaf pine (*Pinus palustris*), and a groundcover dominated by wiregrass (*Astrida beyrichiana*) (Myers & Ewel 1990). The sandhill sites selected for this study were in areas maintained by low intensity fires. High pine sandhill habitats gradually transition downhill to pine flatwoods, which are distinct in vegetation as a result of more poorly-drained soils due to a higher water table and subsequent proneness to flooding (Abrahamson & Hartnett 1990). Sandhill soils are generally categorized into droughty course sands, sandy clays, or loamy sands; our sites were mostly composed of coarse sand classified as Entisols that are generally low in nutrients (Abrahamson & Hartnett 1990). Flatwood soils are usually acidic and hold insignificant amounts of extractable nutrients (Gholz & Fisher 1982, Myers & Ewel 1990). Soil moisture of the flatwoods is usually influenced by soil organic matter content as well as a mulching effect from the litter layer (Myers & Ewel 1990).

Design: A stratified-random sampling design was used in both habitat types, where habitat type boundaries were first identified in the field (based on vegetation) using a handheld GPS. These coordinates were used to generate polygons representative of the two habitat types in ArcMap (Esri 2017). Coordinates for our sample plots (16 per habitat type) were then randomly generated in ArcMap within the habitat type polygons. A minimum distance of 36 meters between sample plots avoided site overlap. Sample plots were randomly assigned a sampling depth of 10 cm or 20 cm. As a result, eight plots in each of two habitat types were sampled at each of two depths (32 total sample plots) (Fig. 1).

Baits: Baits were made using plastic capped vials 70 mm tall and 30 mm in diameter. A ~ 5 mm diameter hole was made near the bottom edge of the vial and covered with 1 mm screening to exclude larger animals (e.g., fire ants) but permit entry by subterranean ants. This was done to specifically target small-bodied subterranean ants as well as other non-subterranean ants that may forage opportunistically within the subterranean environment. Each bait was loaded with ~ 3 - 4 cm^3 of sugar cookie (Pecan Sandies). To deploy the baits a battery-powered 24v drill and a 24-inch auger-bit was used to drill into the soil to a specified depth. The baits were then placed in the holes and covered up with the previously extracted soil. Baits

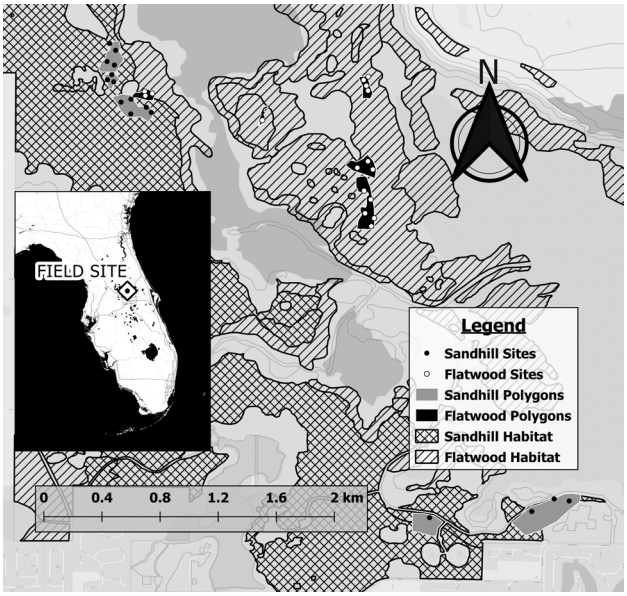

Fig. 1: Map of study site (Wekiva Springs State Park) with sampling sites.

were deployed in the morning and retrieved using a hand trowel ~ 72 hours later. Specimens were kept in sandwich bags and stored in a freezer.

Habitat variables: Soil temperature and soil moisture were recorded at each bait site. Soil temperature was recorded for the entirety of the 72-hour baiting period using data loggers (iButton, Maxim). Each plot had two data loggers installed on both east and west sides at 10 cm below the soil surface to record temperatures every hour during the baiting period. Using those data, we extrapolated average minimum, maximum, and temporal changes in soil temperatures per site. Soil moisture was collected by using a soil moisture sensor at 10 cm depth (Procheck, Decagon Devices). Ten readings were taken from each plot at the time of retrieval and averaged to represent the soil moisture level of the plot.

Sorting: All ants collected from the baits were sorted to species utilizing identification pointers from Deyrup (2016). Additional reference specimens from J. R. King's personal collection were used to confirm identifications.

Aboveground sampling: Aboveground ant communities were sampled previously in the same area and habitat types of the park as the belowground sampling. In August 2012, three 100 m linear transects were established in each habitat type (a total of six transects), separated by at least 100 meters from one another or forest roads. In each transect,

Tab. 2: Pseudo-R^2 values for most plausible logistic regression model of successfully modeled species collected in the subterranean sampling (eight of 15 possible species). Model predictor variables are also displayed.

Species	Pseudo-R^2 of most plausible model	Model
Solenopsis carolinensis	0.16	Occurrence ~ Avg. Soil Moisture + Avg. Soil Temp.
Solenopsis pergandei	0.59	Occurrence ~ Avg. Soil Moisture * Avg. Minimum Soil Temp.
Solenopsis tennesseensis	0.23	Occurrence ~ Avg. Soil Temp.
Solenopsis tonsa	0.33	Occurrence ~ Avg. Soil Moisture + Avg. Soil Temp.
Brachymyrmex depilis	0.74	Occurrence ~ Avg. Minimum Soil Temp. * Avg. Soil Moisture
Pheidole floridana	0.11	Occurrence ~ Avg. Maximum Soil Temp.
Pheidole metallescens	0.12	Occurrence ~ Avg. Minimum Soil Temp + Avg. Soil Moisture
Pheidole morrisii	0.21	Occurrence ~ Maximum Soil Temp.

sampling was performed using pitfall traps placed at 5-meter intervals for a total of 20 traps per site and 120 traps for the two habitat types. Pitfall traps were 85 mm long plastic vials with 30 mm internal diameter partially filled with ~ 15 ml of non-toxic, propylene-glycol antifreeze. Traps were buried with the opened end flush with the surface of the ground and operated for seven days. Traps were installed using a hand-held, battery-powered drill using an auger bit.

Analyses: Each occurrence of a species in a baited vial was considered an occurrence of one colony of that species based on the spatial distances between baits (King & Porter 2007, King 2010). Potential differences in community composition between habitats and depths were evaluated with nonmetric multi-dimensional scaling (NMDS), which is a nonparametric ordination method. Subsequent permutation-based analyses of variance (PERMANOVAs) were used to test for significant differences between detected clusters. The NMDS utilized beta diversity distances based on the Bray-Curtis index, a measure of dissimilarity that allowed for the separation of sites based on differences in species composition (while also accounting for species abundance as measured by frequency of occurrences). Bray-Curtis distances are also robust to sampling errors and preferred to other beta diversity measures (Schroeder & Jenkins 2018). Potential effects of environmental gradients on ant diversity were modeled using both linear mixed-effect models and linear regressions. Species estimators were also calculated using Chao1 estimators (all values listed in Appendix S1, as digital supplementary material to this article, at the journal's web pages) to provide further evidence of the robustness of sampling methods. The response variable for all models was the Jost Diversity index ($D = e^{H'}$; Jost 2006) per site calculated using number of species occurrences per site. Independent variables included depth of the baited vial, soil temperature (averages of maximum, minimum, and daily range), and average soil moisture (Tab. 1). Model assumptions were evaluated based on residual diagnostic plots (Appendix S2). Finally, the occurrence of all species in the baited vials was modeled using logistic regressions, where the occurrence of each ant species was predicted by soil parameters. All regressions were compared and ranked using corrected Akaike Information Criterion weights (AICc w_i) from the R package "bbmle" (Bolker & R Development Core Team 2017) as they allowed an appropriate comparison for model parsimony compared to evaluating individual R^2-values (Tab. 1). Logistic regressions were also evaluated with pseudo-R^2 values calculated by subtracting the null deviance of the model from the residual deviance and dividing the total by the residual deviance (Tab. 2). All soil environmental variables were standardized during analyses and all statistical analyses were conducted using R 3.4.1 statistical software (R Development Core Team 2017). Mixed-effect models were computed using the R package "lme4" (Bates & al. 2015) and the "vegan" package (Oksanen & al. 2017) was used to compute NMDS ordinations and PERMANOVAs. All graphics for regressions and ordinations were done using the R package "ggplot2" (Wickham 2009).

Results

Ant diversity and abundance: A total of 15 species encompassing six genera were captured and identified from all our belowground baits (full species list in Appendix S3). 98% of the 1152 baited vials deployed were recovered; 23 baited vials were lost during sampling. Species-sampling estimates indicate that all existing species were observed in most samples (Appendix S1). We assessed relative abundances as the occurrence of a species at each baited vial. The most common genus was *Solenopsis* (in 70% of baits), followed by *Pheidole* (21.5%) and *Brachymyrmex* (8.3%). The last three genera, *Forelius*, *Hypoponera*, and *Nylanderia* occurred in one baited vial, each. *Solenopsis* was the most species-rich genus with six species (all thief ants except for the introduced fire ant, *S. invicta*). The 8 most common species were *Solenopsis pergandei* (occurring in 209 baited vials, 27.6% of total), *Solenopsis carolinensis* Forel, 1901 (98, 12.9%), *Solenopsis nickersoni* Thompson, 1982 (93, 12.3%), *Pheidole floridana* Emery, 1895 (69, 9%), *Brachymyrmex depilis* Emery, 1893 (63, 8.3%), *Solenopsis tennesseensis* (50, 6.6%), *Solenopsis invicta* (40, 5.3%), and *Pheidole morrisii* Forel, 1886 (39, 5.2%).

Soil strata composition: Most ant taxa other than *Solenopsis*, *Nylanderia wojciki* (Trager, 1984), and *Pheidole dentata* Mayr, 1886 were less frequently sampled at the greater depth (20 cm). *Nylanderia wojciki* and *Pheidole dentata* were relatively rare and were only detected at 20 cm (Appendix S3). Among the *Solenopsis* species, *S. carolinensis* occurrence decreased 42% from 10 cm to 20 cm soil depth and *S. nickersoni* occurrence decreased (25%), but *S. pergandei* occurrence increased (78%), *S. tennesseensis* occurrence had no change, and *S. tonsa* occurrence increased (145%). The most frequently captured species at both depths

was *S. pergandei*. Based on an NMDS analysis and a subsequent PERMANOVA, depth did not significantly affect species compositions (PERMANOVA, P > 0.05).

Habitat-based community structure: *Brachymyrmex* (one occurrence in high pine sandhills, 62 occurrences in pine flatwoods), was more prevalent in the flatwoods than in sandhill habitats. *Forelius* (1, 0), *Hypoponera* (1, 0), and *Nylanderia* (1, 0) were present in flatwoods but absent in the sandhills. *Pheidole* (125, 38) and *Solenopsis* (359, 169) were more common in the sandhills. Within *Solenopsis*, *S. nickersoni* was found more commonly in flatwoods than in sandhill habitats. However, all other thief ant species (*S. carolinensis*, *S. pergandei*, *S.tennesseensis*, *S. tonsa*) were more prevalent in the sandhills.

The NMDS analysis (Fig. 2, Fig. 3) showed a distinct separation between communities of the two habitat types along with the separation of species that was congruent with our raw data. A subsequent PERMANOVA verified significant separation of centroids in this analysis ($P < 0.05$). All thief ant and *Pheidole* species, except for *Solenopsis nickersoni*, *P. dentata*, and *P. morrisii*, were clustered tightly within the sandhill cluster. Positions for *S. tonsa* and *Pheidole adrianoi* Naves, 1985 in the NMDS were furthest away from the flatwood cluster. The species within and around the flatwood cluster had a higher degree of spread, most likely due to several species (*Forelius pruinosus* (Roger, 1863), *Hypoponera opacior* Forel, 1893, *N. wojciki*, and *P. dentata*) having been collected only once. *Brachymyrmex depilis*' position in the NMDS mirrors *S. tonsa* and is one of the few frequently collected species in the flatwoods. Finally, the fire ant, *S. invicta*, is positioned more along the upper edge of the flatwood cluster and towards the center between both habitat clusters. To further validate these results, we removed singletons from the species by site matrix (four total species / columns removed) and ran the NMDS at the same dimensions ($k = 2$) with the same number of starting iterations (1000) and found no differences in patterns. The stress value remained the same at ~ 0.127.

Modeling species diversity: Although not all species caught at our baits are truly subterranean ants, for the purposes of this study, we included species captured in belowground samples as part of the subterranean community as these species were clearly actively foraging belowground. Subterranean ant diversity was most effectively explained in regression models as an interaction between habitat types and average daily soil temperature range (*AICc* $w_i = 0.34$, Tab. 1). This model represented a majority of variance in ant diversity ($P = 0.02$, $R^2 = 0.60$). Residuals met assumptions of the model. The simple linear regression model outperformed the random-intercept model, and conditional pseudo *R*-squared values indicated that random intercepts explained very little variation and both models indicated approximately the same effect sizes. A second linear model also included an interaction between habitat type and average soil maximum temperature (*AICc* $w_i = 0.14$). However, the model using average daily temperature ranges accounted for more variation and was more plausible. In all our initial models we added soil depth as a covariate but the differences between the top-ranked models with and without the covariate was negligible as effect sizes and adjusted-R^2 values barely differed.

Predicting species occurrences: Logistic regression models of thief ant species occurrence per site using soil environment variables significantly predicted four of five

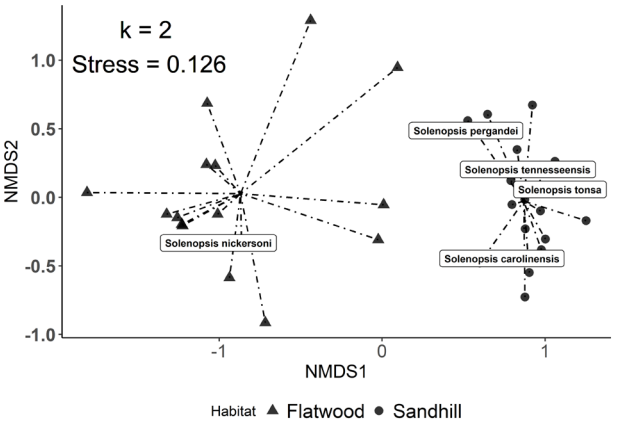

Fig. 2: Nonmetric multi-dimensional analysis of the species by site matrix from the subterranean sampling. Triangles represent pine flatwood sites and circles represent high pine sandhill sites. Lines connect the sites to each habitat's respective centroid in multivariate space. Labels for thief ant species represent the position of species within this space. The analysis had acceptable stress values of 0.126 at two dimensions (k = 2).

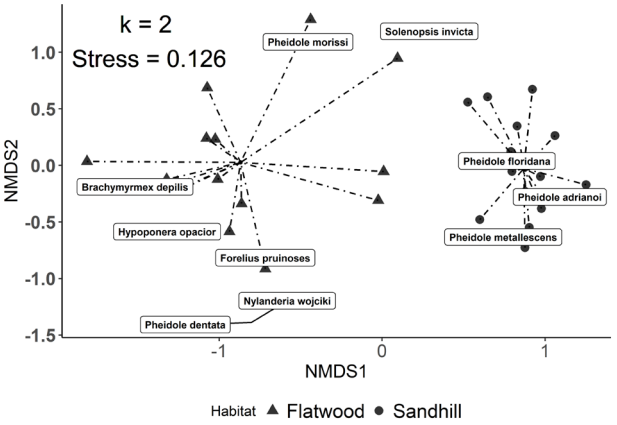

Fig. 3: A replicate nonmetric multi-dimensional analysis visual of Figure 2. Labels differ here to show the position of non-thief ant species. Flatwood species labels have a higher degree of spread due to extreme low occurrences of some species (e.g., *Pheidole dentata*, *Nylanderia wojciki*).

thief ant species and helped in further understanding the NMDS result (full models listed in Appendix S4); only *Solenopsis nickersoni* occurrence was not predicted. *Solenopsis pergandei*'s most plausible model was a function of the interaction between average soil moisture and average minimum soil temperature ($P = 0.02$, Pseudo-$R^2 = 0.59$, Tab. 2). *Solenopsis tonsa*'s most plausible model was a function of the additive effects of average soil moisture and temperature ($P = 0.02$, 0.01 respectively, Pseudo-$R^2 = 0.33$). *Solenopsis carolinensis*' most plausible model was also a function of the same predictors ($P = 0.04$, P = 0.04, Pseudo-$R^2 = 0.16$). Finally, *S. tennesseensis*' most plausible model was a function of average soil temperature ($P = 0.01$, Pseudo-$R^2 = 0.23$).

Other co-occurring ant species found in our samples were also modeled by logistic regression, though not all species had sufficient occurrences to model (Tab. 5, models

Tab. 3: Simple linear model coefficients and their 95% confidence intervals for the top five most plausible models in predicting diversity based on AICc (Akaike Information Criterion corrected for small sample sizes) rankings. Coefficients represent changes in the Jost diversity index relative to different soil abiotic variables. Bolded coefficients were significant at $P < 0.05$. All coefficients are based on the flatwood habitat as being the reference level in the model and all quantitative predictor variables were standardized.

Independent Variables	D ~ Habitat + Avg. Soil Moisture * Avg. Soil Temp.	D ~ Habitat * Avg. Soil Maximum Temp.	D ~ Habitat * Avg. Change in Daily Soil Temp.	D ~ Habitat + Avg. Soil Moisture	D ~ Habitat + Avg. Soil Minimum Temp.
Intercept	**2.25±0.66**	**2.82±0.53**	**2.73±0.49**	**2.59±0.51**	**2.69±0.50**
Sandhill	**2.55±1.03**	**2.15±0.73**	**2.27±0.69**	**2.25±0.74**	**2.05±0.71**
Avg. Change in Daily Soil Temp.	-	-	-0.23±0.48	-	-
Avg. Soil Maximum Temp.	-	-0.39±0.59	-	-	-
Soil Minimum Temp.	-	-	-	-	-0.20±0.36
Avg. Soil Moisture	0.31±0.48	-	-	0.19±0.37	-
Avg. Soil Temp.	-0.08±0.53	-	-	-	-
Sandhill:Avg. Change in Daily Soil Temp.	-	-	**0.87±0.70**	-	-
Sandhill:Avg. Soil Maximum Temp.	-	**0.85±0.76**	-	-	-
Avg. Soil Moisture: Avg. Soil Temp.	**-0.53±0.43**	-	-	-	-

listed in Appendix S5). *AICc* model selection on the logistic regressions for *Pheidole adrianoi* and *Solenopsis invicta* showed the null model being ranked the best indicating the lack of any statistical signal in their species-respective models. *Brachymyrmex depilis*' most plausible model was a function of the interaction between average soil moisture and average minimum soil temperature ($P = 0.04$, Pseudo-$R^2 = 0.74$). *Pheidole floridana*'s most plausible model was a function of average maximum soil temperature ($P = 0.05$, Pseudo-$R^2 = 0.11$). *Pheidole metallescens*' Emery, 1895 most plausible model was a function of the additive effects of average soil moisture and average minimum soil temperature ($P = 0.07, 0.09$, Pseudo-$R^2 = 0.12$). It is important to note that the next plausible model for *P. metallescens* was the null model, and the two models were only different by a $\Delta AICc$ of 0.2 with similar *AICc* weights (Appendix S5). Therefore, we did not evaluate *P. metallescens* occurrences. *Pheidole morrisii*'s most plausible model was a function of average minimum soil temperature ($P = 0.02$, pseudo-$R^2 = 0.21$).

Overall, subterranean ant diversity was dominated by *Solenopsis* species and different in composition between high pine sandhills and pine flatwoods. Those patterns appeared to be related to soil temperature and moisture, which consistently predicted belowground ant diversity and species' occurrences in the two different habitat types.

Discussion

Differences among habitats: Distinct multivariate differences between sandhill and flatwood sites are consistent with the expectation that ant communities differ between habitat types at local scales (Berman & Andersen 2012, Cross & al. 2016) (Fig. 2 & 3). Distinct species compositions existed between habitats, but sandhill sites were more similar to one another than flatwood sites, indicating the greater homogeneity in soil habitat conditions in the sandhills. This suggests that heterogeneous soil habitat conditions affecting thief ants in flatwoods may result in more variation in the species present in any given area. This clustering also indicates the presence of a potential ecological driver (soil temperature and moisture conditions by regressions) for dissimilar species rosters found in both habitats. Such drivers may be environmental filters resulting in different survivorship or competitive abilities among species, ultimately resulting in different species found in pine flatwoods and high pine sandhills. Results here describe patterns in species composition; elucidating actual drivers of these patterns will require experiments and careful observation of species' natural histories.

The known natural history of most of these species agrees with their positions within the NMDS. Of the sandhill thief ant species, only *Solenopsis tonsa*, one of the few truly subterranean species, is expected to occur strictly in sandhill (Deyrup 2016). *Solenopsis pergandei*, another true subterranean species can be found in other soils but tends to be most common in open sandy areas such as sandhills. *Solenopsis tennesseensis*, a suspected subterranean but also litter-dwelling thief ant, is a supposed habitat generalist but in this case, was closely associated with the sandhill sites. Other species that were tightly clustered to the sandhills were *Pheidole metallescens*, *P. adrianoi*, and *P. floridana*. *Pheidole metallescens* is considered a predominantly upland species that is usually found in high pine sandhills and usually co-occurs with *P. adrianoi*. *Pheidole floridana* is associated with drier habitats, like the sandhills, and is less likely to be found in moist forested areas (Deyrup 2016). Flatwood species other than *S. nickersoni* included *B. depilis* and *P. morrisii*. *Brachymyrmex depilis*, predominantly sampled in the flatwoods, is considered a generally

Tab. 4: Logistic regression model coefficients and their 95% confidence intervals for the most plausible model for every successfully modeled thief ant species. Coefficients represent the log odds of the occurrence of the ant species relative to different soil abiotic conditions. Bolded coefficients were significant at $P < 0.05$. All predictor variables were standardized for the models.

Independent variables	*Solenopsis carolinensis* coefficients	*Solenopsis pergandei* coefficients	*Solenopsis tennesseensis* coefficients	*Solenopsis tonsa* coefficients
Intercept	0.02±0.79	**2.76±2.49**	0.37±0.84	-0.57±0.94
Avg. Minimum Soil Temp.	-	**-2.40±2.20**	-	-
Avg. Soil Moisture	**-1.04±0.99**	**-3.61±3.06**	-	**-1.85±1.37**
Avg. Soil Temp.	**-1.04±1.01**	-	**-1.42±1.07**	**-1.71±1.50**
Avg. Soil Moisture: Avg. Minimum Soil Temp.	-	**2.36±2.02**	-	-

Tab. 5: Logistic regression model coefficients and their 95% confidence intervals for the most plausible model for every successfully modeled non-thief ant species. Coefficients represent the log odds of the occurrence of the ant species relative to different soil abiotic conditions. Bolded coefficients were significant at $P < 0.05$. All predictor variables were standardized for the models.

Independent variables	*Brachymyrmex depilis* coefficients	*Pheidole floridana* coefficients	*Pheidole metallescens* coefficients	*Pheidole morrisii* coefficients
Intercept	-3.26±0.91	-0.27±0.75	-0.77±0.82	-0.75±0.89
Avg. Maximum Soil Temp.	-	**-0.86±0.85**	-	**-1.48±1.26**
Avg. Minimum Soil Temp.	**4.49 ±1.06**	-	-0.83±0.97	-
Avg. Soil Moisture	5.56±1.08	-	-1.01±1.07	-
Avg. Soil Temp.	-	-	-	-
Avg. Soil Moisture: Avg. Minimum Soil Temp.	**-3.70±1.02**	-	-	-

subterranean species like subterranean thief ants except they are usually found in a wider variety of habitat types across North America (Deyrup 2016). However, its general absence in the sandhills may be indicative of it preferring mesic conditions or being out-competed by the thief ants or species of small-bodied *Pheidole*. The fire ant, *S. invicta*, is a known invasive and weedy species, capable of surviving in inundation-prone habitats (Tschinkel 2006). Its position in the NMDS analysis indicates its prevalence in both habitats (Figs. 2 & 3) which would be logical considering its ability to establish in a variety of conditions, especially if there are forest roads or other disturbances nearby.

Environmental gradients with diversity and species occurrence: Local scale ant diversity is often weakly correlated with abiotic conditions and is usually more strongly associated with local vegetation (Cross & al. 2016). However for subterranean communities, gradients of abiotic conditions such as soil moisture and temperature may heavily influence their distribution at local scales (Thompson 1980, Lubertazzi & Tschinkel 2003, Wilkie & al. 2010). Teasing apart how local scale abiotic conditions affect diversity can be useful in discerning drivers of diversity. Here we found that diversity was predicted by an interaction between habitat types and average daily soil temperature range, where subterranean ant diversity increased with average daily temperature range in sandhill habitats but decreased slightly in the flatwoods (Fig. 4, Tab. 3). Flatwood sites also experienced higher variation in average daily soil temperature range than sandhill sites. This result may indicate a more dynamic environment in the flatwoods, where soil temperature can be influenced by flooding events due to poorly drained soils. Flooding

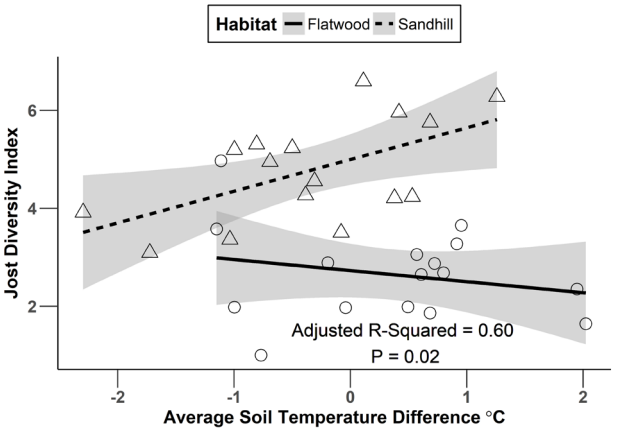

Fig. 4: Most plausible simple linear regression model for subterranean diversity. Y-axis represents the Jost diversity index; X-axis represents average daily soil temperature range (standardized) of the 3-day baiting period. Triangles represent high pine sandhill sites and circles represent pine flatwoods sites. Grey shading represents the 95% confidence intervals of the model. The dashed line represents the interaction of sandhill habitat with soil temperature range while the solid line represents the interaction of flatwood habitats with soil temperature range ($P = 0.02$). Adjusted-$R^2 = 0.60$.

events in these areas as well as shallow water tables may strongly constrain habitat space for these ants (Lammers 1987, Lubertazzi & Tschinkel 2003, Tschinkel & al. 2012). Another possible explanation is that some ant species may

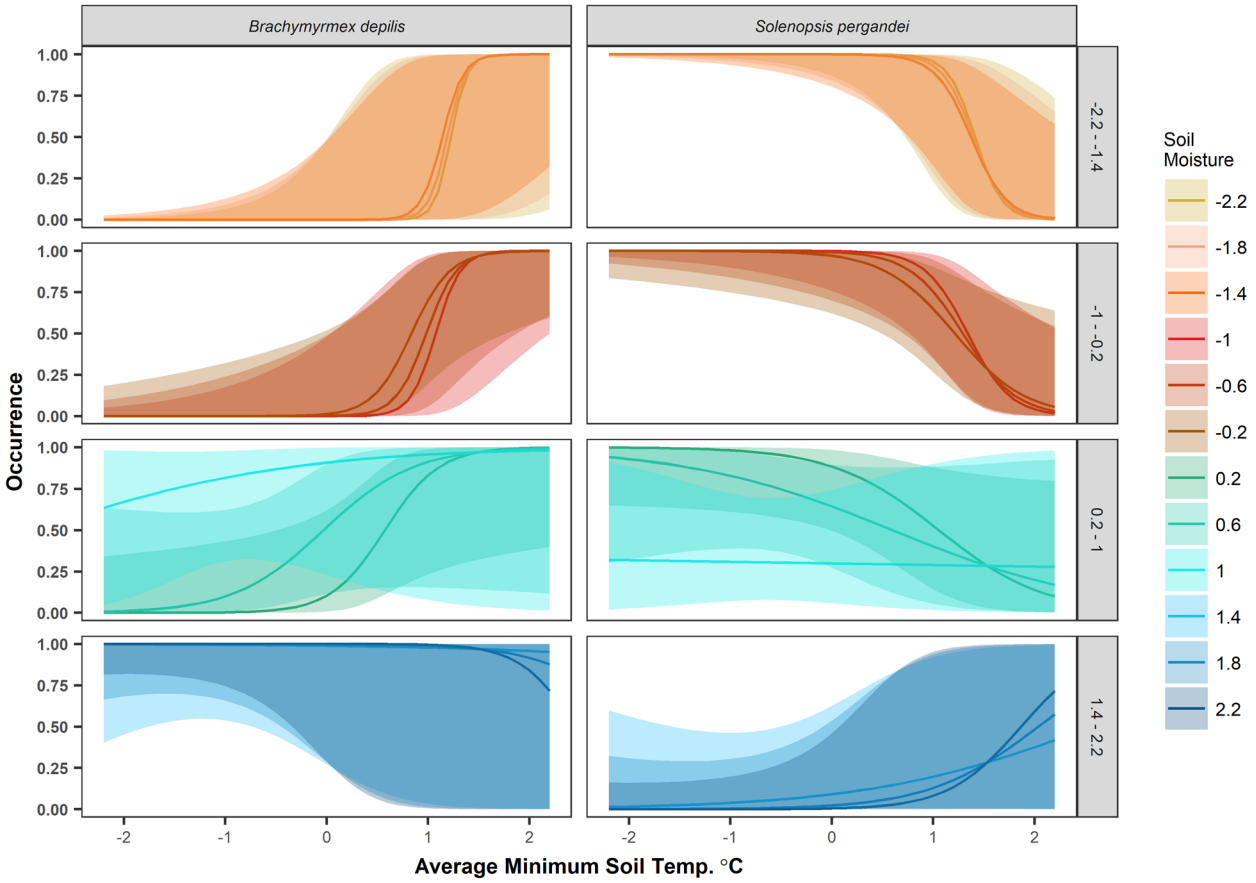

Fig. 5: Logistic regression models of *Brachymyrmex depilis* (left) and *Solenopsis pergandei* (right). Y-axis represents occurrence; X-axis represents average minimum soil temperature (standardized). The interaction between average minimum soil temperature and average soil moisture (standardized) is represented through 4 facets (labels on right). Each facet shows the model at three different average soil moisture levels and average soil moisture increases from the top to the bottom facet. Colors differ for each average soil moisture level and colored shading represents the 95% confidence intervals of the model at various levels of moisture. Pseudo-R^2 values for *B. depilis* and *S. pergandei* models were 0.74 and 0.59 and P-values for each model's interaction were 0.04 and 0.02, respectively.

not be able to tolerate the wide temperature differences and therefore prefer the lower soil temperature variation. It was surprising to find no significant effects on diversity from soil moisture as it could be a better proxy for indicating periodic flooding. However, the relatively brief study did not collect moisture data throughout a wet-dry season cycle, so the full variation of soil moisture that may affect colony distributions was not fully evaluated.

Depths to water tables and inundation dynamics may not drive species composition and diversity differences between the two habitat types. Logistic regressions showed that environmental soil gradients serve a significant role in the occurrence of thief ants and co-occurring ants found in our sampling. For example, in low soil moisture, cooler minimum soil temperature increases the chance of *Solenopsis pergandei* occurrence but in high moisture soils, lower minimum soil temperature decreases the chances of occurrence. This suggests that *S. pergandei* might be sensitive to the synergistic effects of both soil moisture and temperature.

The logistic regression for *Solenopsis carolinensis* showed significant negative effects on the chances of its occurrence as soil temperature and moisture increased. The same significant effect on the same parameters were also observed for *S. tonsa*. Finally, *S. tennesseensis* occurrence was negatively affected by increasing soil temperature. Across these four thief ant species there is thus a trend of decreasing occurrence as soil moisture or temperature increases (Tab. 4). These four species were also all positioned tightly within the same sandhill cluster from the NMDS analysis suggesting, again, soil abiotic conditions as a potential driver for that thief ant clustering. This is congruent with previous assumptions found from Thompson (1980) that highly moist and inundation-prone areas may not be suitable for the persistence of these species as well as a study from Texas (Lammers 1987) where it was suggested that subterranean foraging by thief ants may be limited by soil moisture.

When considering the occurrence of other non-thief ant species in flatwoods within the context of the NMDS analysis, only *B. depilis* occurrence was modelled successfully in the flatwoods. A sandhill species, *Solenopsis pergandei*, was modeled with the same predictors but responded in opposite directions (Fig. 5, Tabs. 4, 5). These contrasting patterns suggest environmental filtering as potential mechanism explaining their occurrence in disparate habitats. *Brachymyrmex depilis* could be more sensitive to xeric conditions as indicated by lower occurrences at lower

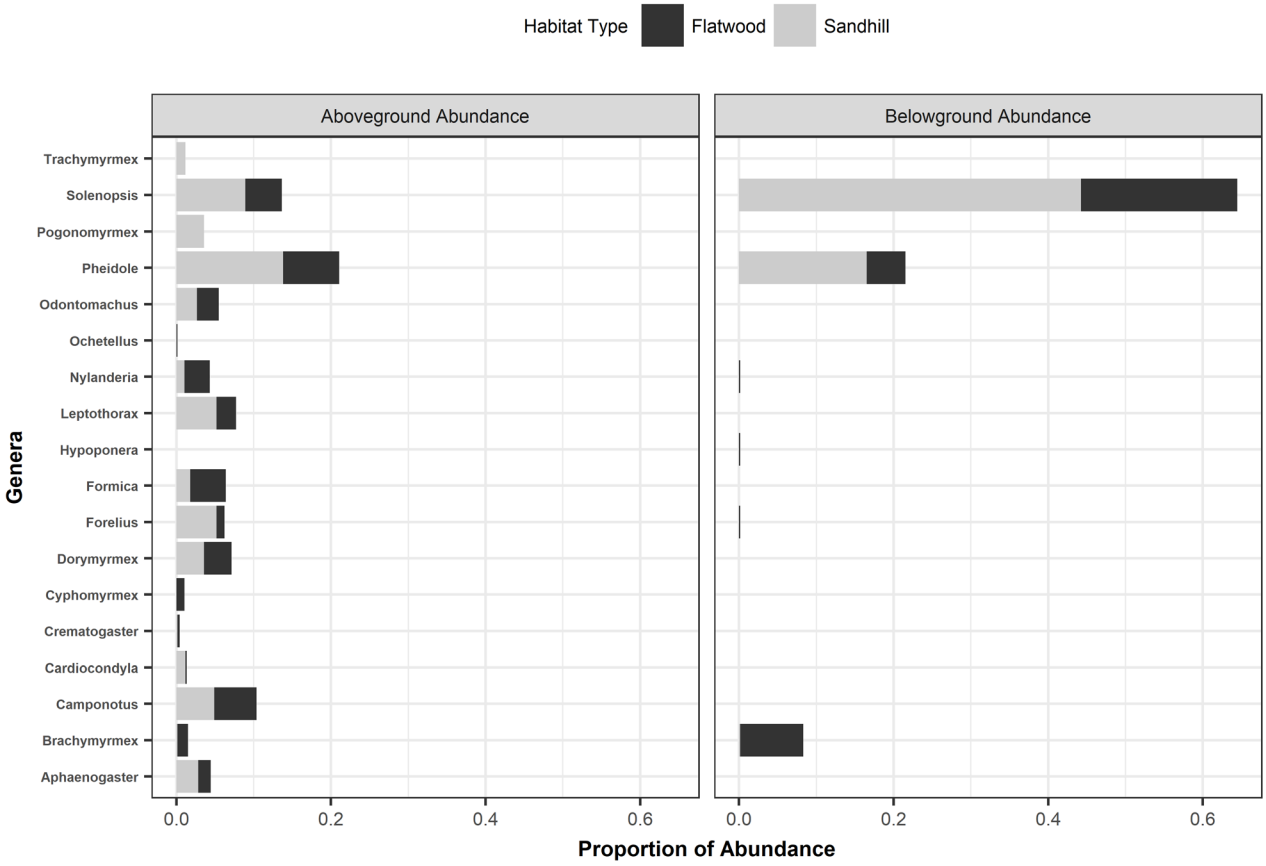

Fig. 6: Abundance of aboveground and belowground sampling. Y-axis represents ant taxa at the genus level. X-axis represents the proportion of total abundance per sampling type. Dark sections of the bars represent abundance found in pine flatwood areas and lighter sections represent abundance found in high pine sandhill areas. Note: *Solenopsis invicta* has been removed from the datasets represented in the figure.

levels of soil moisture while *S. pergandei* tends to show the opposite trend. These results support previous suggestions that Florida's subterranean thief ants may occur more frequently in well-drained soils (e.g., high pine sandhill ecosystems) (Thompson 1980, Lammers 1987). A wider range of environmental conditions in other habitats and locations should also be considered to verify the patterns observed here, in sandy soils.

Although our models show evidence indicative of environmental filtering in certain subterranean species, patterns of occurrence of thief ants may also be affected by the occurrence and distributions of potential prey in the context of the purported lestobiotic interactions that thief ants have with other ants, especially larger-bodied ant species. To further understand the role that species interactions may play in shaping subterranean ant distributions, there is a need for detailed information on, for example, the local distribution of thief ant colonies in relation to other colonies. Unfortunately, no such data exists but we can cautiously infer patterns of co-occurrence from aboveground pitfall data.

A comparison of studies: This study showed the dominance of thief ants among small-bodied ants in the subterranean environment of central Florida's sandy soils. Furthermore, our community analyses indicate significantly distinct subterranean ant communities between flatwood and sandhill habitat types. Moreover, the diversity of these communities can be predicted using soil abiotic conditions. Subterranean thief ant diversity patterns remain largely enigmatic in most regions of the world, so the results of this study are the first quantitative assessments of the diversity and distribution of an abundant group of subterranean ants and the abiotic predictors of that diversity.

This study complements two other subterranean sampling studies in Florida (Tallahassee and Gainesville) and is one of few studies globally to assess abiotic predictors of subterranean ant diversity patterns (Thompson 1980, Lubertazzi & Tschinkel 2003). Ants in the *Solenopsis* genus dominate the subterranean thief ant communities in both north and central Florida. 15 total species were found in belowground samples here while 20 species were captured in north Florida (Lubertazzi & Tschinkel 2003). *Solenopsis pergandei*, was the most dominant species in our study, but not in north Florida. Thompson (1980) described *S. pergandei* as an "occasional dominant" species in north-central Florida (Gainesville). The dominant thief ant in both the Tallahassee and Gainesville studies was *S. carolinensis*. This indicates a transition between *S. pergandei* and *S. carolinensis* as dominant thief ants between central and north Florida. Other species occurrences, including *Pheidole dentata*, *P. floridana*, *P. metallescens*, and *Brachymyrmex depilis* were found in studies of Thompson (1980), Lubertazzi & Tschinkel (2013), and results here. Our study provides further evidence of the widespread, high abundances of thief ants in this region. It is also clear

that the subterranean ant communities of semi-tropical and temperate Florida are not as diverse as subterranean communities in the Neotropics (Thompson 1980, Lubertazzi & Tschinkel 2003, Wilkie & al. 2007) where as many as 47 species were recorded at local scales.

Sampling methods differed between 2012 aboveground sampling (pitfall traps) and belowground baits in this study; comparisons are made with caution. Aboveground samples collected more species (37 species in 18 genera), and abundances were more evenly distributed than in our belowground sampling. Aboveground, the genus *Pheidole* is most abundant followed closely by *Solenopsis* and *Camponotus*. *Solenopsis pergandei* and *S. tonsa*, two truly subterranean species, were not recorded in any of the aboveground traps. However, belowground, *Solenopsis* remains dominant by quite a large margin (Fig. 6). Aboveground species richness remains relatively the same with 32 species in the flatwoods and 35 in the sandhill. The aboveground ant community seems to have a higher abundance of individuals across the genera present in sandhill habitat when compared to flatwoods habitat. However, several genera show the opposite trend, including *Formica* and *Nylanderia*. Considering the temporal difference in the pitfall data and the subterranean data we suggest that it is possible that sandhill habitats may serve as areas of higher abundance of larger-bodied ants that can serve as potential prey for thief ants.

Lestobiosis and subterranean ant communities: This study affirms the general dominance of thief ants in Florida upland soils (Thompson 1980, Lubertazzi & Tschinkel 2003). If thief ants are truly lestobiotic, then their widespread abundance, now shown by three studies in Florida (including this one), suggests potential for substantial effects on co-occurring ants, including direct and indirect effects via brood raiding and generalist predation (Thompson 1980, Buren 1983, Lammers 1987, Nichols & Sites 1991, Yamaguchi & Hasegawa 1996, Vinson & Rao 2004). Further sampling is needed to evaluate subterranean ant communities among various ecosystems, and the environmental conditions that may potentially predict the diversity and distributions of these lesser-known ant communities.

Acknowledgments

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Symbiotic partnerships and their chemical interactions in the leafcutter ants (Hymenoptera: Formicidae)

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Abstract

Leafcutter ants are indigenous to Central and South America and the southern US and are noticeable for their active herbivorous behaviours, collecting mostly fresh plant parts to manure underground gardens of their *Leucoagaricus gongylophorus* fungal cultivars. These gardens contain a single clone of the cultivar but are also susceptible to pathogens, most notably the specialised mycopathogen *Escovopsis*. Pathogen pressure led to the evolution of intensive grooming behaviours assisted by antimicrobials produced in endocrine glands and by domesticated antibiotic-producing actinobacteria grown on the integument of workers. The most notable of these are *Pseudonocardia* species that are abundant in *Acromyrmex* but have been lost in *Atta* leafcutter ants. The leafcutter ant symbiosis represents a fascinating example of chemical warfare of which the details are becoming increasingly known. Here, we review recent progress in understanding the complex interactions that take place between the mutualistic and parasitic symbionts, particularly between the ants, their mutualistic fungal cultivars and cuticular actinobacteria, and their *Escovopsis* parasites.

Key words: Leafcutter ants, antibiotics, *Pseudonocardia*, *Escovopsis*, *Streptomyces*, review.

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Introduction

A leafcutter ant colony represents a remarkable example of multipartite symbiosis, including a mutualism between an ant colony, a clonal cultivated food fungus and, in the genus *Acromyrmex*, a predominantly single strain of *Pseudonocardia* actinobacteria (Fig. 1, Box 1) (Weber 1966, Hölldobler & Wilson 1990, Currie 2001, Andersen & al. 2013). The food fungus provides the ants with the ability to break down fresh plant material, which opened up a food source that was inaccessible to ancestral attine ants, a development that significantly enhanced larval provisioning so that colonies could become large (Mueller & al. 1998, Schultz & Brady 2008, De Fine Licht & al. 2010, Aylward & al. 2013). There is also a parasitic symbiont, the specialised mycoparasite *Escovopsis*, which kills and feeds on the hyphae of the *Leucoagaricus gongylophorus* cultivar (Currie & al. 1999a, Yek & al. 2012, de Man & al. 2016). The *Pseudonocardia* bacteria maintained by *Acromyrmex* leafcutter ants have a positive effect on the fitness of the ant colonies by producing antifungal compounds that are antagonistic towards *Escovopsis* (Samuels & al. 2013). While it is generally agreed upon that the cultivars of the higher attine ants, including the leafcutters, evolved the special hyphal tips called gongylidia that the farming ants feed on in response to full domestication, the extent to which mutualistic and antagonistic coevolution has shaped the details of extant interactions between the higher attine and leafcutter ants and their fungal and bacterial symbionts has remained remarkably controversial.

Attine ants are found throughout Central and South America with around 250 species belonging to 15 recognised genera. 95% of these are located in South and Central America and 5% are from the nearctic region (Mayhé-Nunes & Jaffé 1998, Branstetter & al. 2017). Across the genera, there are five grades of farming: lower agriculture, coral fungus agriculture, yeast agriculture, higher agriculture, and leafcutter agriculture (reviewed in Schultz & Brady 2008). Here, we focus on the positive and negative interactions between mutualists and parasites in the leafcutter ants (*Atta* and *Acromyrmex*) as these are the most conspicuous and evolutionarily derived genera and have been studied most intensively. *Atta* leafcutter ants develop large colonies capable of collecting enough leaf material to equal consumption of

Box 1: Important players in Attini-symbiont relationships.

Leafcutter ants (Order Hymenoptera: Family Formicidae) comprise the genera *Atta* and *Acromyrmex*. They actively cut leaves to manure gardens of their mutualist food fungus *Leucoagaricus*, which they groom and weed particularly to control the spread of *Escovopsis* mycopathogens.

Leucoagaricus gongylophorus (Order Agaricales: Family Agaricaceae) is a vertically transmitted obligate fungal cultivar of *Atta* and *Acromyrmex* species. The ants house and feed this fungus and in return it provides nutrients in the form of hyphal tips called gongylidia that are rich in lipids and sugars.

Pseudonocardia spp. (Order Actinomycetales: Family Pseudonocardiaceae) is a vertically transmitted bacterial mutualist that grows on the cuticles of *Acromyrmex*, but not *Atta* workers. It provides antifungal compounds used by the ants to control *Escovopsis* and antibacterials that prevent most other bacteria from invading its niche.

Escovopsis spp. (Order Hypocreales: Family Hypocreaceae) are co-evolved parasites of leafcutter ants that feed on the *Leucoagaricus* fungus and can cause colony collapse.

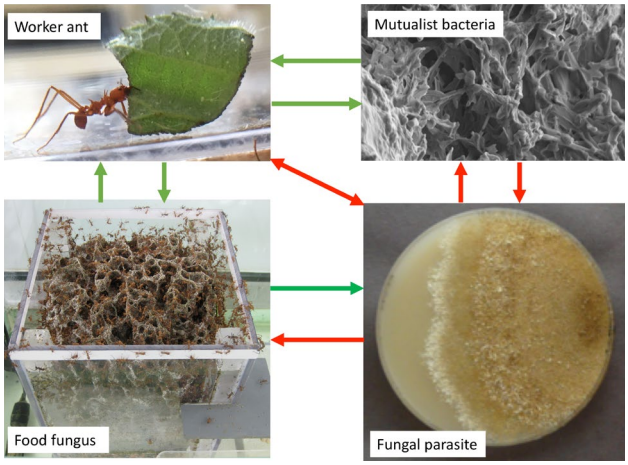

Fig. 1: Summary of the symbiotic relationships between *Acromyrmex* leafcutter ants and their partners. The ants provide freshly cut leaves to their clonal food cultivar *Leucoagaricus gongylophorus*, which in return is the sole food source for the ant larvae. Parasitic mycopathogens of the genus *Escovopsis* can infect the *Leucoagaricus* food fungus, an antagonism that the farming ants can control by weeding and grooming infected fungus-garden patches. *Acromyrmex* ants also have a mutualism with filamentous actinomycete bacteria of the genus *Pseudonocardia* that grow on their cuticle and have been inferred to be maintained via secretions of tiny subcuticular glands. These bacteria provide antifungal compounds to control *Escovopsis* and antibacterials to monopolise their cuticular niches. However, the mycopathogens have also evolved antibacterials to neutralise *Pseudonocardia* defences and neurotoxins to induce incoherent behaviour and enhanced mortality in the farming ants.

a large terrestrial mammal (Hölldobler & Wilson 1990, Herz & al. 2007). Colonies of *Acromyrmex* can number up to 50,000 workers and *Atta* up to 5 million. These ants are thus a considerable pest to farmers whose crops they consume (Hölldobler & Wilson 1990).

Attines first began to farm fungus from a single origin in the Neotropics approximately 55 - 60 million years ago (Currie 2001, Schultz & Brady 2008, De Fine Licht & al. 2010, Nygaard & al. 2016). At the outset of fungus farming, the ants appear to have farmed a variety of fungal cultivars, all belonging to a paraphyletic clade within the Leucocoprineae, which may have been regularly acquired *de novo* from their environment (Mueller & al. 1998, Currie 2001, De Fine Licht & al. 2010). The emergence of the obligately dependent, "higher attine" lineages occurred approximately 30 million years ago and coincided with a shift in garden substrate, from the debris collected by lower attines to supplementation with other plant material, and with a shift to drier habitats (Schultz & Brady 2008, Branstetter & al. 2017). This trajectory culminated in the evolution of the leafcutter ant genera (consisting of *Atta* and *Acromyrmex* species) approximately 15 million years ago (Schultz & Brady 2008, Nygaard & al. 2016, Branstetter & al. 2017). These genera almost exclusively collect fresh plant substrates for their fungal gardens that only ever consist of related clones of the fungus *Leucoagaricus gongylophorus* (Schultz & Brady 2008, De Fine Licht & al. 2014). Recently, it has been suggested that the fungal cultivar of leafcutters can be divided into two clades: one comprising *L. gongylophorus* which is often grown using dicotyledonous plants (though it has also been observed to be cultivated on grasses) found distributed in North America, Central America and South America, and another which is often grown using grasses found only in South America (Mueller & al. 2017).

Processing plant material: fungal enzymes

The primary importance of the attine fungal cultivar is to convert otherwise indigestible foraged plant material into nutrients that can be consumed by the ants, thus allowing them to make use of a food source that is not available to other ant species (De Fine Licht & al. 2010). These nutrients are available to the ants in the form of lipid and carbohydrate rich hyphal swellings, called gongylidia (De Fine Licht & al. 2014). These specialised feeding structures first evolved in the fungus when it became an obligate symbiont of the higher attines, approximately 30 million years ago (Schultz & Brady 2008, De Fine Licht & al. 2014). Gongylidia cluster together to form structures known as staphylae that are the primary food source for the colony, particularly the larvae, which exclusively feed on the fungus (Currie 2001, De Fine Licht & al. 2014).

The necessity to break down plant substrates in order to fuel fungal growth and supply important nutrients to their ant symbionts, has resulted in substantial changes to the metabolic and enzymatic capabilities of the fungal cultivar over evolutionary time (De Fine Licht & al. 2010). The fungal cultivars of lower attine species, which receive predominantly dead plant material and debris, are known

to have a similar enzymatic profile to those of free-living saprotrophic fungi which break down plant material by attacking the complex polysaccharide components of the cell wall such as hemicellulose (cross-linking glycans) and pectin, before fully degrading other cellular components such as cellulose and intracellular starch (De Fine Licht & al. 2010). However, an investigation of the *Leucoagaricus gongylophorus* fungal cultivar associated with leafcutting *Atta* and *Acromyrmex* species has revealed several differences in its metabolic profile (De Fine Licht & al. 2010, 2014). The *L. gongylophorus* genome encodes approximately 145 lignocellulase enzymes, including many pectinases, xylanases and amylase enzymes (Aylward & al. 2013). Comprehensive microarray polymer profiling of the garden strata of *Acromyrmex echinatior* colonies has suggested a rapid degradation of pectin in the upper layers of the garden where fresh leaf substrate is integrated by the ants (Moller & al. 2011). This initial disassociation of the pectin cell wall matrix causes disruption of plant cell walls and potentially allows the fungus better access to intracellular resources such as starch and protein (De Fine Licht & al. 2010, Schiøtt & al. 2010, Moller & al. 2011, Aylward & al. 2013, Kooij & al. 2014). Metaproteomic data and expression analysis also suggest a much higher level of endo-protease and alpha-amylase activity in the fungus compared to free-living counterparts and lower attines (De Fine Licht & al. 2010, Aylward & al. 2013). Cellulose and some xylans, on the other hand, are still found in high abundance in the lower garden strata as well as the waste dump material (Moller & al. 2011) and cellulases are expressed at much lower levels than in free-living fungi, suggesting that this is only partially degraded by the leafcutter crop fungus (De Fine Licht & al. 2010, Kooij & al. 2014). In support of this, expression analysis has suggested that cellulose conversion primarily occurs only towards the end of the plant decomposition process (at the bottom of the fungus garden) and may be more important in supporting the continued growth of the fungus and avoiding the build up of decaying mycelia in this region, rather than for providing nutrients to the ants (Grell & al. 2013). Together, these results suggest that *L. gongylophorus* has evolved to target superior plant cell resources, such as protein and starch, for the generation of fungal biomass and gongylidia, the primary nutrients source for the ants, rather than recalcitrant cell wall polysaccharides such as cellulose (De Fine Licht & al. 2010, Moller & al. 2011). This increasingly targeted use of specific plant cell material by *L. gonglyophorus* is thought to be a logical corollary of the much higher nutritious quality of the garden substrate in leafcutter ants (Kooij & al. 2014), because fresh plant material contains higher levels of protein than dead leaf litter, and increasing protein availability to the ants is desirable as this is normally a major factor limiting insect growth (Grell & al. 2013, Kooij & al. 2014). Down-prioritizing recalcitrant polysaccharides may also explain why increasingly larger quantities of leaf material are often collected by the more derived leafcutter genera since only a fraction of the molecular components are actually used by the fungus for biomass generation and the remainder is deposited as waste (Moller & al. 2011).

Fecal droplet enzymes

An additional layer of complexity in the degradation of plant material and further evidence of the emerging complementarity between the ants and their fungal symbiont is the finding that leafcutter ants are also able to concentrate key plant degrading enzymes in their faecal droplets, including cell wall degrading pectinases, proteases and laccases (Martin 1970, Schiøtt & al. 2010, De Fine Licht & al. 2014, Kooij & al. 2014). Laccases are thought to be involved in the degradation of lignin as well as the detoxification of plant secondary metabolites (De Fine Licht & al. 2014). When adding new material to the fungus garden the ants first masticate the plant material into tiny fragments to enhance fungal entry into the plant cell matrix as hyphae exclusively colonise the cut leaf edges (Erthal & al. 2009). The ants then apply the enzyme-containing fecal droplets to the newly inserted leaf fragments before inoculating them with fungal hyphae (Currie 2001, Erthal & al. 2009). However, although these enzymes are applied by the ant, studies have revealed that they initially derive from the gongylidia of the fungal cultivar (Martin 1970, Martin & Martin 1971, Schiøtt & al. 2010, Kooij & al. 2014). Substituting the ants' fungal diet with glucose eliminates enzymatic activity in fecal droplets (Schiøtt & al. 2010, Kooij & al. 2014) and several of the genes encoding the production of fecal fluid enzymes are upregulated in the gongylidia (Schiøtt & al. 2010, De Fine Licht & al. 2014, Kooij & al. 2014). In fact, of the seven protease enzymes identified in the fecal droplets of *Acromyrmex echinatior*, only a single one is produced by the ants (Kooij & al. 2014). These findings imply that coevolution between leafcutter ants and their fungal cultivar has enabled the ants to vector crucial enzymes from the more prolific central parts of the garden where there is an abundance of gongylidia, to the newly established and fast growing peripheral parts where no gongylidia are as yet produced (Kooij & al. 2014). At the garden periphery (usually mostly the top layer), these enzymes primarily aid in the initial breakdown of plant material (Schiøtt & al. 2010, De Fine Licht & al. 2014, Kooij & al. 2014). Evidence from the sequences of these fungus-derived enzymes also suggested signatures of positive selection, possibly to allow passage and survival through the ant gut (De Fine Licht & al. 2014). This, along with reciprocal benefits provided by the ants, may explain the evolution of gongylidia which represent a major metabolic investment by the mutualistic fungal cultivar (De Fine Licht & al. 2014).

Division of labour: the production of essential amino acids

The fungal cultivar plays a key role in providing ants access to carbohydrate and protein resources, allowing them to fill a niche not occupied by non-herbivorous species. However, another common outcome of an obligate mutualistic symbiosis is a close dependency of partner organisms upon one another due to the production and exchange of essential amino acids (Fisher & al. 2017). This can subsequently result in the reduction of the genomes of symbiotic partners (Martinez-Cano & al. 2014). A study of the *Acromyrmex echinatior* genome has revealed that this species lacks two key genes involved in arginine biosynthesis (Nygaard & al. 2011, 2016), similar to *Atta* (Suen & al. 2011). It is postulated that the ants receive all of their arginine from the fungal cultivar, since *Leucoagaricus gonglyophorus* encodes and expresses the full set of genes for arginine biosynthesis in its gongylidia (De Fine Licht & al. 2014). Genes for phenylalanine and tyrosine are also upregulated here, both of which are essential amino acids that are required in high abundance for cuticle production and growth of immature

insects (De Fine Licht & al. 2014). Phenylalanine and tyrosine are amongst the most expensive amino acids to produce in terms of ATP requirements (Barton & al. 2010), implying a division of labour between the ants and their cultivar (De Fine Licht & al. 2014).

Growing a fungal monoculture: ant and fungal defence strategies

Attine ant colonies and their fungal cultivars have a tight dependency upon one another in terms of accessing and exchanging essential nutrients and enzymatic compounds. However, it is vital that processes are in place to ensure the fungal cultivar is maintained free of parasitic microorganisms, and has preferential access to the leaf material supplied by the ants. Numerous studies of the makeup of ant fungus gardens have shown that, although the gardens are predominantly made up of the fungal cultivar, a diversity of other microorganisms can also be observed in the fungus-garden habitat. For example, several species of microfungi and yeast have been identified (Rodrigues & al. 2005, 2008a, 2009 & 2011) as well as a low diversity core group of bacteria, particularly consisting of gamma proteobacteria (Scott & al. 2010, Suen & al. 2010, Aylward & al. 2012a). Since the fungus is the sole food source of the ant brood it is expected that there will be a strong selective pressure to maintain the purity of the fungal cultivar. This has resulted in a suite of defensive and prophylactic adaptations in both the ants and the garden cultivars, many of which have attracted interest given that this fungal farming symbiosis appears to have suffered little from emerging resistance problems against its defences to pathogens and parasites (Poulsen & al. 2010). Monocultures of clonal fungal cultivars appear to be actively maintained, because chimeric fungus gardens have not been observed in field colonies (Green & al. 2002, Poulsen & Boomsma 2005, Dentinger & al. 2009, Mueller & al. 2010b, Mehdiabadi & al. 2012, Kooij & al. 2015), which appears to be mediated by incompatibility compounds in the mycelia and cultivar-derived fecal droplets that tend to eliminate newly introduced cultivars from sympatric colonies, particularly in *Acromyrmex* (Poulsen & Boomsma 2005, Kooij & al. 2015).

Prophylactic ant behaviour

In *Atta* and *Acromyrmex* species, waste is carried away to underground compost chambers of spatially separate waste piles downstream in order to minimise the spread of infection (Bot & al. 2001, Hart & Ratnieks 2002). The ants also try to minimise the introduction of foreign microbes into fungal gardens through a behaviour called "licking", which implies processing all freshly cut substrates through a filtering device within their oral cavity, known as the infrabuccal pocket, which acts to selectively remove microbes and hazardous debris from the newly collected forage-material (Eisner & Happ 1962, Currie & Stuart 2001). These are later expelled as compressed pellets onto the waste dump (Eisner & Happ 1962, Currie & Stuart 2001, Little & al. 2006). The ants also carry out "grooming" behaviours by pulling pieces of the growing fungus though their mouth parts to remove foreign microbes and their spores (Currie & Stuart 2001). Studies of the composition of infrabuccal pellets show that they contain non-viable fungal spores and tissue implicating the role of the pocket in detoxification of foreign material (Little & al. 2006). Several viable actinomycete bacterial species can also be isolated from pellets, particularly in colonies infected with the specialised fungal parasite *Escovopsis* (see Little & al. 2006). Bioassays have shown that these bacteria can inhibit *Escovopsis in vitro* suggesting that antibiotics produced by these bacteria may be partially responsible for the detoxification of fungal spores within the infrabuccal pockets (Little & al. 2006). It remains unclear whether these actinobacteria are permanently housed within the pocket or continuously acquired from the ants' cuticle (discussed later) in response to infections (Little & al. 2006). All of these protective behaviours increase upon the introduction of invasive spores to ant sub-colonies (Currie & Stuart 2001). These behaviours are even more intense when the cultivar is infected with *Escovopsis* but are not observed when irradiated non-viable *Escovopsis* spores are applied to the nest. This suggests that ants have the ability to detect foreign fungal infections, an observation which is supported by the fact that workers rapidly move into infected parts of the garden, possibly in response to chemicals signals (Currie & Stuart 2001, Ugelvig & Cremer 2007). Attine ants regularly carry out "weeding" of the fungus garden which can include the removal of infected areas of the cultivar (Currie & Stuart 2001).

Chemical defences of the ants

Leafcutter ants have a number of chemical adaptations to protect their fungal cultivar from aggressive microbes. One of these, the metapleural glands (MGs), is located on the posterior lateral end of the metathorax and continuously secrete a mixture of chemicals onto the surface of ants (Yek & Mueller 2011, Vieira & al. 2012). Such secretions are used by almost all major lineages of ants to ward off infection, but leafcutter ants groom their MG openings using specific foreleg movements and then spread the secretions to their cultivar (Fernandez-Marin & al. 2006, Aylward & al. 2012b, Vieira & al. 2012). MG secretions contain an abundance of hydroxyacids, including indolacetic acid and myrmicacin, which negatively influence bacterial and fungal growth and spore germination (Do Nascimento & al. 1996, Ortius-Lechner & al. 2000, Bot & al. 2002). In addition, a study of the MG secretions of *A. octospinosus* identified 20 other compounds including several fatty acids and alcohols, many of which have general antimicrobial properties against an array of bacteria and fungi (Ortius-Lechner & al. 2000, Bot & al. 2002). One of the most abundant secretions from the MGs of *Atta* is phenylacetic acid (PAA), which is absent in *Acromyrmex* species (Vieira & al. 2012, Fernandez-Marin & al. 2015). It has been demonstrated that this compound can act to inhibit mitosis and also the germination of fungal spores (Vieira & al. 2012, Fernandez-Marin & al. 2015). In addition to the direct antimicrobial functions seen *in vitro* for many MG compounds, the acidic nature of the combined set of secreted compounds is thought to play a primary role in maintaining a low pH in the fungal garden (Ortius-Lechner & al. 2000, Bot & al. 2002). This is likely to maintain an optimal pH for the growth of the *Leucoagaricus gongylophorus* cultivar, and has been hypothesised to inhibit the spread of unwanted microbes in the garden – for example, the growth of several pathogenic bacteria is detrimentally affected by low pH (Ortius-Lechner & al. 2000, Bot & al. 2002).

Beyond bioassays of individual MG compounds, several attempts have been made to determine the extent to which the secretions have hygienic functions *in vivo*. Some authors

have suggested that the greater number of MG cells per unit biomass and the greater relative size of MGs of minor garden workers, which are more important in the maintenance of the crop fungus, is circumstantial evidence for their explicit role in the protection of the fungus garden (Poulsen & al. 2003, Vieira & al. 2012). Poulsen & al. (2003) used a more direct approach and showed that, although the secretions impose substantial metabolic costs on the ant, they are continuously secreted under natural conditions. Active secretion was also important in protecting the ants against an entomopathogenic fungus *Metarhizium anisopliae*. When glands were experimentally closed there was a much greater level of mortality caused by these infections, suggesting that the antifungal activity of MG secretions was important for the protection of the ants themselves (Poulsen & al. 2003). Another study showed that the observed level of MG grooming and the transfer of compounds to the fungus significantly increased when *Atta* colonies were exposed to fungal infection (Fernandez-Marin & al. 2015). PAA could be detected in these gardens, as well as on the forelegs of ants, but only in infected fungal gardens (Fernandez-Marin & al. 2015). This selective and pointed application on demand, rather than continuous prophylactic use, of PAA has been suggested to be a key factor in reducing the evolution of resistance to metapleural gland secretions in the ant-fungus system (Fernandez-Marin & al. 2015). Other authors have also suggested that ants may be able to alter the composition and relative concentrations of their MG secretions to specifically target individual pathogenic species, increasing the specificity of this process and minimising unnecessary exposure to preserve the efficacy of these antimicrobials (Bot & al. 2002, Fernandez-Marin & al. 2006, Yek & Mueller 2011, Fernandez-Marin & al. 2015).

In addition to MG secretions, mandibular secretions and compounds in the fecal fluid of *Atta* ants also reduce spore germination of particular species of microfungi found in their fungal gardens (Rodrigues & al. 2008b). Together, these chemical adaptations act in a synergistic fashion with protective ant behaviours and likely reinforce protection provided by symbiotic actinobacteria in *Acromyrmex* species. Such a multifaceted and adaptable set of defences is believed to have been a key factor underlying the success of leafcutter ants, and likely fungus-growing ants in general, in preventing the overgrowth of fungal cultivars and the evolution of resistance against defensive compounds. The prudency of operating with multiple lines of defence that can each be applied specifically rather than prophylactically is now beginning to take hold in human medical endeavours (Fortman & Mukhopadhyay 2016).

The evolutionary dynamics of ant defences against disease

In general, ants are remarkably efficient in disease defence, which has meant that very few specialised diseases of ants are known (Cremer & al. 2018). This appears to be mediated by extremely efficient combinations of individual and social immune defences that have remained robust over evolutionary time. With that perspective it is perhaps not surprising that the leafcutter ants were able to both maintain these general defences, and to extend them to include active control of diseases in their fungus gardens, where as far as known their only specialised and co-evolving diseases occur. More efficient defence against disease pressure has likely been one of the selective forces shaping the evolution of obligate multiple insemination of queens in the *Atta* and *Acromyrmex* leafcutter ants (Villesen & al. 2002). Multiple queen mating implies that colonies become "chimeric" in the sense that workers are an assembly of full-sisters (patrilines) that are half-sisters to each other. The ensuing higher genetic diversity of colony workers as collective likely gave a series of advantages related to division of labour and disease defence in the leafcutter ants with their large and long-lived colonies, relative to the basal attine genera with smaller colonies that retained ancestral single insemination of queens (Boomsma & al. 2009). The leafcutter ants also evolved higher degrees of caste polymorphism, that is, distinct small and large workers in *Acromyrmex* and even higher worker caste diversity in *Atta*, which also evolved a specialised soldier caste. Several studies have shown that this enhanced social diversity boosted colony-level social immunity of *Acromyrmex* colonies (Hughes & Boomsma 2004, 2006, Hughes & al. 2008). Another study showed that there is significant genetic variation in the relative size of metapleural glands, particularly in small workers who are most active in disease defence. This suggests that some worker-patrilines specialise on specific defence activities not shared by other patrilines, consistent with the hypothesis that genetically diverse colonies are more robust in their collective social immune defences (Hughes & al. 2010).

Fungal defence compounds

Although the ants play an integral role in providing protection to their fungal cultivars, the crop-symbionts have also evolved their own set of defences that work in concert with the ants and respond to a variety of infections. Basidiomycete fungi are generally known to encode a diversity of secondary metabolites including several antimicrobial compounds (Aylward & al. 2012b). Despite this, relatively few studies have actually investigated the bioactive potential of the ant fungal cultivar and there have often been variable results. Several of the attempts to demonstrate antifungal activity of ant cultivar extracts have been negative (Martin & al. 1969, Weber 1972, Hervey & al. 1977) but two compounds, 7-Chloro-4,6-dimethoxy-1(3H)-isobenzofuranone and basidalin, have been isolated from the fungal species *Leucoagaricus carneifolia* (a close relative of *L. gongylophorus*), which demonstrated weak to strong bioactivity against several bacterial and fungal species *in vitro* (Huff & al. 1994). Another study has shown that fungal cultivars isolated from the nests of a basal attine ant of the genus *Apterostigma* can, at least to some extent, suppress the growth of a variety of morphologically distinct *Escovopsis* lineages on agar plates, suggesting the cultivars may also have some of these defensive capacities *in vivo* (Gerardo & al. 2006a). A study of the uniquely unicellular yeast form of the cultivar maintained by *Cyphomyrmex minutus* ants, also showed that the fungus was capable of strong antifungal activity when grown on plates (Wang & al. 1999). Organic extracts from the fungus revealed three diketopiperazines which, when isolated, showed moderate inhibition of fungi in bioassays (Wang & al. 1999). It was hypothesised that this fungal cultivar may actually secrete a more potent mixture of these compounds *in vivo* leading to a greater degree of inhibition when live cultivars rather than extracts would be used. Diketopiperazine compounds have additionally been implicated to have antibacterial properties (Arnone & al. 1966). Leafcutter cultivars are also able to inhibit *Escovopsis* strains isolated from sym-

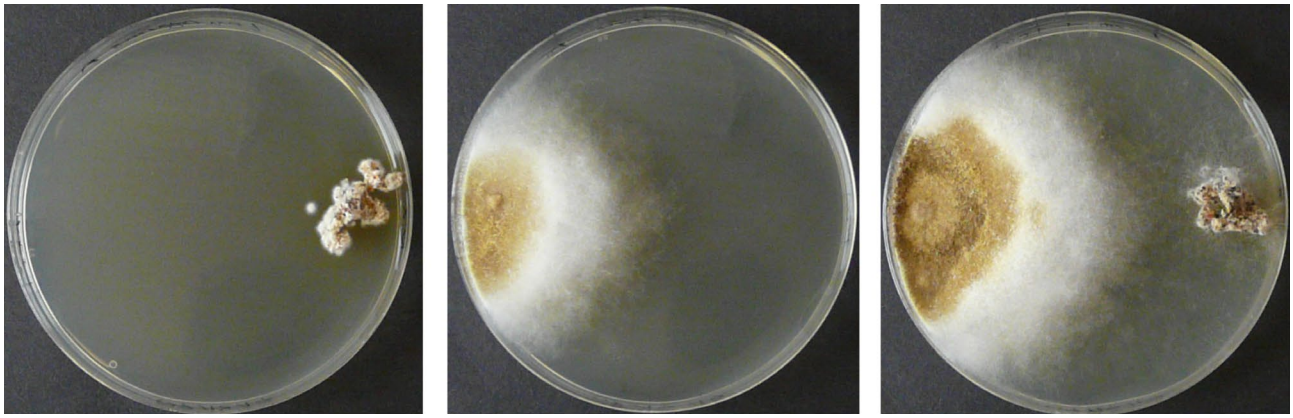

Fig. 2: *Escovopsis* fungi are attracted by and feed upon *Leucoagaricus gongylophorus* cultivars. *Escovopsis* grown on potato glucose agar plates with pieces of *Leucoagaricus gongylophorus* (right panel) grow faster than when grown alone (centre panel). The left panel displays a culture of *Leucoagaricus gongylophorus* growing alone.

patric colonies of phylogenetically more basal attine ants, especially those isolated from *Apterostigma* colonies with pterulaceous fungal cultivars (Birnbaum & Gerardo 2016). These small colonies are often found in considerable numbers in the close proximity of *Atta* and *Acromyrmex* colonies, so further work on the chemical interactions underlying these patterns of defence specificity would be of interest.

Diversity and virulence of *Escovopsis*

Parasitism of attine ant nests by *Escovopsis* fungi is an ancient symbiosis that evolved shortly after the ancestral attine ants adopted a farming lifestyle 55 - 60 million years ago (Yek & al. 2012, Nygaard & al. 2016). *Escovopsis* belongs to the order Hypocreales, which comprises a number of mycoparasitic fungi, including the biocontrol agents *Trichoderma* and *Clonostachys* and the specialised *Tolypocladium* species that are insect pathogens and can also attack ectomycorrhizal truffle fruiting bodies (for a recent review of necrotrophic mycoparasites see Karlsson & al. (2017). Traditionally there were just two species of *Escovopsis* recognised, *E. weberi* and *E. aspergilloides*, based on the formation of cylindrical or globose vesicles on their spore-bearing cells. However, a recent phylogenetic study suggests there are in fact nine lineages of *Escovopsis* belonging to five species (Meirelles & al. 2015). A sixth species, *E. trichodermoides*, was subsequently isolated from a nest of the lower attine ant, *Mycocepurus goeldii*, and does not have vesicles and phialides, but instead develops spores on a trichoderma-like conidiophore (Gerardo & al. 2006b, Masiulionis & al. 2015). Although *Escovopsis* evolution is heavily dependent on its ability to parasitise attine ant cultivars, the phylogeny of *Escovopsis* strains does not match the phylogeny of the attine ants they were isolated from (Currie & al. 2003b). This may be due to rare horizontal transfers of *Leucoagaricus* cultivars across attine ant genera or may suggest that closely related *Escovopsis* strains are capable of infecting a wide variety of attine ant cultivars (Currie & al. 1999a).

Escovopsis hyphae can be observed to grow towards *Leucoagaricus in vitro* (Fig. 2) and direct contact between their hyphae can be an important factor in causing the degradation of *Leucoagaricus* (Marfetán & al. 2015, Varanda-Haifig & al. 2017). However, this process does not necessarily require contact between hyphae since *Escovopsis* can also produce soluble factors, including a number of toxins and enzymes, which may diffuse towards *Leucoagaricus* from a distance and contribute to cultivar degradation (Reynolds & Currie 2004, Marfetán & al. 2015, Varanda-Haifig & al. 2017). The 27 Mbp genome of *Atta*-derived *Escovopsis weberi* is small relative to its closest free-living relatives consistent with specialisation and gene loss in this parasite (de Man & al. 2016). This was further supported by sequencing of *Escovopsis* genomes from *Acromyrmex echinatior* (~30 Mbp) and additional *Atta* colonies (~27 Mbp) (Heine & al. in press). Primary metabolism genes are still present in *E. weberi*, but the number of carbohydrate metabolism genes has been reduced and genes necessary for sexual reproduction have also been lost. *Escovopsis* has not been isolated from environments outside attine ant nests, consistent with this genus being fully specialised on its attine host cultivars and being unable to reproduce away from attine ant colonies (Seifert & al. 1995, Currie & al. 1999a).

Attine ants suppress *Escovopsis* using behavioural mechanisms, as well as bioactive molecules produced by their MGs and their bacterial mutualists. Anecdotal evidence suggests that when infections are severe the ants abandon their garden after which it is rapidly overgrown by *Escovopsis* (Currie & al. 1999a). This implies that weeding and grooming behaviours of worker ants are essential to keep *Escovopsis* at bay. *Escovopsis* is consistently present in the waste dumps of uninfected nests and spores can thus be picked up by worker ants (Augustin & al. 2017). A possible infection mechanism suggested by these observations is that spores of *Escovopsis* are washed from waste dumps of attine ants into the soil of nearby foraging tracks where they may be picked up by foraging workers and carried back to the nest. Unlike the mutualist cultivar fungus, *Escovopsis* is not vertically transmitted by founding queens and the claustral founding colonies of *Atta* do not contain *Escovopsis,* so that infections only emerge after the first foraging workers start to leave the nest (Currie & al. 1999a). More detailed studies on the spread of *Escovopsis* species between attine colonies are sadly lacking and should be the focus of future research.

***Escovopsis* compounds**

The first *Escovopsis weberi* genome sequence, from a strain isolated from an *Atta cephalotes* colony, revealed that it

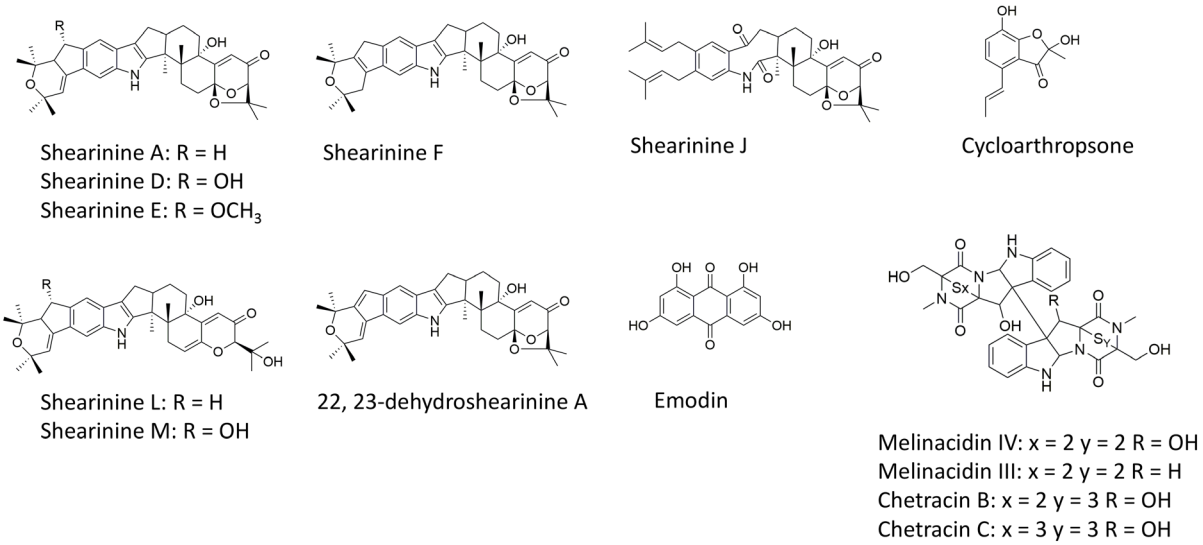

Fig. 3: Bioactive compounds produced by *Escovopsis* fungi. *Escovopsis* mycopathogens synthesise triterpenic indole alkaloid shearinine compounds as well as emodin, cycloarthropsone, and epipolythiodiketopiperazine alkaloids of melinacidins and chetracins.

encodes a number of putative secondary metabolites (de Man & al. 2016). Furthermore, RNA sequencing of this *E. weberi* strain growing towards its host revealed that some of the genes for these compounds were upregulated, implying a role in pathogenesis. A separate study attempted to identify secondary metabolites produced by an *Escovopsis* species growing alongside an ant-associated *Streptomyces* strain using imaging mass spectrometry (Boya & al. 2017). The *Escovopsis* species was not identified but the study showed that this isolate produced shearinines D, F and J (Fig. 3). The shearinines are triterpenic indole alkaloids with activity against calcium-activated potassium channels (Xu & al. 2007) and were first isolated from an endophytic fungus *Penicillium janthinellum*. More recently, cultivation of an *E. weberi* strain on solid agar led to the detection of five shearinines (A, E, D, L & M), cycloarthropsone and emodin (Fig. 3) (Dhodary & al. 2018). These compounds were also detected on plates with *Leucoagaricus gongylophorus* and two of the shearinines, L and M, are novel compounds. The shearinines are part of a larger group of metabolites, the penitrems, which are mycotoxins associated with insecticidal and tremorgenic activity (Staub & al. 1993). Similar fungal alkaloids are encoded by *Ophiocordyceps* fungi (de Bekker & al. 2015) and induce "zombie" behavior in infected ants (details below). Provision of oat flakes coated with shearinine L to subcolonies of *A. octospinosus* eventually led to them being rejected for incorporation into the fungus garden (Dhodary & al. 2018), presumably due to shearinine L being repellent as a substrate for *L. gongylophorus*. Shearinine L didn't directly lead to a zone of inhibition against *L. gongylophorus* whereas emodin and cycloarthropsone both displayed antifungal activity against the nest cultivar (Dhodary & al. 2018). Emodin has previously been known to have a number of activities including antibacterial (Levin & al. 1988), antifungal (Izhaki 2002), antiviral (Barnard & al. 1992), anticancer (Liu & al. 2011), anti-inflammatory (Park & al. 2009) and antiulcerogenic (Goel & al. 1991) effects. It is also known to be an insecticidal compound against mosquitoes (Yang & al. 2003), caterpillar larvae (Trial & Dimond 2012) and adults of the white fly *Bemisia tabaci* (Georges & al. 2008). Cycloarthropsone has been isolated previously as a fungal metabolite from *Arthropsis truncata* (Ayer & Craw 1992), but a functional role for this compound has not been determined. More recently, artificial infection of *Leucoagaricus* nest material with *E. weberi* led to the identification of two upregulated compounds; shearinine D and melinacidin IV (8.9- and 3.4-fold, respectively), when *E. weberi* was grown on *Leucoagaricus* (Heine & al. 2018). However, monocultures of *E. weberi* also produced shearinine D, shearinine A, 22,23-dehydroshearinine A, melinacidin IV, melinacidin III, chetracin B and C and emodin (Fig. 3) (Heine & al. 2018). Shearinine D was shown to reduce the mobility of *A. echinatior* worker ants, leading to uncoordinated behaviour, spasmodic leg movements, and ultimately death. These observations are consistent with the roles of shearinines as ion channel modulators and potential neurotoxins. Finally, shearinine D was also shown to be active against the two *Pseudonocardia* species associated with *A. echinatior ants*, *P. echinatior* and *P. octospinosus*, previously known as Ps1 and Ps2 (Poulsen & Boomsma 2005, Andersen & al. 2013, Holmes & al. 2016), suggesting that this might be used by *E. weberi* to antagonistically suppress the antifungal producing mutualist *Pseudonocardia* bacteria (Heine & al. 2018). Melinacidins have antibacterial activity against Gram-positive bacteria (Reusser 1968) and also inhibit the *Pseudonocardia* strains associated with *A. echinatior* ants, suggesting they also play a role in counteracting the inhibitory effects of *Pseudonocardia* on *Escovopsis* (Heine & al. 2018). The chetracins are cytotoxic compounds, previously identified from the fungus *Oidiodendron truncatum* (Li & al. 2012), but their effects on the mutualists in the *A. echinatior* system have not been tested.

The *Pseudonocardia* mutualists

Many attine worker ants have a visible white bloom on their cuticles, often concentrated on their laterocervical plates (Fig. 4). This white covering was eventually identified

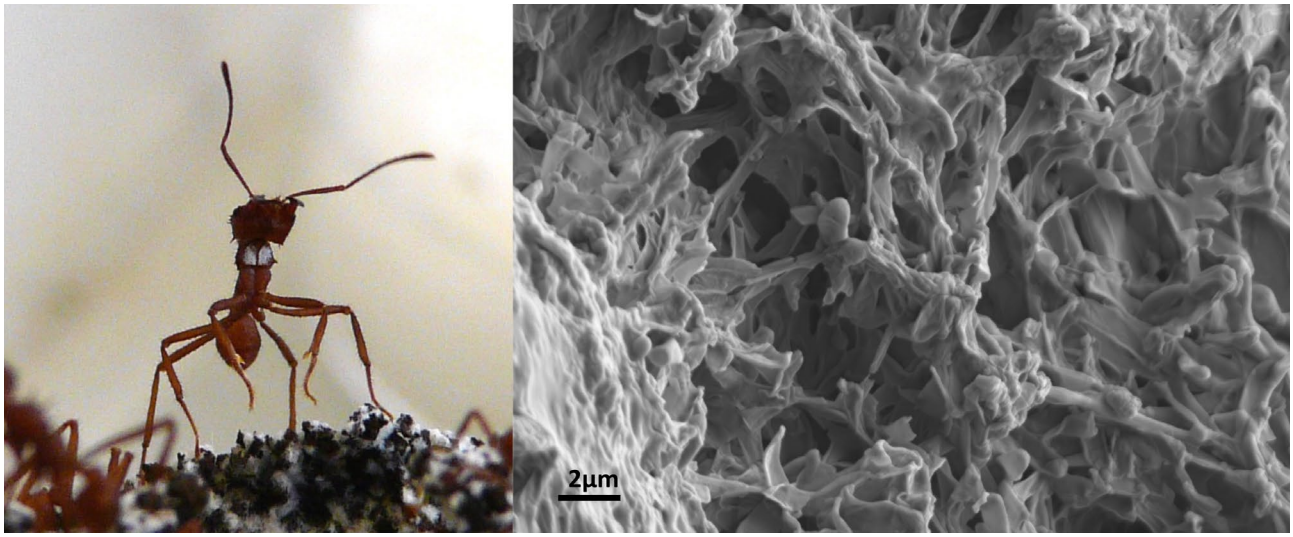

Fig. 4: Filamentous actinobacterial mutualists growing on *Acromyrmex echinatior* ants. *Pseudonocardia* bacteria can be seen growing on the integument of *Acromyrmex echinatior* ants (left panel) where they appear as a white covering on young callow ants or on the laterocervical plates of more mature workers. The filamentous growth of this actinobacterial mutualist can be readily observed using electron microscopy (right panel).

as a dense filamentous growth of actinomycete bacteria, mostly belonging to the genus *Pseudonocardia* which can be isolated from the cuticles of *Acromyrmex* but not *Atta* leafcutter ant workers (Currie & al. 1999b). On a smaller scale, these bacterial patches concentrate in species-specific cuticular regions where subcuticular exocrine glands are hypothesised to provision the bacteria (Currie & al. 2006). The bacteria were shown to produce antifungal antibiotics, presumably in return for ant resources, that inhibit the growth of *Escovopsis* (Fig. 5) (Currie & al. 1999b, Currie & al. 2003a). These defensive compounds, in addition to the ants' behavioral defences, are likely to be important in providing protection to the fungus gardens, which may be particularly prone to infection because they grow as monocultures and have low genetic diversity (Kost & al. 2007, Mueller & al. 2010b). *Pseudonocardia* has since been found to be faithfully transmitted between generations within a colony with newly eclosed workers being inoculated by older workers within 24 hours after hatching (Marsh & al. 2014). Partner Fidelity Feedback (PFF) has therefore been suggested to help maintain this mutualism since the fitness interests of both partners remain aligned during the life of a colony; ant colony productivity increases due to the bacterially-derived antimicrobial substances and this, in turn, improves the likelihood of bacterial transmission to the next generation via dispersing virgin queens that found new colonies (Foster & Wenseleers 2006, Barke & al. 2011). A tight relationship between the *Acromyrmex* worker ants and their *Pseudonocardia* mutualist strains may also have facilitated the on-going dynamics of a co-evolutionary arms race between these bacterial symbionts and the *Escovopsis* pathogen, possibly helping to explain the apparently low levels of observed antimicrobial resistance (Mueller & al. 2005, Currie & al. 2006). It is noteworthy that *Atta* leafcutter ants lack the cuticular crypts that maintain the *Pseudonocardia* symbionts in *Acromyrmex* and that isolation of *Pseudonocardia* in *Atta* yields negligibly low results which are comparable to levels expected for contaminations (Mueller & al. 2008, Marsh & al. 2013). The loss of *Pseudonocardia* might be attributed to the gain of PAA produced by the MG secretions, a powerful antifungal compound that may have made the antifungals produced by *Pseudonocardia* redundant (Vieira & al. 2012, Fernandez-Marin & al. 2015).

Strains of mutualistic *Pseudonocardia* produce antifungal antibiotics with novel structures, such as the cyclic depsipeptide dentigerumycin in the lower attine *Apterostigma*, and the polyene nystatin P1 (Fig. 5) in *Acromyrmex* (Oh & al. 2009, Barke & al. 2010). Further evidence of the interaction specificity between *Pseudonocardia* and leafcutter ants has emerged from the finding that colonies of *Acromyrmex echinatior* tend to maintain a single strain of *Pseudonocardia*, either Ps1 or Ps2 (Poulsen & al. 2005, Andersen & al. 2013). Cross-fostering experiments have suggested some degree of co-adaption between each particular *Pseudonocardia* strain and the ants that vertically-transmit them (Andersen & al. 2015). A population of each of the two *Pseudonocardia* strains was genome sequenced, which allowed them to be identified as two distinct species, named *P. octospinosus* (Ps1) and *P. echinatior* (Ps2) (Holmes & al. 2016).

Construction of a wider attine ant derived *Pseudonocardia* phylogeny revealed that the predicted model of vertical transmission which would result in coevolution and codivergence does not fit observed taxonomic trees based on 16S rRNA gene sequencing (Mueller & al. 2010a). This suggests that *Pseudonocardia* strains either occur in other microbiomes of the ants (e.g., the guts) or that they can be free-living in the environment before they are acquired as ant symbionts, possibly to replace an existing *Pseudonocardia* symbiont whose secondary metabolites *Escovopsis* has become resistant to (Poulsen & al. 2010). *Pseudonocardia* strains have also been predicted to set up a complex partner choice mechanism using the antibacterial antibiotics that they produce to defend their primacy of place in the ant cuticular biofilms (Scheuring & Yu 2012). That scenario implies that only other actinobacteria with similar antibiotics production can invade *Pseudonocardia*

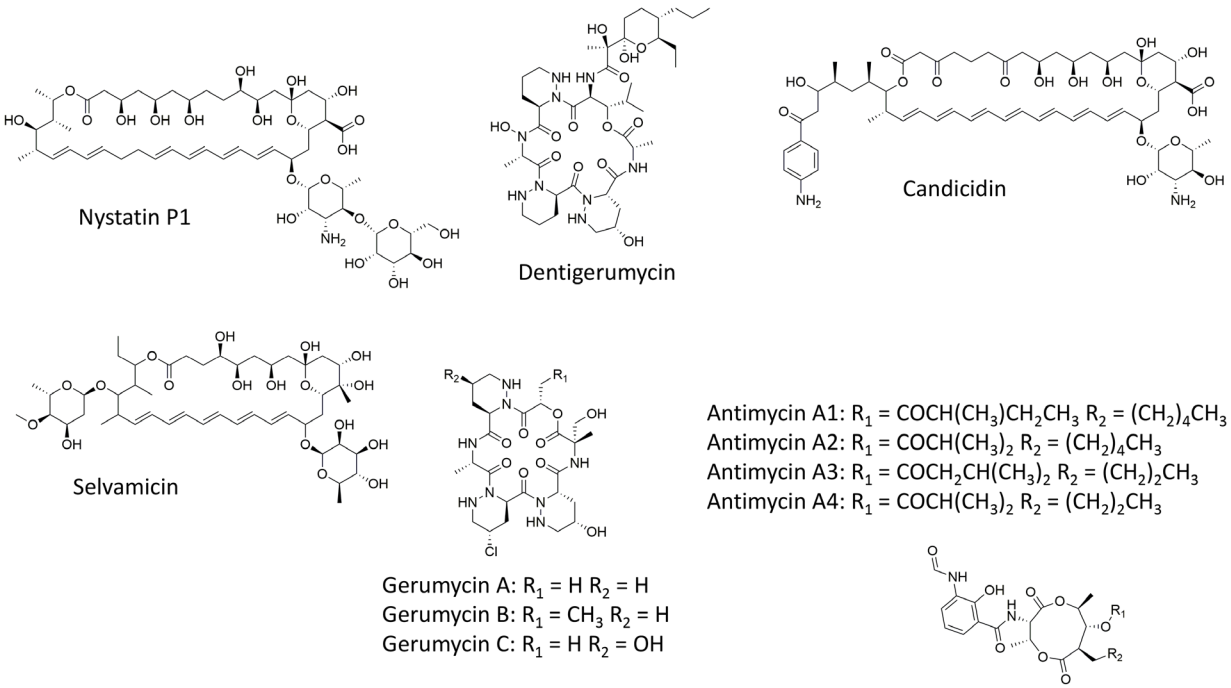

Fig. 5: Bioactive compounds produced by filamentous actinobacteria isolated from attine ants. *Pseudonocardia* isolated from various attine ants have been demonstrated to make antifungal polyenes such as nystatin P1 and selvamicin as well as the antifungal gerumycins. *Streptomyces* isolated from lab colonies of *Acromyrmex octospinosus* and *Trachymyrmex cornetzi* make the antifungals candicidin and the antimycins.

biofilms established by vertical transmission within colonies, because these producers are usually also resistant to multiple antibiotics (Barke & al. 2011).

Pseudonocardia-produced antifungal compounds

The first compound to be detected from a *Pseudonocardia* mutualist of attine ants was the antifungal dentigerumycin (Oh & al. 2009). Dentigerumycin is a cyclic depsipeptide made by a *Pseudonocardia* strain associated with the lower attine *Apterostigma dentigerum* in Panama. It is active against the nest pathogen *Escovopsis* and can be actively used by the ants to keep their fungus garden clear of *Escovopsis* infection (Oh & al. 2009). Dentigerumycin was later shown to be produced by another *Pseudonocardia* strain associated with Panamanian *Trachymyrmex cornetzi* ants (Sit & al. 2015), suggesting this molecule might also occur in fungus-farming ants with cuticular *Pseudonocardia*. Variants of dentigerumycins called gerumycins A-C are produced by another *Pseudonocardia* strain associated with *A. dentigerum* ants (Sit & al. 2015). Gerumycins A-C contain an almost identical depsipeptide to dentigerumycin but are distinguished by the absence of the PKS derived side chain (Fig. 5). The arrangement of genes in the biosynthetic gene clusters that encode these molecules show hallmarks of recent evolution (Sit & al. 2015). A full suite of PKS and NRPS genes are present in one chromosomal location for dentigerumycin but the PKS genes are absent and do not contribute to the final molecule for the gerumycins. Instead, the clusters are situated on mobile elements and are either spatially distributed on plasmids and split into pieces or located in so-called recently acquired "genomic islands" on the chromosome (Sit & al. 2015). The genes are likely under direct selective pressure to keep up in an arms race with the *Escovopsis* strains that infect the fungus gardens that the ants maintain.

Pseudonocardia octospinosus (Ps1) isolated from a captive colony of *Acromyrmex octospinosus* ants was discovered to produce a nystatin-like polyene (Barke & al. 2010). Nystatin A1 was originally discovered from the terrestrial strain *Streptomyces noursei* and is widely used as an antifungal drug (Brautaset & al. 2000). *P. octospinosus* (Ps1) was demonstrated to make a similar molecule with an additional hexose sugar that was subsequently named nystatin P1 (Fig. 5). More recently, genome sequencing of *P. echinatior* and *P. octospinosus* populations associated with captive colonies of *A. echinatior* ants demonstrated they have matching nystatin polyene biosynthetic clusters (Holmes & al. 2016). *P. echinatior* strains also have nystatin-like biosynthetic gene clusters that do not resemble those encoding nystatin A1 or P1 biosynthesis and potentially encode as yet unidentified novel nystatin-like polyenes. Furthermore, a *Pseudonocardia* strain isolated from *Trachymyrmex cornetzi* also contained a biosynthetic gene cluster that encodes a polyene resembling nystatin P1 (Sit & al. 2015). A free-living terrestrial strain of *Pseudonocardia autotrophica* was also shown to make the polyene molecule NPP, which is similar to nystatin P1 (Kim & al. 2009), and it was shown that a similar additional hexose sugar made NPP 100x more soluble in water (Lee & al. 2012). Deletion of the additional glycosyl transferase gene in *Pseudonocardia autotrophica* and heterologous expression of the *nypY* glycosyl transferase gene from *Pseudonocardia* P1 resulted in addition of D-mannose which demonstrated lower antifungal activity than nystatin A1, but is presumably more soluble in aqueous solutions (Kim & al. 2017). This raises interesting questions as to the functional relevancy of these molecules

in different ecological settings (e.g., dry and wet habitats) that may be associated with different *Escovopsis* strains. Further evidence that polyenes are important antifungals in the attine fungus farming symbiosis comes from the discovery of the polyene selvamicin from a *Pseudonocardia* strain associated with *Apterostigma* ants (Van Arnam & al. 2016). Selvamicin is a structurally distinct (Fig. 5) and shorter polyene than nystatin A1, P1 or NPP. It has a 6-deoxymannose sugar added at the canonical glycosylation site, but it also has a distinctive 4-*O*-methyldigitoxose on the other side of the molecule. The genomes of *P. echinatior* and *P. octospinosus* strains also suggest evolutionary divergence in their nystatin-like biosynthetic gene clusters with a set of representative strains demonstrating subtle variations and individual clusters sometimes being split into separate loci (Holmes & al. 2016). The variety of polyenes and their encoding biosynthetic gene cluster arrangements are generally consistent with ongoing evolutionary selection pressure and may also suggest a certain degree of variation in the compounds that can be produced across different colonies of the same attine ant species.

Other ant-associated symbionts

A diversity of other antibiotic-producing bacterial species have been isolated from the cuticles of attine ants, some of which have also been shown to inhibit the growth of *Escovopis* and other pathogenic microbial species (Kost & al. 2007, Haeder & al. 2009, Sen & al. 2009, Barke & al. 2010, Schoenian & al. 2011). In particular, several members of the genus *Streptomyces*, which are common in soil, and produce ~55% of the antibiotics used in human medicine and husbandry, have been shown to associate with *Acromyrmex* leafcutter ants, although with a high level of variability across different colonies of the same ant species (Andersen & al. 2013). Such findings have led to the suggestion that leafcutter ants may be actively and dynamically recruiting other antibiotic-producing bacterial symbionts from their environment, following the initial colonisation of their cuticular chest plates with vertically acquired *Pseudonocardia* (Kost & al. 2007, Mueller & al. 2008, Barke & al. 2010, Andersen & al. 2013). The external morphology of the ant cuticular crypts may have evolved to facilitate this process (Mueller & al. 2008), but it remains to be seen how common such secondary acquisitions are under natural field conditions since most studies that have isolated sctinobacterial symbionts have been conducted using laboratory-maintained colonies of *Acromyrmex*. Secondary acquisitions of non-*Pseudonocardia* actinobacteria may give the ants access to a more diverse set of antimicrobial compounds, possibly enabling defence against a larger variety of pathogens (Barke & al. 2011), but it has also become clear that *P. echinatior* (Ps2) and *P. octospinosus* (Ps1) biofilms on the cuticles of Panamanian *Acromyrmex* workers differ in the degree to which they allow secondary invasion by other actinobacteria (Andersen & al. 2013).

It is not known how the ants accumulate, regulate and maintain their cuticular microbiome, allowing antibiotic producers to dominate, whilst preventing colonisation by non-producers. A recent theoretical model suggests that the cuticular microbiomes are regulated via a screening process, whereby the nutrient rich conditions surrounding the cuticular crypts on the surface of the ant sets up a highly competitive environment for colonising microorganisms (Scheuring & Yu 2012). This would select for greater antibiotic production by both the native *Pseudonocardia* symbionts and any other bacteria able to invade. Since antibiotic producing bacteria also carry antibiotic resistance genes they are likely to preferentially survive in this demanding environment so the ants will always end up being covered with antibiotic-producing actinomycetes independent of their taxonomic identity (Scheuring & Yu 2012). Consistent with this, the *Pseudonocardia* symbionts have been shown to encode and produce a broad spectrum of antibacterial compounds (Holmes & al. 2016). *Streptomyces* found on leafcutter ants have been shown to make various antimicrobials including candicidin and antimycins (Fig. 5)(Haeder & al. 2009, Barke & al. 2010, Schoenian & al. 2011, Seipke & al. 2011), both of which have antifungal activity and are active against *Escovopsis*. A study using matrix-assisted laser desorption ionization (MALDI) on *A. echinatior* ants and their *Streptomyces* symbionts detected the production of antimycins, valinomycins and actinomycins (Schoenian & al. 2011). Valinomycin was also detected directly on the exterior of the ants, whereas valinomycin and actinomycins were identified from the waste dump. To our knowledge this is the only report showing antibiotics being produced *in situ* in a leafcutter ant colony, but whether *Streptomyces* species are associated with *Acromyrmex* colonies in the field remains to be confirmed.

In addition to cuticular symbionts, Panamanian *Acromyrmex* leafcutter ants also harbor a relatively simple community of gut bacteria. Four bacterial taxa (a *Wolbachia* species, a species from the order Rhizobiales and two Mollicutes species from the order Entomoplasmatales) dominate the gut microbiome (Sapountzis & al. 2015). *Wolbachia* bacteria appear to be obligate symbionts that are maintained across all developmental stages of *Acromyrmex* but not *Atta* leafcutter ants, and to be maternally transmitted because they could be retrieved from the eggs (Zhukova & al. 2017). They exist intracellularly, interacting closely with mitochondria in the ant cytoplasm (Zhukova & al. 2017), but can also be found extracellularly. Further work is required to clarify their functional significance. The functions of the Entomoplasmatales species are also unknown but they appear to be facultative symbionts as there is variation in their abundance across and within *Acromyrmex* colonies (Zhukova & al. 2017). Rhizobiales bacteria are confined to the gut lumen where they form biofilms along the hindgut cuticle (Sapountzis & al. 2015). These bacteria have been shown to produce bacterial NifH proteins that are normally associated with the fixation or preservation of nitrogen (Sapountzis & al. 2015). Although further confirmation is needed, it is hypothesised that these highly compartmentalised symbionts somehow help to alleviate the nutritional constraints that emerge from an exclusive fungal diet which, in turn, is provisioned solely with leaf material (Zhukova & al. 2017). Finally, the bacterial genus *Enterobacter* was shown to be abundant in larval guts of both *Acromyrmex* and *Atta* but to be absent in the guts of adult workers. It has been suggested that these may be involved in immune priming, reducing the susceptibility of larvae to pathogens that they may encounter later in life (Zhukova & al. 2017).

In addition to symbionts found on or within the ants, strains of antibiotic producing bacteria have also been isolated directly from the fungus gardens of leafcutter ants. For example, a member of the bacterial genus *Burkholderia* was isolated from the garden material of *Atta sexdens*

rubropilosa (Santos & al. 2004). Isolates of this species had potent anti-fungal activity against entomopathogenic fungi as well as the specialist pathogen *Escovopsis weberi* (Santos & al. 2004), while not inhibiting the growth of the mutualistic cultivar fungus (Santos & al. 2004). These results suggest that a broad consortium of bacteria may contribute to fungal garden defence including both those associated with the ants and the fungus gardens.

Other putative pathogens

As well as *Escovopsis*, there are additional reports of other exploiters of the attine fungus-farming symbiosis, despite the production of antibiotics by mutualistic bacteria associated with the ants and defensive MG secretions. Black yeast in the genus *Phialophora* were shown to live on the body of attine ants exploiting the resource base meant to nourish mutualistic actinobacteria (Little & Currie 2007). Their presence matches a wider observation of black yeasts in the order Chaetothyriales that have been isolated from across the family of ants (Formicidae), including in species that maintain carton nests or domatia (Voglmayr & al. 2011, Vasse & al. 2017). *In vitro* experiments suggested that the black yeast associated with *Apterostigma* can outcompete *Pseudonocardia* for nutrients as well as directly parasitise the cuticular actinobacteria (Little & Currie 2008), although the interaction seems complex. The black yeast was unable to directly affect the ants or the *Leucoagaricus* fungus garden, but their presence made the colony as a whole more susceptible to *Escovopsis* infection, presumably because *Pseudonocardia* defence was compromised. Black yeasts have predominantly been found in extreme environments (De Hoog 2014) and their ability to withstand those environments may allow them to survive on attine ants where they are likely to encounter metapleural gland secretions and symbiont derived secondary metabolites (Little & Currie 2008).

Other yeasts have also been documented to be associated with attine ants (Craven & al. 1970, Carreiro & al. 1997, Pagnocca & al. 2008, 2010, Rodrigues & al. 2009, Mendes & al. 2012). These yeasts likely play a role in detoxification of the plant material, which would benefit both the *Leucoagaricus* cultivar and the farming ants (Mendes & al. 2012). They may also play a protective role in preventing other fungal pathogens from getting a foothold in the nest (Rodrigues & al. 2009). Yeasts and filamentous fungi were also found on the body of gynes (dispersing virgin queens), whereas *Escovopsis* was not present, suggesting that they are transported in a passive fashion to new nests (Pagnocca & al. 2008).

Filamentous fungi other than *Escovopsis* have also been observed in the nests of attine ants (reviewed by Pagnocca & al. 2012). When the farming ants are removed from nests the fungus gardens are quickly overgrown by other fungi. This is believed to involve mainly opportunistic species, whereas *Escovopsis* endures as a specialised infectious agent for a greater length of time in the presence of ants tending the nest. The presence of additional fungal species is most likely connected to the live plant material used to grow *Leucoagaricus* cultivars, suggesting that the colony-wide microbiome of fungus gardens is not fully static and determined to some extent by the forage material available to the colony (Fisher & al. 1996). Another potential pathogen of attine ants are filamentous *Syncephalastrum* fungi, which have been shown able to infect laboratory colonies of *Atta sexdens rubropilosa* (Barcoto & al. 2016), though the importance of these fungi in infecting wild attine ant colonies is unknown.

Finally, *Ophiocordyceps* fungi have been well documented to infect ants of the Camponotini tribe causing the well-known "zombie ant" phenomenon (Hughes & al. 2011, de Bekker & al. 2014), but they have also occasionally been found to infect leafcutter ants both in natural settings and laboratory experiments (Hughes & al. 2009). Infections were documented from several Panamanian isolates, some of which induced the characteristic *Ophiocordyceps* stroma growing out of the back of the head. Although rare, it does suggest that *Ophiocordyceps* may be able to overcome taxonomic barriers to infect a wider range of ant genera. As we mentioned above, both *Ophiocordyceps* and *Escovopsis* share the ability to produce alkaloids, which can act as ion channel blockers during infection (de Bekker & al. 2015, Heine & al. 2018).

Summary of recent insights in symbiotic partnerships of leafcutter ants

The attine ant fungus-farming symbiosis is one of the best-known examples of obligate multipartite mutualism and has been intensively studied for more than 25 years. During the last ten years, advanced molecular techniques have allowed a series of novel insights in the chemical characteristics of the evolutionary arms race between the mutualistic partners and their fungal parasites in the genus *Escovopsis*. It is becoming increasingly clear that these arms race dynamics are likely to drive complex co-adaptation processes even though most studies continue to be based on two-partner interactions. The intricacy of novel discoveries leave little doubt that the attine fungus-growing ants will continue to provide a cutting edge and experimentally tractable model, not only for studying mutualisms and microbiome formation, but also for analysing multi-partite co-evolutionary interactions. The recent work that this review is primarily focused on shows that the specialised cultivars and *Pseudonocardia* mutualists make potent antifungals that can be effective in controlling parasitic *Escovopsis* mycoparasites. However, the *E. weberi* strains that specialise on infecting the gardens of the most derived genera, *Atta* and *Acromyrmex*, have streamlined genomes able to maintain virulence factors and toxins such as shearinines. These compounds can kill the cuticular *Pseudonocardia* bacteria that assist *Acromyrmex* species in controlling *Escovopsis* infections while also adversely affecting foraging, grooming and weeding behaviour of *Atta* and *Acromyrmex* workers. Despite these recent advances there is still much to be clarified. Just a handful of the secondary metabolites encoded by *Escovopsis* species have been characterised and we still know nothing about the putative secreted or volatile compounds produced by the *Leucoagaricus gongylophorus* cultivar that inadvertantly induce accelerated growth of *Escovopsis* hyphae towards host fungus gardens. Neither do we know what compounds the tiny subcuticular glands that nourish cuticular actinobacteria produce. In addition, much of the knowledge gained about the chemical ecology of this complex mutualism has been obtained via *in vitro* experiments and needs to be validated under field conditions. Recent technological advances, including stable isotope probing combined with next generation sequencing and imaging mass spectrometry, should now allow the study of these molecules *in vivo* to exactly determine the chemical

interactions that occur between these multiple symbiotic partners. We expect that future studies will reveal an even greater breadth of chemical compounds produced by the various mutualists and parasites involved in the attine ant fungus-farming symbiosis, and will help to unravel their adaptive functions in much greater detail than possible at present.

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Temporal variation in social structure and worker reproduction in the temporary social parasite *Lasius fuliginosus* (Hymenoptera: Formicidae)

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Abstract

Ant societies exhibit striking diversity in their social systems, including variation in the number of queens and mating partners. Knowledge on the number of breeders in a colony is crucial for a better understanding of the evolution of social insect life history traits such as reproductive skew or worker reproduction. Little is known about the breeding system of the formicine ant *Lasius fuliginosus* (Latreille, 1798), even though it is widely distributed in the Palearctic and able to compete ecologically with dominant genera like *Formica*. Moreover, *L. fuliginosus* has a particularly interesting life history in that it is a temporary social parasite of several *Lasius* species, which themselves are temporary social parasites. We determined the number of (reproductive) queens and mating partners of *L. fuliginosus* colonies and queens, respectively, from a population in Münster, Germany. Workers from 33 colonies and males from 12 of these colonies were genotyped for four polymorphic microsatellite markers. Our results show that 29 of these colonies were monogynous and monandrous and that two colonies were monogynous and polyandrous. Workers of the remaining two colonies were derived from multiple queens, possibly due to adoption of unrelated queens after the original queen's death. Furthermore, genotyping of male offspring provided evidence for worker reproduction in three colonies, potentially also in response to queen orphanage in two of these. We estimated the mutation rate at one microsatellite locus in *L. fuliginosus* to be 1.46×10^{-3} mutations per generation, which is similar to what has been observed in *Apis mellifera* Linnaeus, 1758 and *Drosophila melanogaster* Meigen, 1830. To our knowledge, this is the first study to provide molecular insights into the breeding system of *L. fuliginosus*, which appears to be characterized by facultative polyandry and monogyny. In addition, *L. fuliginosus* now represents the second species in the genus *Lasius* for which worker reproduction has been documented.

Key words: Ants, *Lasius*, microsatellites, genetic structure, worker reproduction, facultative polyandry, monogyny, queen adoption, mutation rate.

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Introduction

Ants exhibit great intra- and interspecific diversity in their social systems (Hölldobler & Wilson 1977, 1990, Bourke & Franks 1995). An important feature of ant social systems is the breeding system, which includes the number of breeders (female and male), their relatedness, and reproductive skew between breeders (Goodisman & Hahn 2005). The breeding system determines the sociogenetic structure of a colony (Ross 2001) and is associated with several life history traits (e.g., worker reproduction) that likely shape evolutionary processes (Keller 1993, Bourke & Franks 1995, Ross 2001). Therefore, the evolution of life history traits in ant species can only be understood with knowledge of the breeding system.

The ancestral state of the ant breeding system is thought to be monogyny and monandry, i.e., a colony is headed by one singly mated queen (Hughes & al. 2008, Boomsma & Gawne 2018). Therefore, multiple-queen societies (polygyny) or multiple mating by queens (polyandry) represent derived states. The evolution of multiple breeders is still not fully understood. It poses a potential conceptual problem to kin selection theory (Hamilton 1964) because the presence of multiple breeders in a colony dilutes the relatedness among workers and hence also reduces the inclusive fitness advantage that workers obtain from rearing their siblings. Certain ecological advantages associated with polygyny and polyandry must clearly have facilitated the secondary evolution of multiple breeders in a colony.

Polygyny has evolved many times (Hölldobler & Wilson 1977, Bourke & Franks 1995) among ant social systems. Likely, the most common route to polygyny is queen adoption, where mature and queenright colonies adopt newly inseminated queens after their mating flight. This so-called secondary polygyny might be favourable if dispersal costs are high (Bourke & Franks 1995). An alternative route to polygyny is through pleometrosis. That is, several newly mated queens found a colony together. Such foundress associations can evolve when increased group survivorship outweighs the disadvantage of sharing reproduction in a colony (Bourke & Franks 1995). In most cases, all but one queen are killed after the colony founding period, but

in a few species, several queens stay alive to share colony reproduction (primary polygyny) (e.g., Mintzer 1987, Hölldobler & Carlin 1989, Rissing & al. 1989, Overson 2011, Helms & Cahan 2012). Ecological factors promoting polygyny are for example heavy predation (Rosengren 1983, Bolton 1986), habitat patchiness (Rosengren 1983, Bourke & Heinze 1994), or habitat saturation (Herbers 1986). Moreover, Boulay & al. (2014) showed that in 149 Palearctic ant species polygyny was significantly correlated with ecological dominance and larger colony size, suggestive of the potential advantage of polygyny in interspecific competition.

Many ant species show some multiple mating (Bourke & Franks 1995). However, polyandry in ants is mostly facultative (Page & Metcalf 1982, Starr 1984, Hölldobler & Wilson 1990, Keller & Reeve 1994) and only few genera are known to exhibit obligate multiple mating (see Villesen & al. 2002, Gadau & al. 2003, Denny & al. 2004, Kronauer & al. 2004, Pearcy & al. 2004, Rheindt & al. 2004, Wiernasz & al. 2004, Pol & al. 2008). The benefit of multiple mating might include a more diverse workforce, which would be more efficient (Starr 1984, Crozier & Page 1985) and less prone to parasites (Sherman & al. 1988, Schmid-Hempel 1997), a reduction in variance of diploid (sterile) male production (Page 1980, Page & Metcalf 1982, Crozier & Page 1985, Page 1986), and a reduction of kin conflicts over sex allocation between queens and workers (Starr 1984, Woyciechowski & Łomnicki 1987, Sundström 1993). Costs and benefits of multiple mating in ants were reviewed by Baer (2016).

When studying ant breeding systems, it should be considered that in many species workers represent potential breeders as well. The production of reproductive offspring by workers remains possible if the worker caste has functional ovaries. Since worker-laid eggs are generally not fertilized, worker reproduction is restricted to producing male offspring. In fact, workers are reported to have totally lost functional ovaries only in nine out of over approximately 300 ant genera (Oster & Wilson 1979, Hölldobler & Wilson 1990, Villet & al. 1991) and there is evidence for worker reproduction in more than 40 ant species across 23 genera (Bourke 1988, Choe 1988, Hölldobler & Wilson 1990). However, this number is most likely very conservative since nobody has systematically searched for worker reproduction. Data on more species will be necessary to understand the selective factors that favour and disfavour worker reproduction in ants.

In this study, we provide insights into the breeding system of the formicine ant *Lasius fuliginosus* (Latreille, 1798), which is widely distributed in the Palearctic region (Collingwood 1979, 1982) and shapes local ecosystems due to its territorial and aggressive behaviour and large colonies. *Lasius fuliginosus* populations can have a significant influence on the local assemblage of species in the genera *Formica*, *Lasius* and *Myrmica* (see Savolainen & al. 1989, Czechowski 1999, Czechowski & al. 2013, Markó & al. 2013, Ślipiński & al. 2014). Moreover, *L. fuliginosus* has a particularly interesting life history as a temporary social "hyperparasite". That is, it is a temporary social parasite of several species in the subgenus *Chthonolasius* which also found their colonies by temporary social parasitism (Collingwood 1982, Seifert 2007, Markó & al. 2013). *Lasius fuliginosus* is reportedly polygynous (Donisthorpe 1915, Mattheis 2003, Seifert 2007) alongside only two other species in the genus *Lasius* (see Yamauchi & al. 1981, van Loon & al. 1990). However, the origin of this hypothesis is uncertain and molecular evidence for it is still lacking.

To get a better understanding of the breeding system of *Lasius fuliginosus*, we determined queen numbers and mating frequencies for 33 colonies in a German *L. fuliginosus* population by genotyping workers at four highly polymorphic microsatellite markers. Moreover, males of twelve colonies were genotyped to test whether worker reproduction occurs in this species. This is the first study to provide molecular insights into the breeding system of *L. fuliginosus*.

Material and methods

Sampling: Around 30 *Lasius fuliginosus* workers were hand-sampled from the nest entrance of a total of 33 colonies between June and July 2016 and between April and June 2017 in the vicinity of Münster, Germany. Two colonies were sampled in both 2016 and 2017 (see Tab. S1 in Appendix, as digital supplementary material to this article, at the journal's web pages). Furthermore, males were sampled from the nest entrance of twelve of these colonies as shown in Table S1. The samples were preserved in 100% ethanol. The geographic distribution of the colonies is shown in Figure S1.

Ant species identification: Ants were identified morphologically using the key to European ant species by Seifert (2007). Voucher specimens are reposited at the zoological collection of the Westphalian Museum of Natural History (WMNZ).

DNA extraction: A standard Chelex extraction protocol was used for the extraction of genomic DNA (Gadau 2009). The gasters of females were removed prior to DNA extraction. The specimens were placed into tubes with 100 µl 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 2 µl Proteinase K (10 mg / ml) and 2 metal beads. After grinding on a Mixer Mill for 20 seconds (28 Hz), 100 µl 10% Chelex in 1 × TE were added. The solution was incubated at 57 °C for one hour followed by a 5 min step at 95 °C. After centrifugation at 14000 rpm for 10 min, 100 µl of the extracted DNA were transferred to a new tube. The extracted DNA was stored at -20 °C.

Microsatellite genotyping: For genotyping, fluorescent-labelled primers for four loci (Lf 03, Lf 04, Lf 05, Lf 06) developed by Falk & al. (2004) were used. To infer the number of queens and mates in the sampled *Lasius fuliginosus* colonies, all four loci were genotyped in approximately 20 workers of each colony, except for colonies Lfu 1 and Lfu 18, where twice as many workers were genotyped. For colonies Lfu 6 and Lfu 8, around 20 workers sampled in both 2016 and 2017 were genotyped. The exact numbers of workers genotyped are given in Table 1.

Polymerase chain reactions (PCRs) contained 1 µl of extracted *Lasius fuliginosus* DNA (diluted 1 : 2.5 with TE buffer), 2.5 mM $MgCl_2$, 1x colourless GoTaq® Flexi buffer, 0.1 mM dNTPs, 4 picomole of each forward and reverse primer and 0.3 U GoTaq® Flexi DNA Polymerase. For each DNA sample one PCR (multiplex) was conducted with primers for the loci Lf 03, Lf 04, Lf 05 at an annealing temperature of 63 °C. Another PCR was conducted with primers for locus Lf 06 at an annealing temperature of 57 °C. The PCRs were carried out on Eppendorf thermocyclers. The initial denaturation step was 94 °C for 3 min, followed by 30 cycles of [40 s at 94 °C, 40 s at the respective annealing

Tab. 1: Sample size and genotyping statistics for the analysis of queen number. Note that queen number refers to the number of queens necessary to explain observed worker genotypes, which might deviate from the number of physically present queens during sampling. *Doubly mated queen.

Colony	No. of workers genotyped	Genotyping failure / missing data				No. of queens
		Lf 03	Lf 04	Lf 05	Lf 06	
Lfu 01	42	0.02	0	0	0	3
Lfu 02	20	0	0	0	0	1
Lfu 03	20	0	0	0	0	1
Lfu 04	20	0	0	0	0	1
Lfu 05	20	0	0	0	0	1
Lfu 06 (2016)	18	0	0	0	0	1
Lfu 06 (2017)	20	0.05	0.15	0.1	0	2
Lfu 07	19	0	0	0	0	1
Lfu 08 (2016)	20	0	0	0	0	1
Lfu 08 (2017)	17	0.41	0.29	0.24	0	1
Lfu 09	19	0	0	0	0	1
Lfu 10	20	0	0	0	0	1
Lfu 11	20	0	0	0	0	1
Lfu 12	19	0	0	0	0	1
Lfu 13	20	0	0	0	0.13	1*
Lfu 14	20	0	0	0	0	1
Lfu 15	20	0	0	0.15	0	1
Lfu 16	19	0.05	0	0.11	0	1
Lfu 17	20	0	0	0	0	1
Lfu 18	40	0.03	0	0	0.05	1
Lfu 19	20	0.05	0	0.05	0	1
Lfu 20	20	0	0	0	0	1
Lfu 21	20	0.05	0	0.1	0	1
Lfu 22	20	0	0	0	0	1
Lfu 23	20	0.05	0	0	0.05	1
Lfu 24	20	0	0	0	0.05	1
Lfu 25	20	0.25	0	0.05	0	1
Lfu 26	20	0.65	0	0.05	0	1
Lfu 27	20	0.15	0	0.05	0.05	2
Lfu 28	20	0.2	0	0.15	0.05	1
Lfu 29	20	0.35	0	0.25	0.05	1
Lfu 30	20	0.05	0	0.05	0	1
Lfu 31	20	0.1	0	0.18	0.05	1
Lfu 32	20	0.6	0.05	0.05	0	1
Lfu 33	20	0.1	0	0.05	0.05	1*
Total	733	0.09	0.01	0.04	0.02	

temperature as stated previously and 40 s at 72 °C]. The final extension step was 72 °C for 5 min.

0.5 µl of both PCR products (multiplex PCR and the PCR for Locus Lf 06), 0.25 µl GeneScan™ - 350 ROX™ size standard and formamide to a total volume of 10 µl were placed together in one well of a 96-well plate so that each well contained the fluorescent-labelled amplified loci Lf 03, Lf 04, Lf 05 and Lf 06 for one DNA sample. After a denaturation step of 5 min at 95 °C and subsequent cooling down to 4 °C, the 96-well plate was placed onto an ABI 3130xl Genetic Analyzer for capillary separation in the POP-7™ polymer by Applied Biosystems™. Scoring was done manually with GeneMapper v4.0 (Applied Biosystems™). For loci that were not amplified, the microsatellite geno-

Tab. 2: Sample size and genotyping statistics for the analysis of worker reproduction. Males were only genotyped for loci at which the queen and its mate did not share a genotype because only then worker reproduction can be identified. Loci for which a queen and its mate shared an allele in a colony are marked with "n.a.". The probability to identify a male as worker-derived in a monogynous and monandrous colony depends on the number of microsatellite loci genotyped (0.5, 0.25, 0.125, 0.0625 for one, two, three and four loci, respectively).

Colony	No. of males genotyped	Genotyping failure / missing data				Worker reproduction
		Lf 03	Lf 04	Lf 05	Lf 06	
Lfu 06	4	0	0	0	0	YES
Lfu 08	31	n.a.	0	0	n.a.	YES
Lfu 09	15	0	0	n.a.	0	NO
Lfu 10	5	0	0	0	n.a.	NO
Lfu 11	8	0.25	0.13	n.a.	0	NO
Lfu 14	20	n.a.	0	0.1	0.1	NO
Lfu 16	20	n.a.	0	0.55	0.1	NO
Lfu 17	16	n.a.	0	0	0	NO
Lfu 19	20	n.a.	0	0	0.05	YES
Lfu 21	11	0.09	0	n.a.	0.18	NO
Lfu 29	20	0.1	0	0.4	0	NO
Lfu 33	20	0	0	0	n.a.	NO
Total	190	0.06	0.01	0.13	0.06	

typing procedure was repeated. In this case, a single PCR was done for each locus at the optimal primer annealing temperature as stated by Falk & al. (2004).

In addition to workers, males from twelve colonies were genotyped to detect worker reproduction. The exact numbers of male specimens genotyped are given in Table 2. Since males are produced parthenogenetically, they cannot inherit alleles of the queen's mate (paternal alleles). However, workers carry the paternal allele and can pass it on to their offspring. Hence, males carrying a paternal allele are strong evidence for worker reproduction. This also implies that worker reproduction is only detectable by means of male genotyping in case the queen and her mate carry different alleles at a given locus because only then males with the paternal allele can be identified. Additionally, since workers carry one maternal and one paternal allele, only half of their offspring will inherit an allele of the queen's mate. Therefore, males were only genotyped for loci for which the queen and her mate showed different alleles (see Tab. 2). This information was inferred from worker genotypes as described in the subsequent section. Males were genotyped for these loci following the protocol for worker genotyping.

Microsatellite data analysis: For population genetic analyses, ten datasets were created by taking the alleles for all four loci of one randomly selected worker of each colony, respectively. Parameters were calculated for each dataset and then averaged (arithmetic mean). In this way bias due to the relatedness of workers belonging to the same colony was avoided.

For each locus, allele frequencies, observed heterozygosity (H_O) and expected heterozygosity/genetic diversity (H_E) (Nei 1973) were calculated from worker genotypes of all sampled colonies. Genepop v4.2 (Raymond & Rousset 1995) was used to test all loci for deviations from Hardy-Weinberg and genotypic linkage equilibrium.

To test for the degree of isolation by distance among colonies, a Mantel test estimating the correlation between genetic distance according to Nei (1987) and spatial distance was conducted with the programme GeneAlEx v6.503 (Peakall & Smouse 2006).

The narrow deduction method of Matesoft v1.0 (Moilanen & al. 2004) was used to infer the most parsimonious number of mating partners from worker genotypes at the loci Lf 03 to Lf 06 for each colony in case worker genotypes could be explained by a single queen. For colonies with worker genotypes that required multiple reproductively active queens, likely matrilines were separated manually and then also tested for the number of mating partners.

The effective paternity was estimated for each polyandrous colony and population-wide according to Boomsma & Ratnieks (1996) and Starr (1984), respectively.

Microsatellite locus quality: A frequent source of error using microsatellite markers are null alleles, which are the result of polymorphisms at the binding sites of microsatellite primers. Such a polymorphism might cause a primer not to bind so that the respective allele would not be amplified during PCR. This can result in misinterpreting heterozygote diploid workers as homozygotes (Jones & Ardren 2003). Consequently, estimates of queen number and mating frequency would be incorrect. A homozygote excess at any locus would be a strong indicator for the presence of null alleles at this locus. We tested for homozygote excess and null allele frequency at each locus using the programme ML-NullFreq (Kalinowski & Taper 2006). No significant homozygote excess was detected at any locus ($P > 0.25$ for each locus, 30000 randomizations). Assuming null alleles were present nonetheless, their frequency was estimated to be extremely low (Lf 03: 0.018; Lf 04: 0; Lf 05: 0.008; Lf 06: 0.004). Therefore, it is unlikely that null alleles affected our estimates of queen number and mating frequency.

Two types of errors can lead to an underestimation of mating frequencies based on microsatellite genotypes of worker offspring. Nonsampling errors occur when a colony contains several patrilines but not a single worker was sampled from one of these because of insufficient sampling sizes. Nondetection errors occur when queen mates possessed identical genotypes so that their offspring would be genetically indistinguishable.

The probability of not detecting a patriline in a set of 20 workers because of nonsampling errors is $(1\text{-}p)^{20}$ where p is the proportion of workers derived from this patriline among workers in the given colony. For example, the probability of a nonsampling error to occur in a set of 20 workers would exceed 0.05 only if one patriline contributed more than 86% to offspring. Such high skew is not likely and even if it was present in the studied population, it would not affect the population-wide effective mating frequency substantially.

The probability of nondetection errors can be calculated as $\Pi\ (\Sigma\ q_i^2)_j$ where q_i describes the allele frequency of the ith allele at the jth locus (Boomsma & Ratnieks 1996). Thus, the probability of nondetection errors is equal to the product of expected homozygosities at each locus. In our dataset the probability of nondetection errors was estimated to be 0.0004. Therefore, we excluded that nonsampling and nondetection errors substantially affected our results.

Results

Mutations: A recent paper by Schlick-Steiner & al. (2015) reported a lack of awareness of recent insertion / deletion (reINDEL) mutations in animal microsatellite studies. We checked our data for mutated alleles and identified two alleles at locus Lf 06 (GenBank acc. no. AY616195) that were only present in a single worker per colony (Tab. 3). In both cases, the allele was lacking one repeat motif compared to another allele that was present in the same colony. Therefore, we suggest a deletion event (loss of one repeat motif) that happened during parental meiosis as the most likely explanation for the observed rare alleles. In total 685 workers (diploid) were successfully genotyped at locus Lf 06. Two mutations at this locus would correspond to a mutation rate of $2 \times (685 \times 2)^{-1} = 1.46 \times 10^{-3}$ mutations per generation. This matches estimations for microsatellite mutation rates in *Apis mellifera* Linnaeus, 1758 (between $\mu = 1.5 \times 10^{-4}$ and $\mu = 1.14 \times 10^{-3}$) by Estoup & al. (1995) and in *Drosophila melanogaster* Meigen, 1830 ($\mu = 3 \times 10^{-4}$) by Schlötterer & al. (1998). We could exclude errors during allele calling and PCR artefacts by re-genotyping the individuals and manual inspecting of the chromatograms. Therefore, both alleles were considered as mutations and were not included in the analysis of queen number and mating frequency. No mutations were identified at the loci Lf 03, Lf 04 and Lf 05.

Tab. 3: Details of identified mutations in two workers for microsatellite locus Lf 06.

Colony	Worker	Sex of allele donor	Ancestral allele	Derived allele
Lfu 18	W25	Male	228	226
Lfu 32	W01	Female	244	242

Statistical considerations: All four loci were highly polymorphic in the studied population with allele numbers ranging from 12 to 15 (for allele frequencies, see Tab. S2). Observed (80 - 92%) and expected (79 - 93%) heterozygosity (Tab. 4) were similarly high to the findings of Falk & al. (2004) for another *Lasius fuliginosus* population. No significant heterozygote deficit ($P > 0.3$ for each locus), excess ($P > 0.1$ for each locus) or genotypic linkage disequilibrium were detected for any locus.

No significant correlation between Nei's genetic (1987) and spatial distance was detected (Mantel test, $r = 0$, $P = 0.497$, 9999 permutations).

Queen number and mating frequency: Queen number and mating frequency were estimated for 33 colonies in total (Tab. 1). Worker genotypes of 29 of the colonies could be explained by one, singly mated queen (monogyny and monandry), as only two or three genotypes were detected per locus (excluding the two mutations, see above) (see Tab. S3 for genotypes). Colonies Lfu 1, Lfu 13, Lfu 27 and Lfu 33 consistently showed genotypes deviating from the monandry / monogyny-pattern.

For colony Lfu 1, the observed worker genotypes could not be explained by polyandry. Hence, it clearly contained workers derived from multiple queens. To explain worker genotypes in this colony with only two reproductively active queens, required for one of these queens to be mated with at least six mates. This seemed not likely regarding the high rate of monandry in the population and the rare occurrence of high mating frequencies in ants in general (Boomsma & Ratnieks 1996). Alternatively, the observed worker genotypes were in agreement with the more likely scenario of three singly inseminated reproductively active queens in the colony.

Matesoft assumes monogyny to explain worker genotypes whenever possible. However, in some cases this assumption is not suitable because it can result in artificially high mating frequencies. For instance, it was possible to explain worker genotypes for colony Lfu 27 with just one queen that mated with seven mates. Again, such amounts of mating partners were unlikely (given the results of the remaining colonies). Two singly mated queens were a more likely explanation for observed worker genotypes (see Tab. S3).

Tab. 4: Number of alleles and observed / expected heterozygosity of four microsatellite loci for the *Lasius fuliginosus* population around Münster, Germany.

Locus	GenBank acc. no.	No. of alleles	H_O	H_E
Lf 03	AY616192	13	0.797	0.785
Lf 04	AY616193	15	0.871	0.815
Lf 05	AY616194	15	0.924	0.926
Lf 06	AY616195	12	0.883	0.861

Our data suggested that colonies Lfu 13 and Lfu 33 were monogynous and polyandrous with two predicted mating partners for each queen. The effective paternities in colonies Lfu 13 and Lfu 33 were $m_e = 1.724$ and $m_e = 1.246$, respectively. In colony Lfu 33 however, a second mating partner carrying an allele "117" at locus Lf 03 was only necessary to explain the observed genotyping pattern because of two workers' genotypes at locus Lf 03 (see Tab. S3). Consequently, the proposed mating partners only differed in the allele "117" at locus Lf 03 but were genetically identical at the loci Lf 04, Lf 05 and Lf 06. This raised the question whether the queen was indeed doubly mated (with high skew between the reproductive contributions of mates making the effective mating frequency almost 1) or whether the allele "117" was a genotyping artefact. The population-wide effective paternity would have been $m_e = 1.017$ assuming double mating due to allele 117 and $m_e = 1.012$ under the assumption that the allele "117" was a genotyping artefact. Therefore, whether the queen of colony Lfu 33 was singly or doubly mated did not have a strong effect on the mode of mating in this *Lasius fuliginosus* population.

In the two colonies Lfu 6 and Lfu 8, workers of two consecutive years were genotyped (see Tab. S4 for genotypes). In colony Lfu 8, worker genotypes found in 2017 were identical to worker genotypes of 2016. Thus, workers of both years were likely derived from the same singly mated queen. In contrast, three workers with new genotypes at each locus were found in 2017 compared to 2016 in colony Lfu 6. These workers could not have been produced by either queen proposed by Matesoft for 2016 since alleles were present in these workers that are absent in both the queen and its mating partner.

In summary, the majority of studied *Lasius fuliginosus* colonies (88%) was monogynous and monandrous. Only two colonies contained workers derived from more than one queen (6%), which were all single mated. Two monogynous colonies contained a doubly mated queen (6%). Hence, polygyny and polyandry did not occur together.

Worker reproduction: To check for worker reproduction in *L. fuliginosus*, we genotyped males for twelve colonies (see Tab. S5 for genotypes). According to our previous findings, all these colonies were monogynous and only colony 33 was not monandrous but was headed by a doubly mated queen. Workers of three of these colonies, i.e., colonies Lfu 6, Lfu 8 and Lfu 19, carried paternal alleles, which is strong evidence for worker reproduction (Tab. 2). Four males were sampled from colony Lfu 6, of which three possessed paternal alleles at the loci Lf 05 and Lf 06. For colony Lfu 8, one male out of 31 possessed paternal alleles at the loci Lf 04 and Lf 05. For colony Lfu 19, 17 of 20 genotyped males possessed a paternal allele at one or more of the loci Lf 04, Lf 05 and Lf 06.

Worker-produced males inherit either the paternal allele or the maternal allele of their worker mother. Hence, only 50% of worker-produced males are expected to carry the paternal allele and can be identified as worker-derived regarding a single locus. Regarding three loci, the probability of a worker-derived male to possess only maternal alleles would be $0.5^3 = 0.125$, so that we would not have been able to identify about two to three males as worker-derived in a set of 20 samples. This fit our data for colony Lfu 19, since three males possessed alleles at all loci that could be either queen- or worker-derived. Therefore, it is quite possible that all genotyped males were worker-derived in colony Lfu 19. A similar calculation for colony Lfu 6 would not be meaningful because only four males were genotyped for this colony. However, the fact that three of four genotyped males were clearly worker-derived also suggests that the majority of males in this colony was produced by workers. Males of colony Lfu 8 could only be genotyped for two loci, so that 0.25 of the samples, i.e., about eight samples, would likely not be identifiable as worker-derived. However, we only found one worker-derived male for this colony. Therefore, it is likely that most males in this colony were produced by the queen.

Discussion

Queen number: Except for *Lasius sakagamii* (Yamauchi & Hayashida, 1970) and the invasive ant *L. neglectus* van Loon, Boomsma & Andrasfalvy, 1990 (Yamauchi & al. 1981, van Loon & al. 1990), all *Lasius* species for which queen number is known are monogynous (e.g., Tanquary 1913, Waloff 1957, Seifert 2007). We found that the majority of colonies in the studied *L. fuliginosus* population were also monogynous. Hence, we could not confirm the previous findings on queen number, i.e., polygyny, in *L. fuliginosus* (see Collingwood 1979, Mattheis 2003, Seifert 2007). This discrepancy can be explained in two ways. First, colonies that contain multiple queens but are effectively monogynous can lead to a difference between molecular (i.e., worker genotyping) and observational data on the number of queens. It is not known what studies the polygyny hypothesis for *L. fuliginosus* is based on since Collingwood (1979), Mattheis (2003) and Seifert (2007) did not provide proper citations for their claims but, most likely, these findings are based on personal observations. Secondly, intraspecific polymorphism of the number of functional queens between populations has been shown for a number of ant species including *L. sakagamii* (see Yamauchi & al. 1981, Ross & Fletcher 1985, Chapuisat & al. 2004, Schlick-Steiner & al. 2007, Gill & al. 2009, Overson 2011, Helms & Cahan 2012). Variation of social organization could therefore also be present among *L. fuliginosus* populations. Such variation might be due to differing environmental conditions, i.e., conditions that favour polygyny (e.g., habitat saturation, predation, inter- / intraspecific competition) are present in one (sub-)population but not in another. Interestingly, variation of queen number can go along with restricted gene flow and genetic differentiation between social forms (e.g., Ross & al. 1997, Gyllenstrand & al. 2005). Considering the conflicting accounts on queen number in *L. fuliginosus*, it will therefore be worthwhile to use molecular methods to estimate the number of queens in colonies of different *L. fuliginosus* populations. It is also worthy of note that Collingwood (1979) and Seifert (2007) reported polydomy for *L. fuliginosus*, which we did not observe in a single colony in the studied population.

Only two of 33 colonies in the studied population, i.e., colonies Lfu 1 and Lfu 27, contained workers that were derived from multiple queens. In the following, we discuss five possibilities that can explain the presence of worker genotypes derived from multiple queens in *Lasius fuliginosus* colonies (see Gadau & al. 1998): (1) Intraspecific brood or worker raiding; (2) Primary polygyny as a result of pleometrosis; (3) Intranidal mating of related individuals; (4) Adoption of related queens after the mating flight; (5) Adoption of unrelated queens by orphaned colonies.

(1) By means of intraspecific brood or worker raiding, genotypes of foreign queens could be present in a monogynous colony. However, intraspecific raiding is not known for any *Lasius* species. Moreover, no other *Lasius fuliginosus* colonies, on which raids could have been conducted, were found in proximity to colony Lfu 1. Although colony Lfu 27 was located only about 30 meters apart from colony Lfu 28, no worker genotypes present in colony Lfu 28 were found in colony Lfu 27 (we assumed that colonies Lfu 27 and Lfu 28 were distinct colonies because no trail connection existed between them, they did not share any genotypes among workers, and alleles were present in both colonies that could not be found in the other one). Therefore, it is unlikely that intraspecific raids were conducted by *L. fuliginosus* in our study population.

(2) Pleometrosis can lead to the presence of several queen genotypes in a colony as a result of primary polygyny. However, in most species that exhibit pleometrosis only one queen remains in the colony after the emergence of the first workers (Bourke & Franks 1995). Hence, primary polygyny is rare in ants and has unlikely occurred in colonies Lfu 1 and Lfu 27. However, even if pleometrosis occurred but did not lead to primary polygyny in the colony, it would be possible that we saw genotypes of several queens because of it. This would be the case if we sampled workers right after the colony founding, and before or shortly after the killing of all but one queen took place. In this way, workers derived from multiple founding queens could still be present in the colony. A newly founded colony would show relatively few *Lasius fuliginosus* workers and could be recognized by the presence of host workers. Since the colonies Lfu 1 and Lfu 27 were large, exhibited extensive trails and did not show any host workers, they were not incipient but fully-grown colonies. In addition, it is not clear if pleometrosis occurs in *L. fuliginosus* at all. For example, we found *L. umbratus* (Nylander, 1846) workers in the nest of colony 18 foraging with *L. fuliginosus* workers, which might indicate that this colony was newly founded. However, the colony was clearly monogynous and monandrous. Therefore, pleometrosis does not seem to be the reason for genotypes of multiple queens in colonies Lfu 1 and Lfu 27. Nevertheless, we do not exclude that parasitic pleometrosis could facilitate colony founding in *L. fuliginosus* as indicated by Mattheis (2003). Regarding the high amount of monogynous colonies in the studied population, all colonies would have to reduce functional queen number to one after the founding period. Such queen reductions might include killing of queens by workers, fighting among queens or the renouncement of reproduction by some queens. The latter, however, is only exhibited in few ant species (Bourke & Franks 1995). To test for pleometrosis in *L. fuliginosus*, studies on the sociogenetic structure of more newly founded colonies will be necessary.

(3) Sexuals in ant species do not necessarily participate in nuptial flights to mate. For several formicine species intranidal mating is known (e.g., Rosengren 1983, van Loon & al. 1990, Sundström 1993). That is, sexuals do not leave the natal nest but mate with related individuals inside the nest. However, in *Lasius fuliginosus*, extensive nuptial flights were observed by Mattheis (2003). The prevalence of independent colony foundation and nuptial flights is also supported by the absence of isolation by distance among colonies in the study population. Intranidal mating often results in high rates of homozygosity due to inbreeding. We calculated homozygosity rates for all colonies (see Tab. S6). Colony Lfu 01 indeed showed elevated homozygosity rates compared to the average rates across monogynous colonies. However, this was not the case for Lfu 27. In summary, intranidal mating in *L. fuliginosus* seems unlikely but remains a possible explanation (especially in Lfu 01).

(4) Re-adoption of daughters is usually associated with changes in life history and mating behaviour. In species that show queen adoption, mating often occurs in, on or close to the nest so that the possibility of predation while relocating and re-entering the nest is minimized (Bourke & Franks 1995). *Lasius fuliginosus* sexuals participate in extensive nuptial flights during which they cover considerable distances. This would make returning to their natal nest difficult and costly. On the contrary, adoption of colony daughters in *L. fuliginosus* was reported by Donisthorpe (1915). However, it was not described by other authors and Donisthorpe (1915) did not give evidence for his claims. Although we cannot exclude daughter adoption to be present in *L. fuliginosus*, it seems unlikely in the studied population. Mitochondrial haplotype analyses might prove useful to shed light on the descent and relatedness of matrilines in polygynous colonies.

(5) Finally, we propose the adoption of unrelated queens as the most likely explanation for multiple queen genotypes in colonies Lfu 1 and Lfu 27. When the queen in a monogynous colony dies, the colony is usually no longer able to produce sexual offspring. However, the production of sexuals can continue either if workers start to reproduce or if new queens are adopted into the colony. Mattheis (2003) observed that *Lasius fuliginosus* workers show aggression towards foreign conspecific queens. After the queen's death in a monogynous colony these aggressions might decrease. Subsequently, there could be a period in which *L. fuliginosus* colonies would accept new queens to be adopted into the colony. Such a mechanism is described in *Solenopsis* and *Myrmica* (see Tschinkel & Howard 1978, Seppä 1994). Queen adoption would be in accordance with the longevity of *L. fuliginosus* colonies despite the small queen size (Donisthorpe 1915, Mattheis 2003). Moreover, genotyping of colony Lfu 6 revealed that new genotypes were present among workers in 2017 compared to 2016, suggestive of the presence of a new reproductively active queen in the colony. It is unlikely that this new queen was related to the old one because, based on worker genotypes, it showed alleles at the loci Lf 04 and Lf 05 that were not present in the old queen. Therefore, the adoption of an unrelated queen into colony Lfu 6 has likely occurred.

If adoption of unrelated queens evolved in *Lasius fuliginosus*, it should yield fitness advantages to both the adopted queen and adopting workers. Adoption is advantageous for adopted queens because *L. fuliginosus* colonies are large and have elaborate nests in tree cavities that are constructed with at least two mutualistic fungal species (Schlick-Steiner & al. 2008). By means of adoption, queens take advantage of this costly resource and avoid the critical stage of colony founding. However, it is worthy of note that an intruding gyne also faces complications such as worker hostility and a lowered expected fertility (Keller 1993). Workers would obtain an inclusive fitness advantage adopting a newly mated queen under queenless conditions if they were related to her. Even if this was not the case, adoption could result in a fitness benefit for workers. Gadau & al. (1998) proposed this for *Camponotus ligniperda* (Latreille, 1802), in which

sexual brood overwinters twice. If maturation time of sexuals is long as in *C. ligniperda*, sexual brood of an old but dead queen, which was related to the workforce, would still be present in the colony some time after the queen's death. This brood's survival rate and thus the inclusive fitness of workers could be increased if a newly adopted queen produced additional workers. However, there is no information on the maturation time of sexuals in *L. fuliginosus* and whether the queens in the studied colonies were related to one another and to the workers.

Unrelated *Lasius fuliginosus* queens could also enter a colony against its fitness interests as intraspecific parasites. Such a scenario is likely since *L. fuliginosus* is well adapted to a parasitic lifestyle because of its mode of colony founding (temporary social parasitism in the *Chthonolasius* group). It is possible that *L. fuliginosus* queens can enter a foreign but conspecific colony and kill the queen in this colony as they do in colonies of their host species. This would present intraspecific parasitic behaviour. Since Mattheis (2003) observed high worker aggression towards foreign queens under queenright conditions parasitizing a *L. fuliginosus* colony might only be possible in orphaned colonies because defence mechanisms would be weaker there.

Our genetic data suggest that at least two queens would have been adopted into colony Lfu 1 and one queen in colony Lfu 27 (alleles of a dead queen could still be present in worker genotypes). This raises the question whether queen adoption in colony Lfu 1 led to a stable coexistence of multiple queens as shown for *C. ligniperda* by Gadau & al. (1998) or whether eventually all but one queen would be killed. To answer this question, the polygynous colonies need to be genotyped over consecutive years.

Mating frequency: We showed that the majority of queens in the studied *Lasius fuliginosus* population was singly mated. Colonies Lfu 13 and Lfu 33 were the only colonies for which a doubly mated queen was probable. This resulted in a population-wide effective mating frequency of $m_e = 1.017$. Boomsma & Ratnieks (1996) proposed four categories to classify mating frequencies in ants: (s) single (double mating is absent or rare; $m_e < 1.05$); (s - d) single-double (double mating occurs in 20% - 50% of queens; $m_e = 1.05$ to 1.25); (s-m) single-multiple (mating frequency above two occurs regularly; $m_e = 1.4$ to 2); and (m) multiple (mating frequency greater than two; $m_e > 2$). Even though over half of the ant species investigated show multiple mating to some degree (Bourke & Franks 1995), most species belong to categories (s) or (s-m) including *Lasius flavus* (Fabricius, 1782), *L. neglectus* and *L. niger* (Linnaeus, 1758). Instances of obligate multiple mating have been shown in the genera *Acromyrmex* (see Boomsma & al. 1999), *Atta* (see Boomsma & Ratnieks 1996), *Cardiocondyla* (see Lenoir & al. 2006), *Cataglyphis* (see Pearcy & al. 2009), *Pogonomyrmex* (e.g., Wiernasz & al. 2004) and in army ants (e.g., Kronauer & al. 2004, 2006), but have not been reported for *Lasius*. Therefore, it is not surprising that *L. fuliginosus* queens are generally mated once (category (s)).

Worker reproduction: There is evidence for worker reproduction in more than 40 species across 23 genera including several species within the Formicinae (mostly *Formica*) (reviewed by Bourke 1988). However, in the genus *Lasius* only *L. niger* workers were shown to produce males. We found evidence for worker reproduction in three of twelve *L. fuliginosus* colonies, now presenting the second *Lasius* species for which worker reproduction is documented.

Queen presence / absence is key for considering fitness costs and benefits associated with worker reproduction. In queenright colonies there is potential conflict over male parentage between workers and queens, particularly in monogynous and monandrous systems where workers are more closely related to their sons ($r = 0.5$) and nephews ($r = 0.375$) than to their brothers ($r = 0.25$) and should therefore favour their own male offspring over the queen's male offspring. However, male production could also have indirect negative effects on worker fitness by decreasing colony-level productivity (Keller & Nonacs 1993). Hence, queens are likely selected to prevent worker reproduction. Whether this occurs against the fitness interests of workers (manipulation) or not (honest signalling) is controversial (Keller & Nonacs 1993, Brunner & al. 2011). For queenless colonies, however, worker reproduction is the only remaining possibility to increase the fitness of both, the absent queen and the workers (Alexander 1974). Therefore, reproductive conflicts are alleviated under queenless conditions, rendering worker reproduction potentially adaptive. Indeed, most cases of worker reproduction are reported under queenless conditions (Hölldobler & Wilson 1990). Because of the potential conflict over male parentage and the higher probability of queen orphanage in monogynous systems, Bourke (1988) suggested that worker reproduction might be more frequent in monogynous than in polygynous species. Since all colonies with worker-derived males in the studied population, i.e., colonies Lfu 6, Lfu 8 and Lfu 19, were monogynous (and monandrous), our findings support this hypothesis. It remains to be seen whether worker reproduction has occurred under queenless or queenright conditions in these colonies.

In colonies Lfu 6 and Lfu 19 it was likely that all sampled males were produced by workers. Under queenright conditions worker reproduction in ants is commonly suppressed behaviourally or through pheromones (Fletcher & Ross 1985). This means that even if worker reproduction took place in a queenright colony, the fraction of worker-derived males should be low. Therefore, we hypothesized that colonies Lfu 6 and Lfu 19 were orphaned. If this was the case, either worker numbers would decline, or a new queen would be adopted in the years after male sampling. We genotyped workers of colony Lfu 6 that were sampled one year after initial worker and male sampling to see whether queen adoption took place in this colony. Indeed, new worker genotypes were found at all loci, suggesting that worker reproduction is a possible temporary stage between queen orphanage and queen adoption. To finally see whether colonies Lfu 6 and Lfu 19 were orphaned at the time of male sampling, it will be necessary to monitor these colonies in the years to come.

Queen orphanage is no possible explanation for worker reproduction in colony Lfu 8 since it was likely that most male offspring was produced by a queen. Only one male out of 31 was clearly worker-derived. This would be expected under queenright conditions because of the suppression of worker reproductive behaviour by the queen. As done for colony Lfu 6, we genotyped workers of colony Lfu 8 sampled one year after initial sampling. We did not find any new alleles at the loci Lf 03 to Lf 06 suggesting that no queen adoption took place in this colony (as would be expected).

Considering that in colony Lfu 8 only one male was identified as worker-derived and that the sampling size of males in all the other colonies was only 65% of the sampling

size in colony Lfu 8, it might well be possible that worker reproduction might be a feature of a greater number of colonies but was not detected due to too small sample sizes in this study. We propose that worker reproduction in *Lasius fuliginosus* occurs under both queenright and queenless conditions. With a queen present, only a small fraction of workers successfully produces males. After colony orphanage worker reproduction can become more frequent because behaviour and / or pheromones of the queen that supress worker reproduction are no longer present.

Outlook: Surprisingly, for a species that has large and long-lived colonies, *Lasius fuliginosus* appears to be predominantly monogynous and monandrous. However, we did record some cases of polyandry and workers which were derived from multiple queens in the study population. Latter might be the result of queen adoption by orphaned colonies, but this hypothesis needs further investigation. Our data challenge the polygyny hypothesis in *L. fuliginosus* as stated in the literature. Studies in more *L. fuliginosus* populations will be necessary to unveil the predominant queen number of this species.

We also found evidence for worker reproduction in *Lasius fuliginosus*, making it the second species in the genus *Lasius* for which this phenomenon is described. Worker reproduction might rarely occur in queenright *L. fuliginosus* colonies but might be a strategy to increase colony fitness after orphanage, potentially before new queens would be adopted. In recent years, not much work has been conducted on worker reproduction in ants and not many species have been studied in this regard at all. However, it seems to be a frequent feature (Hölldobler & Wilson 1990), and its implications for evolutionary processes, like retention or loss of worker ovaries, are still not well understood.

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Forum

The Ant Chromosome database – ACdb: an online resource for ant (Hymenoptera: Formicidae) chromosome researchers

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Abstract: Ants are an important and diverse natural insect group exhibiting astonishing variation in chromosome number and structure. Their haploid chromosome numbers range from n = 1 to n = 60 and include diverse karyotypes comprising various different types of chromosome. This marked variation first attracted attention over 40 years ago, and has led to several theories concerning the evolution of chromosome change and the manner in which it may have promoted and contributed to ant species diversification. Despite the significance of ants, their chromosome numbers have been only sporadically documented and assembled. We present here the Ant Chromosome database – ACdb (www.ants.ufop.br), which was conceived as a regularly updated online tool for rapid access to data regarding ant karyology. ACdb is an easy-to-use database which will advance the accession and dissemination of cytogenetic knowledge of the Formicidae. At present, it contains 1080 entries of chromosome and karyotype data for 520 named species from 134 genera in 11 subfamilies. Basic information on karyotypes and chromosome numbers is provided, along with relevant karyotype formulae, which summarize chromosomal structure. ACdb highlights the challenge to ant cytogeneticists to improve the available chromosome knowledge of the Formicidae. Chromosome counts are still lacking in several subfamilies and many genera, while banding data are unavailable for most ant species. We trust that the database will inspire efforts to improve cytogenetic knowledge of these significant insects.

Key words: Genome, chromosome, nucleus, ant, evolution, karyotype.

Introduction

Chromosomes as the carriers of genes are the units of inheritance, which are duplicated and transmitted between cells during cell division. This was understood before the discovery of the DNA molecule, in light of the chromosome theory of heredity by Walter S. Sutton, and acknowledgement of the link between genes and chromosomes (Ganetzky & Hawley 2016). Further, the recognition of linear chromosomes and understanding that they are found in all eukaryotes was an important evolutionary transition, which allowed the partitioning of the genome (Schubert 2007). The basic chromosome mitotic structure appears to be preserved across all plants and animals. It encodes the fundamental information of an organisms' genome. Chromosome numbers have been used extensively in systematics and are important in species delimitation (Bai & al. 2018) and lineage diversification (Cristiano & al. 2013).

Ants are an important insect group which exhibits considerable diversity in chromosome numbers, varying from n = 1 in the Australian bulldog ant *Myrmecia croslandi* (see Crosland & Crozier 1986, Imai & Taylor 1989, Taylor 2015) to n = 60 in the giant neotropical ant *Dinoponera lucida* (see Mariano & al. 2008). The marked variation in chromosome number across species attracted the attention of the first ant cytogeneticists, and several mechanisms regarding the manner in which this diversity has evolved have been proposed. These include the "Minimum Interaction Theory" proposed by Imai & al. (1994), in which rearrangements that involve Robertsonian fissions are important. Karyological evolution in ants generally tends towards an increase in chromosome number apparently serving to reduce the risk of deleterious rearrangements resulting from interactions between chromosomes in the nucleus (Imai & al. 1988, Imai & al. 1994). Nevertheless, this general trend in chromosome increase is probably subject to certain limits and other chromosomal rearrangements also induce changes in ant karyotypes. They include inversions and fusions (Cardoso & al. 2014). Cytogenetic knowledge about ants, as well as other organisms, has been regularly summarized in previously published compendiums and reviews. Lorite & Palomeque (2010) contributed the most recent comprehensive summary of ant chromosome numbers and karyotypes, including a supplement which provides at least 750 records. It is easy to find different online database sites for ants, including Ant Web (www.antweb.org) and Antwiki (www.antwiki.org), but these databases are mainly concerned with literature, species distribution, and systematics. Indeed, none of them specifically include cytogenetic data. Bolton's Catalogue, which has been published electronically (Bolton & al. 2007), reports cytogenetic information for some taxa. Recently, many online databases for cytogenetic information have become available (Peruzzi & Bedini 2014), mainly for plants. Some of them are specific for a taxonomic group or ecosystem (Roa & al. 2017). A comprehensive database of all plants was published by Rice & al. (2015).

The work reported here aims to compile the current cytogenetic knowledge of ants for presentation in a digital

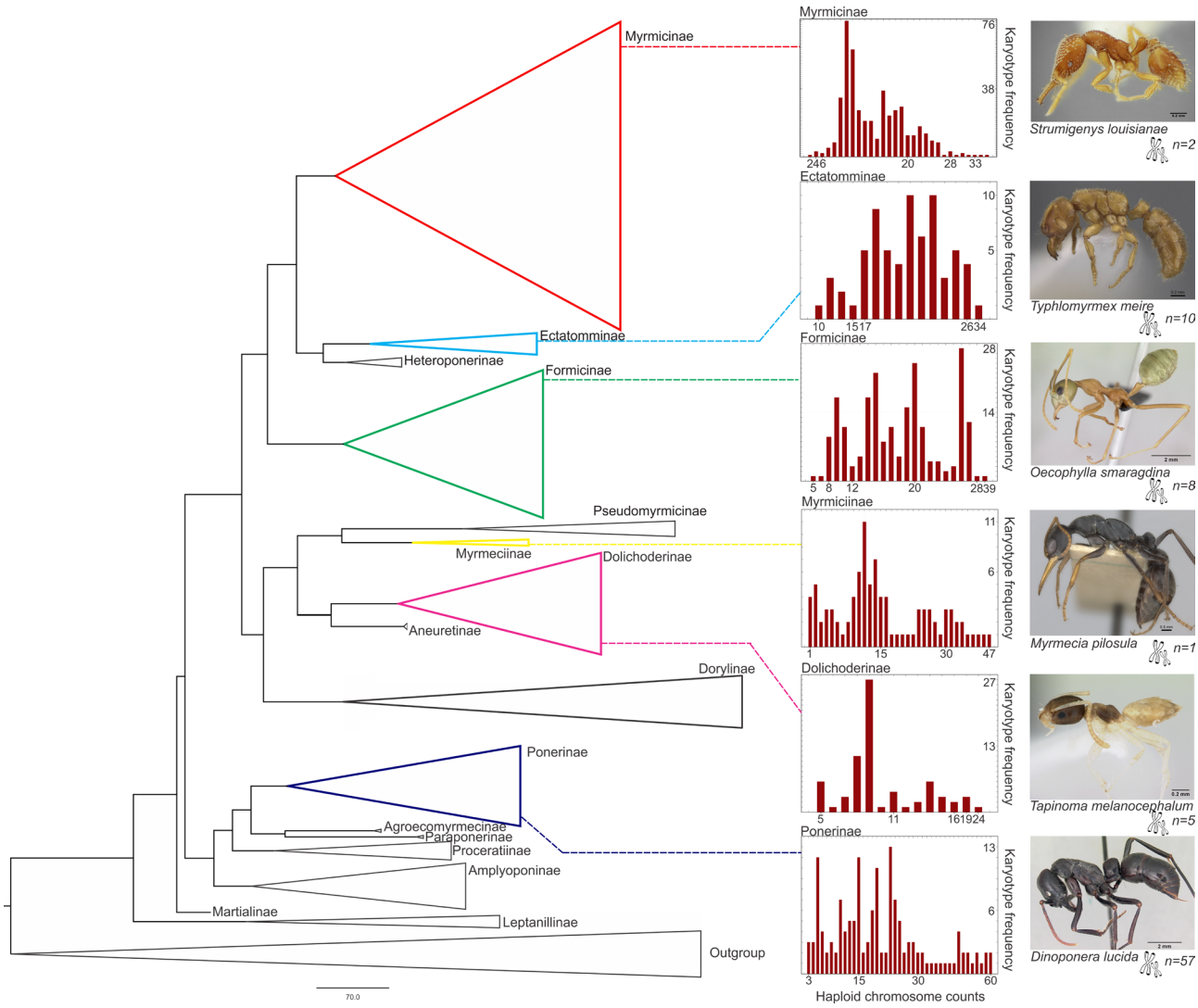

Fig. 1: Phylogenetic relationship between Formicidae subfamilies redrawn from Moreau & Bell (2013) and the haploid chromosome count distribution. The figure shows subfamilies for which a considerable number of studies are available. On the right, representative ant species with a remarkably low or high chromosome count. Ant images from AntWeb (www.antweb.org), top to bottom: *Strumigenys louisianae* (ANTWEB1038234), *Typhlomyrmex meire* (CASENT0915350), *Oecophylla smaragdina* (CASENT0070232), *Myrmecia pilosula* (CASENT0217500), *Tapinoma melanocephalum* (CASENT0173215), and *Dinoponera lucida* (CASENT0104920).

and straightforward online database. We here present the Ant Chromosome database – ACdb (www.ants.ufop.br) (Fig. 1) in recognition that knowledge of ant karyotypes provides important information which will advance our understanding of ant evolutionary biology and systematics.

Cytogenetic data assembly

Several compilations and reviews of ant chromosome counts and karyotypes have been published over recent decades (Crozier 1975, Lorite & Palomeque 2010). Our starting point was the comprehensive publication of Lorite & Palomeque. We have retrieved all ant chromosome counts assembled by these authors by checking all of the publications involved. Additionally, we carried out structured searches in the following scientific databases: ISI Web of Knowledge, SCOPUS, and Google Scholar, using the following search terms: "ant", "chromosome", "cytogenetic", and "karyotype". Manuscripts mentioning or describing ant chromosome counts and karyotypes were accessed and the information was recorded.

The parameters recorded include the species name, diploid chromosome set (2n), haploid chromosome set (n), and karyotype. Karyotype information that provides the number of each type of chromosome (e.g., whether a chromosome was metacentric – M, submetacentric – SM, telocentric – T, subtelocentric – ST, or acrocentric – A) was accessed when it was reported by the original study. Similarly, the number of haploid or diploid chromosomes, or both if applicable, was recorded. Each distinct chromosome count was considered, so that, where counts vary among specimens each is recorded as an individual record (entry) in the database. In addition, varying conspecific chromosome counts from different localities or populations are registered individually. To make ACdb functional, taxonomic names were standardized using only recognized valid names provided by Bolton's catalog version 2.5.4 (www.antcat.org).

Dolichoderinae *Dolichoderus*

Cytogenetic data

Species	haploid(*n*)	diploid(2*n*)	country(ies)	karyotype	notes	references
Dolichoderus quadripunctatus		28	Japan			Imai 1969
Dolichoderus scabridus	14	28	Australia		in Crozier 1966 as Diceratoclinea scrabida	Crozier 1966 Imai et al. 1977
Dolichoderus thoracicus		33	Malaysia		as D. bituberculatus	Imai et al. 1983
Dolichoderus thoracicus		30	Malaysia, Indonesia		as D. bituberculatus	Imai et al. 1983 Imai et al. 1985
Dolichoderus sp.		18	Malaysia			Goni et al. 1982
Dolichoderus lutosus		10	Brazil	4M+6SM		Santos et al. 2016
Dolichoderus bidens		18	Brazil	6M+12SM		Santos et al. 2016
Dolichoderus voraginosus		20	Brazil	14M+6SM		Santos et al. 2016
Dolichoderus diversus		22	Brazil	10M+12SM		Santos et al. 2016
Dolichoderus imitator		38	Brazil	6M+28SM+4A		Santos et al. 2016
Dolichoderus decollatus		38	Brazil	6M+32SM		Santos et al. 2016
Dolichoderus attelaboides		38	Brazil	2M+50SM+6A		Santos et al. 2016

http://ants.ufop.br/dolichoderinaedolichoderus.html

Fig. 2: Table of cytogenetic data generated by the Ant Chromosome database – ACdb. In this example, data were browsed for the genus *Dolichoderus*.

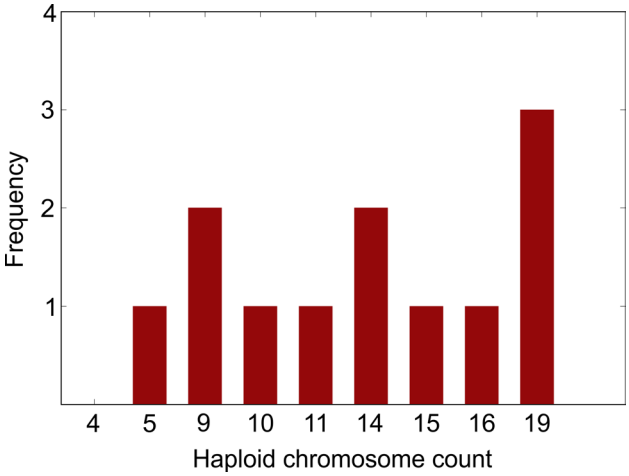

Fig. 3: Frequency histogram of the haploid chromosome count generated by the Ant Chromosome database – ACdb. In this example, data were browsed for the genus *Dolichoderus*, haploid counts are shown; diploid (2n) values are converted for (n).

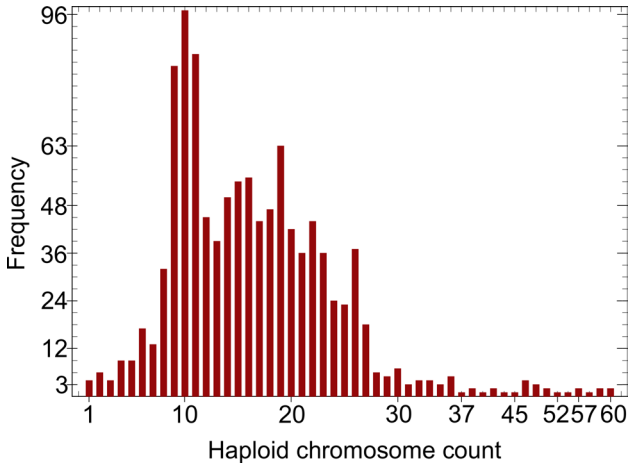

Fig. 4: Frequency distribution of haploid chromosome ant entries in the Ant Chromosome database – ACdb in May 2018.

Ant Chromosome database – ACdb conception and data access

ACdb is available at the address http://www.ants.ufop.br hosted at the Universidade Federal de Ouro Preto – UFOP. Chromosome counts, haploid or diploid, locality and country of individual report, karyotype and the reference are presented in tabular form. All information can be searched or browsed by Subfamily and Genus, and data output can be presented as a table (Fig. 2) or a frequency histogram of the haploid chromosome counts (Fig. 3). When the diploid number alone is known, the histogram automatically represents its haploid conversion. Each entry is referenced by the study or studies which described or reported appropriate details. Further information is provided in the "notes" column. For example, it can include former names of species as used when their chromosome number was published. The query results, by subfamily or genus, can be downloaded by clicking the bottom link generating a csv file. In the "Statistics menu" a general histogram for all species can be generated and the total number of entries reported. Browsing by subfamily locates a histogram at subfamily level. The database will be progressively kept

Tab. 1: Number of chromosome counts for species and morphospecies by subfamily of Formicidae actually in the Ant Chromosome database.

Subfamilies	Species and morphospecies
Amblyoponinae	4
Dolichoderinae	70
Dorylinae	11
Ectatomminae	66
Formicinae	214
Heteroponerinae	2
Myrmeciinae	94
Myrmicinae	460
Ponerinae	144
Proceratiinae	4
Pseudomyrmecinae	11
Total entries	**1080**

up to date by the correction of potential errors or flaws and by the addition of new records. Cytogenetic information can be submitted to the database using the link under the menu "submit" by filling-up the form.

The ACdb data: retrospect and prospect

As of May 2018, a total of 1080 entries in 134 genera and 11 subfamilies have been made available. From these entries, 520 named species have at least one chromosome report (some species show more than one chromosome count) and more than 200 were records from morphospecies. The most frequent haploid chromosome number is n = 10, followed by n = 9 and n = 11 (Fig. 4). The 520 named species represent ~ 3% of the ant species currently known (with ~ 16,000 taxa in all according to AntWeb as of January, 2018). There is information from 11 of the 20 subfamilies of Formicidae. The coverage of available cytogenetic data varies widely across subfamilies. The larger subfamilies comprise the majority of entries: Myrmicinae accounts for 42.60% (460), Formicinae for 19.81% (214), and Ponerinae for 13.33% (144) of all entries (Tab. 1). With respect to the country from which the records were taken, the majority come from Australia, Malaysia, India, and Indonesia (249, 166, 97, and 62 entries, respectively) mainly because most of the studies were effected by Imai and Crozier and associates (e.g., Crozier 1975, Imai & al. 1977, 1988). Other records included 119 from Brazil and 80 from the United States of America.

Details on the karyotypes of ants, such as chromosome morphology according to centromere position, has been incorporated for 234 entries. This information was added to the database when the authors provided the karyotype formula or when it could be determined by evaluating published data. Two different chromosome nomenclatures have been used previously to describe the chromosome morphology of ants. The most frequent was that proposed by Levan & al. (1964), which was based on the centromeric index. Some authors however, described karyotypes following the nomenclature proposed by Imai & al. (1991) which considers heterochromatin patterns.

Conclusions and perspectives

Ants show an astonishing variety of karyotypes that may be related to their huge species diversity, forms, and functions. Changes in chromosomes may play an important role in speciation by promoting reproductive isolation (King 1993). However, the importance of mechanisms that underlie chromosome changes and speciation remains poorly understood. Indeed, a growing number of cytogenetic studies in ants have emphasized that chromosome evolution has accompanied genus and species diversification (Lorite & Palomeque 2010). The application of fluorescence in situ hybridization (FISH) and comparative analyses of chromosome-based phylogenetic trees has been made possible by the advances in the study of ant chromosomes (Cristiano & al. 2013, Cardoso & al. 2014).

Reviews and compilations of ant chromosome counts are generally published periodically. As noted by Lorite & Palomeque (2010), the previous substantial and unique global compilation of ant karyotypes was published by Crozier in 1975. The speed with which the data are currently generated and published demands a new approach to the gathering and dissemination of ant karyotype data. Acdb is a public, straightforward and easily used resource about ant cytogenetics designed for use by interested researchers.

Based on the study of chromosomes in the species complexes of the Australian genus *Myrmecia*, Imai & al. (1994) proposed the "Minimum Interaction Theory". This proposes a mechanism which prevents deleterious rearrangements produced by the interaction of chromosomes in the nucleus. The result is that overall chromosome evolution in eukaryotes proceeds towards increase in the number of chromosomes, and reduction of their average size (Imai & al. 2002). Advances in the cytogenetic knowledge of Formicidae will allow for the identification of patterns and processes underlying chromosome variations, resulting in an improved understanding of ant systematics, evolution, and chromosome biology.

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Cryptic species of the *Myrmica tibetana* complex (Hymenoptera: Formicidae) revealed by integrative taxonomy

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Abstract

Three closely related species, *Myrmica tibetana* Mayr, 1889, *M. bactriana* Ruzsky, 1915 and *M. gebaueri* sp.n., are identified. They are restricted to the Tibetan Plateau and proposed to form the *M. tibetana* species complex. *Myrmica tibetana* and *M. gebaueri* sp.n. are truly cryptic: They showed considerable interspecific overlap in all of the tested 18 shape, pilosity, and sculpture characters and were not safely separable by simple visual inspection by a trained expert. However, all three entities are clearly demonstrated by Nest Centroid (NC) clustering of morphological data which agreed by 100% with the genetic classification based on 11 microsatellite markers. The clusters shown by hierarchical NC-Ward clustering and the partitioning algorithms NC-part.hclust and NC-part.kmeans were coincident in all of the 62 nest samples. A stepwise linear discriminant analysis reducing the set to nine characters achieved a classification error of 0% in 178 investigated worker individuals. All three entities are partially sympatric, and the absence of phenotypically mixed nest samples rejects the hypothesis that they could represent an intraspecific polymorphism. The coincident classification of all three exploratory data analyses of morphology and nuDNA revealed a paraphyly of mtDNA between *M. bactriana* and *M. gebaueri* sp.n. adding another example to the multiple evidence on failures of mtDNA barcoding in biodiversity research. Yet, mtDNA data appeared adequate for rough assessment of divergence times. According to this, the separation of the *M. tibetana* complex from other members of the *M. rubra* group is estimated to have occurred approximately 7.5 Ma Before Present (BP), and the radiation within the *M. tibetana* complex started > 5 Ma BP. A taxonomic description and a differential diagnosis of *M. gebaueri* sp.n. are presented. *Myrmica bactriana* Ruzsky, 1915 is shown as a senior synonym of *M. furva* Ruzsky, 1915 and *M. ruzskyana* Radchenko & Elmes, 2010. Synonymies of either member of the *M. tibetana* complex with the following Central and Middle Asian species are excluded: *M. smythiesii* Forel, 1902, *M. fortior* Forel, 1904, *M. wittmeri* Radchenko & Elmes, 1999, and *M. tenuispina* Ruzsky, 1905.

Key words: Nest centroid clustering, species delimitation, numeric morphology-based alpha-taxonomy, phylogenetic age, Tibetan fauna, microsatellite primer sequence, new species, new synonymies.

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Introduction

There are disparate conceptions of what "cryptic species" might constitute. True crypsis is given when heterospecificity has been clearly demonstrated by some kind of an exploratory data analysis and if an experienced expert is not able to use these samples with pre-established determination to build up in her/his brain pathways for reliable subjective-sensory species recognition. This permanent, true crypsis differs from temporary or historic crypsis. The meaning of the latter can be elucidated by an example from ornithology: The Common Tern *Sterna hirundo* and the Arctic Tern *Sterna paradisaea* were considered inseparable by field observers in the 1950s. Today, however, hundreds of amateur ornithologists in Europe are able to reliably identify these two species in the field because the knowledge on useful characters and individual training have enormously developed. In order to distinguish from temporal crypsis, Seifert (2009) defined true crypsis as follows: "Cryptic species are two or more species which are not separable by primary visual or acoustic perception of an expert. This reflects the immediate sense of the word and restricts such species to truly cryptic cases – i.e., to species not safely separable by training of innate pathways of the human cognitive system. Rather, their reliable identification requires the application of elaborate methods such as numeric recording and analysis of phenotypic characters, DNA analysis, biochemistry or analysis of sound spectrograms …"

Within the context of the Chinese-German research project Pasture Degradation Monitoring System (PaDeMoS) three of the authors conducted field work in grassland ecosystems on the Tibetan Plateau in 2011 and 2012. The ant fauna of this huge, climatically extreme area is poorly studied. This also applies to the genus *Myrmica* Latreille, 1804 of which we collected ten species. Among these was *Myrmica tibetana* Mayr, 1889, originally described from material of the famous 1884 Przewalski expedition near Lake Kuku Nor (Qinghai Lake) in NE Tibet and the closely related *M. bactriana* Ruzsky, 1915, collected during the Kozlov expedition in 1900 / 1901. To our surprise, we discovered a third sympatric species very closely related to *M. tibetana*. Two of these are truly cryptic following the definition given above: They showed considerable interspecific overlap in any of the tested 18 shape, pilosity or sculpture characters and were not safely separable by simple visual inspection of a trained expert. Such non-transparent situations can be solved by application of advanced exploratory data analyses. In eusocial organisms, Nest Centroid clustering (NC clustering) is the method of choice. NC clustering has been introduced by Seifert & al. (2014) and is promising to be a powerful tool for recognition of taxonomic and zoogeographic patterns for any cohesive organism or social system providing repeats of definitely conspecific elements. A dozen of follow-up applications of NC clustering using data of Numeric Morphology-Based Alpha-Taxonomy (NUMOBAT; e.g., Seifert 2013, Csösz & al. 2014, Seifert & al. 2014, Csösz & al. 2015, Seifert & al. 2017a, b) and another one using data of cuticular hydrocarbon chemistry of ants (Guillem & al. 2014) have been published since then. Speed and objectivity of hypothesis formation has recently been improved by introducing Partitioning Algorithms based on Recursive Thresholding (PART) into the NC-clustering protocol (Csösz & Fisher 2015). Here, we present a convincing demonstration of truly cryptic and sympatric *Myrmica* species by NC clustering of NUMOBAT data and support this finding by a classification based on nuclear DNA.

Material

NUMOBAT data were recorded in a total of 62 nest samples and 178 worker individuals collected on the Tibetan Plateau. In the individual species treatments, material examined is listed in following sequence and format: site, date in the format yyyy.mm.dd, field sample number "field No" which is found on the mounted specimens (the sample number of microsatellite DNA analysis "µsat No") (GenBank accession number) [latitude in decimal format, longitude in decimal format, metres above sea level]. The accuracy of coordinates is proportional to the number of decimal points and "xx" in the sampling date sequence mean missing data. In some samples without any direct or derived information on date, the collector is given to allow an approximate conclusion on the time period of collection. Field sample numbers are missing in some historic samples.

The acronyms of depositories are as follows:

NHM Wien – Naturhistorisches Museum Wien, Austria
MHN Genève – Muséum d'histoire naturelle de Genève, Switzerland
SMN Görlitz – Senckenberg Museum für Naturkunde Görlitz, Germany
ZM Berlin – Zoologisches Museum am Museum für Naturkunde Berlin, Germany
ZM Moskow – Zoological Museum of the Lomonossov University Moskow, Russia
ZM St. Petersburg – Zoological Museum St. Petersburg, Russia

Myrmica tibetana Mayr, 1889

A total of 12 nest samples with 45 workers were investigated by linear morphometrics.

Ganzu: Aze Station, riverbank, 2012.07.10, field No 032 (= µsat No M29) (GenBank MG603025), field No 033 (= µsat No M30) (GenBank MG603026) [33.655° N, 101.831° E, 3478 m]; Luqu 8.4 km SW, 2011.08.19, field No 074 (= µsat No M12) (GenBank MG603020) [34.541° N, 102.416° E, 3297 m]. **Qinghai:** Hainan-10 km NE, 2011.09.14, field No 107a (GenBank MG603022) [36.354° N, 100.708° E, 3088 m]; Lake Kuku Nor, 2011.07.28, sample No 027 (= µsat No M07) (GenBank MG603019) [37.144° N, 99.752° E, 3206 m]; Beishan National Park, 1996.05.25 [36.95° N, 102.48° E, 2400 m]; Heka, 1990.07.14 [35.78° N, 99.88° E, 3560 m]; S Kuku Nor Mountains, type *M. tibetana* 1884.04. [36.5° N, 98.7° E, 3700 m]; Xia Zhangshan S, 2012.07.05, field No 023a (= µsat No M25) (GenBank MG603024) [35.592° N, 102.711° E, 3032 m]; Xinghai 2 km NNW, 2011.07.17, field No 56 [35.605° N, 99.978° E, 3314 m]; Xinghai, Stipa, 2012.07.01, field No 010 (GenBank MG603023), field No 011 (= µsat No M23) (GenBank MG603021) [35.605° N, 99.981° E, 3305 m].

Myrmica bactriana Ruzsky, 1915

A total of 20 nest samples with 52 workers were investigated:

Ganzu: Aze Station, rock, 2011.08.14, field No 056b [33.681° N, 101.874° E, 3605 m]; Aze Station, rock, 2011.08.14, field No 058 (= µsat No M11) (GenBank MG603009) [33.681° N, 101.873° E, 3608 m]; Aze Station 3.3 km NNE, 2011.08.12, field No 172, field No 173 (= µsat No M16) (GenBank MG603011), field No 174 (= µsat No M17) (GenBank MG603012), field No 175 [33.702° N, 101.887° E, 3630 m]; Aze Station, above AzAII, 2012.07.09, field No 027, field No 028 (= µsat No M27) (GenBank MG603017), field No 029 (= µsat No M28) (GenBank MG603018) [33.676° N, 101.854° E, 3599 m]; Aze Station, near AzAII, 2012.07.08, field No 025a (= µsat No M26) (GenBank MG603016) [33.667° N, 101.854° E, 3551 m]; Luqu 8.4 km SW, 2011.08.19, field No 078 (=µsat No M13) (GenBank MG603010) [34.541° N, 102.416° E, 3295 m]; Luqu 8.4 km SW, plot LqA, 2011.08.17, field No 177, field No 181 (= µsat No M18) (GenBank MG603013), field No 183 (= µsat No M19) (GenBank MG603014), field No 185a (GenBank MG603015) [34.541° N, 102.418° E, 3296 m]; **Qinghai:** Aba County, wetland, 2011.08.20, field No 081 [33.455° N, 101.840° E, 3520 m]; Sanzin Gömpa Monastery, creek, 2011.07.21, field No 021 (= µsat No M06) (GenBank MG603008) [35.501° N, 99.824° E, 3517 m]; Sanzin Gömpa Monastery -1.7 km E, field No SaA-BPs, 2011.07.18 [35.500° N, 99.817° E, 3588 m]; Sanzin Gömpa Monastery-2.2 km E, field No SaZ-Blg, 2011.07.20 [35.504° N, 99.823° E, 3628 m]; **Sichuan:** river Dza-Chyu, 1901.05, type *M. furva* [32.930° N, 98.770° E, 4080 m].

Myrmica gebaueri sp.n.

A total of 30 nest samples with 81 workers were investigated:

Ganzu: Tjanzhu-0.5 km SSE, 2011.08.03, field No TzA Blg [37.192° N, 102.788° E, 2901 m]; Tjanzhu-0.8 km SSW, 2011.08.04, field No 041 (= µsat No M08) (GenBank MG603000) [37.189° N, 102.783° E, 2963 m]; Tjanzhu-0.8 km

SSW, 2011.08.04, field No TzZ Blg, field No TzZ-R, field No 044 (= µsat No M09) (type series of *M. gebaueri* sp.n. GenBank MG603001) [37.189° N, 102.783° E, 2963 m]; Tjanzhu, 2011.08.05, field No 049 (= µsat No M10) (GenBank MG603002) [37.182° N, 102.776° E, 3095 m]; Tjanzhu, Gipfel, 2011.08.04, field No 84 [37.177° N, 102.773° E, 3146 m]; Xicheng, 2011.08.01, field No 037b [38.038° N, 101.593° E, 3167 m]; **Qinghai:** Heimahe - 4.1 km SSE, 2011.07.26, field No KoA2 - B1 (= µsat No M31) (GenBank MG603006), field No KoA2 - Blg (GenBank MG603007) [36.696° N, 99.802° E, 3294 m]; Heimahe - 4.4 km SSE, 2011.07.24, field No 64, field No KoZ - BPs, field No KoZ-pseu [36.694° N, 99.804° E, 3288 m]; Heimahe - 4.4 km SSE, 2011.07.26, field No KoZ2-Blg, field No KoZ2-R1, field No KoZ2-R2, field No KoZ2-Re [36.691° N, 99.797° E, 3294 m]; Sanzin Gömpa Monastery 6 km NE, 2011.07.18, field No 016 (GenBank MG602998), field No 017 (= µsat No M05) (GenBank MG602999) [35.542° N, 99.840° E, 3435 m]; Sanzin Gömpa Monastery 6 km NE, 2012.07.03, field No 020, field No 021 (= µsat No M24) (GenBank MG603005) [35.541° N, 99.849° E, 3409 m]; Gonghe, 1992.05.08 [36.300° N, 100.683° E, 3400 m]; Xinghai-13 km WSW, 2011.07.19, field No 61, field No 62 [35.542° N, 99.840° E, 3428 m]; Xinghai, Stipa, 2012.07.01, field No 008b [35.610° N, 99.977° E, 3332 m]; Xinghai, Stipa, 2012.07.01, field No 009 (= µsat No M21) (GenBank MG603004) [35.607° N, 99.982° E, 3311 m]; Xinghai, grassland, 2011.07.17, field No 013a (= µsat No M01) (GenBank MG602995), field No 014 (= µsat No M02) (GenBank MG602996), field No 015 (= µsat No M03) (GenBank MG602997) [35.617° N, 99.975° E, 3374 m]; Xinghai 3 km NNW, plot XiZ R, 2011.07.16, field No 158a [35.612° N, 99.968° E, 3349 m].

Detailed information on type material

***Myrmica tibetana* Mayr, 1889**

Mayr (1889) gave the following collecting data in his original description: "April 1884, Jumel-Kuku-Gebirge; Mai-Juni 1884 Tibet septentr." The term "Jumel Kuku Gebirge" is undoubtedly a reading error of the original Cyrillic label. This original label was discarded by Mayr and should have read probably as "Южные Куку Горы" = "Southern Kuku Mountains". If handwritten as a script, "Южные" is easily misinterpreted by a person not familiar with Russian language. Reading the travelling report of Przewalski (1954), we found that the Southern Kuku (Nor) Mountains were reached in April 1884 and we assume as most probable collecting site a place near the pass road – approximately at 36.5° N, 99.7° E and 3700 m.

We have investigated the lectotype worker labeled "Tibet" [handwriting of H. Stitz], "Myrmica tibetana Mayr" [handwriting of H. Stitz], "Forel ded. 1922", "Zool. Mus. Berlin", "Paratypus" [label probably attached by Stitz], "Lectotype *Myrmica tibetana* Mayr, 1889 [published by Radchenko & Elmes (2010), des. Seifert 2014]"; stored in ZM Berlin. Note: Radchenko & Elmes (2010) published a specimen from ZM Berlin museum with the above labelling as lectotype but did not physically designate it and they also gave no morphological data to identify it unambiguously. However, as this type is the only specimen of *M. tibetana* stored in the Berlin collection, we are rather sure to have labeled the right specimen. We further investigated a big series of 13 paralectotype workers, stored in NHM Wien, labeled "Tibet Coll. G. Mayr", "tibetana G.Mayr, Type". One of these specimens shows a label "Lectotype Myrmica tibetana MAYR A.F. -1978" which is invalid as this physical lectotype designation by André Francoeur has not been published.

***Myrmica gebaueri* sp.n.**

Holotype labeled "CHI: 37.1852° N, 102.7844° E Tianshu station-1.2 S, 2939 m moist pasture, under stone R.Schultz 2011.08.04-044" and "Holotype *Myrmica gebaueri* Seifert et al. 2018"; two worker paratypes on a different pin, 21 worker and 14 male paratypes stored in ethanol – all from the same nest sample and with equal collecting data label as the holotype; all material stored in Senckenberg Museum für Naturkunde Görlitz. Three worker paratypes with the same labelling in MHN Genève.

***Myrmica tibetana* var. *furva* Ruzsky, 1915**

We investigated three supposed paralectotype workers from ZM St. Petersburg, labeled "r. Dza-Chyu, Kam', Golubaya, 12 - 12500', Kozlov, nach. V.01" [in Cyrillic] and "Myrmica tibetana Mayr M. Ruzski det." Radchenko & Elmes (2010) published a lectotype with identical locality label. This lectotype was not found in the St. Petersburg museum. Perhaps it was not physically labeled by Radchenko & Elmes. In each of the three paralectotypes some characters could not be recorded due to damage – in one the whole head was missing. However, we constructed complete data sets for two specimens using relational calculations. River Dza-Chyu is a tributary of the upper Yangtse (= Golubaya in the Russian naming of the Kozlov expedition). According to the travelling report of the expedition (Kozlov 1906), the putative collecting site was reached 11 May 1901 and should be situated approximately at 32.928° N, 98.770° E and ca. 4080 m if the lowest point in that region is chosen. The 12 - 12500 feet given on the label are equal to 3750 m. This means no contradiction because altitudinal estimates in Kozlov's time were rather inaccurate.

***Myrmica smythiesii* var. *bactriana* Ruzsky, 1915**

Radchenko & Elmes (2010) published a lectotype worker stored in ZM St. Petersburg and cite its label as "okr. ur. Darindo, Kam, verkh. Goluboj, Kozlov, 1/3.VIII.00" [in Cyrillic]. The term "1/3.VIII.00" stands probably for the first decade of August (I. Kabak, pers. comm.). This site is situated at the upper course of Yangtse at 33.054° N, 96.903° E and 3850 m. No type specimens could be discovered in the collection St. Petersburg during a search by D. Dubovikov in 2013 but the identity of this taxon and of *M. ruskyana* Radchenko & Elmes, 2010 can be concluded with low risk of error from Radchenko & Elmes drawings of the lectotypes and the geographic data (see section Results and Discussion).

***Myrmica ruzskyana* Radchenko & Elmes, 2010**

This is a replacement name for the primary homonym *Myrmica smythiesii* var. *exigua* Ruzsky, 1915. Radchenko & Elmes (2010) published a lectotype labeled "rechka Bachyu, 12.000', Kam, bass. Goluboj r., Kozlov, 2/3. VIII. 00" [in Cyrillic]. "2/3. VIII". means probably the second decade of August (I. Kabak, pers. comm.). Though the label shows another locality name, the travelling report of Kozlov does not allow separating this site geographically from the lectotype locality of *M. bactriana*. According to Kozlov's map, he had been in Darindo (locality of

M. bactriana) on 8 August and in Ba-Tshu River on 9 - 20 August 1900. The linear distance between Darindo and the mouth of Ba-Tshu River is approximately 11 km and that between Darindo and the next station – the confluence of the Ba-Tshu and Dza-Tshu rivers, reached on 21 August – is about 27 km (I. Kabak, pers. comm.). Thus the collecting points are between 11 km and 27 km apart and both in the Yangtse basin close to the present town of Yushu. Type material was not available from ZM St. Petersburg and ZM Moscow.

***Myrmica tenuispina* Ruzsky, 1905**

The combination *Myrmica laevinodis* var. *tenuispina* Ruzsky, 1915 is the first available use of *Myrmica rubra laevinodis tenuispina* Forel, 1904 and the types are those designated by Forel. Four syntype workers from MHN Genève were investigated, labeled "*M.rubra Linné r. laevinodis Nyl. v. tenuispina For type Buchara*" [Forel's handwriting] and a printed label in Cyrillic letters "Tabi dara-Zagyr-desht. v. Bukhara Kaznakov 17 VI. 97". These specimens belong to the lectotype sample because Radchenko & Elmes (2010) published a lectotype worker in the ZM Moscow with the labelling "Tabi-Dara Zagyrdesht V.Buchara, 17. VI. 97, Kaznakov" [in Cyrillic].

Methods

Phenotypic investigation

The optical equipment used, the character recording methods and estimation of measuring errors are given in Seifert (2011). Precise definitions of the following phenotypical characters are given in Seifert & al. (2014). Briefly explained these are: cephalic length CL, cephalic width CW, head size CS (= arithmetic mean of CL and CW), eye size EYE (= arithmetic mean of large and small diameter of the elliptic eye), scape length SL, maximum frontal lobe width FL, minimum frontal carina distance FR, petiole width PEW, postpetiole width PPW, petiole height PEH, petiole length PEL, maximum length of postpetiolar setae PPHL, spine length SP, metapleural lobe height MetL, height of subspinal propodeal excavation MetSp and postocular distance PoOc. We introduced the following new characters in the investigation system of the *Myrmica tibetana* complex and present their precise description:

SPBA – the smallest distance of the lateral margins of propodeal spines at their base. This should be measured in dorsofrontal view, since the wider parts of the ventral propodeum do not interfere with the measurement in this position. If the lateral margins of spines diverge continuously from the tip to the base, a smallest distance at base is not defined. In this case, SPBA is measured at the level of the bottom of the interspinal meniscus.

SPTI – the distance of propodeal spine tips in dorsal view; if the tips are rounded or thick, the centres of spine tips are taken as reference points.

Removal of allometric variance. Removal of allometric variance (RAV) was performed with the procedure described by Seifert (2008). RAV is calculated here for the assumption of all individuals having an identical cephalic size of CS = 950 µm. The parameters of RAV functions were calculated as the arithmetic mean of the species-specific functions of *Myrmica bactriana*, *M. gebaueri* sp.n. and *M. tibetana*. It can be seen from the functions below that allometries of shape are weak in the small and weakly size-variable workers of the *M. tibetana* complex. The RAV functions were as follows:

$CL / CW_{950} = CL / CW / (-0.0515 * CS + 1.1754) * 1.1265$
$SL / CS_{950} = SL / CS / (-0.0480 * CS + 0.8321) * 0.7865$
$EYE / CS_{950} = EYE / CS / (0.0528 * CS + 0.1455) * 0.1966$
$FL / CS_{950} = FL / CS / (-0.0055 * CS + 0.4786) * 0.4734$
$FR / CS_{950} = FR / CS / (-0.0459 * CS + 0.4421) * 0.3985$
$PEW / CS_{950} = PEW / CS / (-0.0605 * CS + 0.3063) * 0.2488$
$PPW / CS_{950} = PPW / CS / (-0.0630 * CS + 0.4377) * 0.3778$
$PEH / CS_{950} = PEH / CS / (-0.0293 * CS + 0.3464) * 0.3186$
$PEL / CS_{950} = PEL / CS / (-0.0862 * CS + 0.5346) * 0.4527$
$PPHL / CS_{950} = PPHL / CS / (-0.0408 * CS + 0.2370) * 0.1982$
$SPBA / CS_{950} = SPBA / CS / (-0.0350 * CS + 0.3182) * 0.2849$
$SPTI / CS_{950} = SPTI / CS / (-0.1597 * CS + 0.4595) * 0.3077$
$SP / CS_{950} = SP / CS / (0.0122 * CS + 0.1610) * 0.1726$
$MetL / CS_{950} = MetL / CS / (0.0001 * CS + 0.2228) * 0.2229$
$MetSp / CS_{950} = MetSp / CS / (-0.0006 * CS + 0.1980) * 0.1975$
$PoOc / CL_{950} = PoOc / CL / (-0.0703 * CS + 0.4774) * 0.4106$
$FL / FR_{950} = FL / FR / (0.1214 * CS + 1.0730) * 1.1882$

Analysis of phenotypic data. Exploratory data analysis was run using three different methods of NC-clustering (Seifert & al. 2014). These were hierarchical NC-Ward clustering and two partitioning algorithms based on recursive thresholding: part.hclust and part.k means (for details see Csösz & Fisher 2015). Checking for misclassified samples was done following the rationale described in Seifert & al. (2014). All linear discriminant analyses were run with the SPSS 16.0 software package.

Genetic investigation

The reduced number of 32 / 26 nest samples for which mtDNA / microsatellite data were evaluated compared to 62 nest samples available for morphological investigation is explained by the fact that DNA was extractable in only a part of these samples currently housed in museum collections. We did not made any attempt to extract DNA from mounted specimens because of the resulting physical damage. In the type specimens of *Myrmica tibetana* and *M. furva* such destructive investigations were even forbidden by the curators.

Analysis of mtDNA. From each genetically evaluable nest sample one worker was chosen for genetic analyses. The complete individual was shred with a mixer mill (Retsch MM 400, Haan, Germany) and genomic DNA was extracted using Qiagen DNeasy blood & tissue kit (Qiagen, Hilden, Germany).

An approximately 2.500 bp fragment of mtDNA was amplified in two segments. The first segment including *ND6* (89 AA), *cytb* and tRNASer was amplified using the primers cytb FeF (Liautard & Keller 2001) + tRS (Jermiin & Crozier 1994). The second segment including *ND1* (269 AA) was amplified using the primers ND6_ND1bF + ND6_ND1cR (Holzer & al. 2009). The second primer pair failed for all *Myrmica tibetana* specimens. Yet, since this taxon was already well separable from both *M. gebaueri* sp.n. and *M. bactriana*, no effort was undertaken to find new primer pairs. The short tRNASer sequence showed little variation and was ignored in this analysis.

Each segment was sequenced on both strands using the same primers. The segments overlapped at ca. 400 bp, and this sequence section was carefully checked for congruence between the two segments to potentially detect pseudogenes. Half of the individuals were additionally amplified with a third primer pair, CB11059 (Goropashnaya & al. 2004) +

Tab. 1: Microsatellite loci developed for *Myrmica tibetana,* which were cross-amplified for *M. bactriana* and *M. gebaueri*. Superscript letters at locus names indicate the combination of loci in multiplex PCR reactions. Annealing temperatures multiplex reactions 1 - 4: 1 at 63 °C; 2 and 3 at 61 °C; 4 at 59 °C.

Locus	Repeat motif	Size range (bp)	Primer sequence
B04[1]	$(TTGG)_{13}$	231 - 252	B04-fwd: 5‘-5AGATCGAGCCGGAGAATCG-3‘ B04-rev: 5‘-TACCTTCTCGTCGCCCAAC-3‘
B09[2]	$(GAAC)_{14}$	247 - 267	B09-fwd: 5‘-CCATTAGCGCGTCCAACAG-3‘ B09-rev: 5‘-ACCGAGGACTTCGTTAGGC-3‘
B10[3]	$(TCGT)_{18}$	263 - 282	B10-fwd: 5‘-GCGACAAGGAGAGCAAGTC-3‘ B10-rev: 5‘-AGAGCAGCATGAGTCTCTAAGG-3‘
C03[2]	$(TTCG)_{13}$	134 - 167	C03-fwd: 5‘-ACCGTGTAAATCCAGTCGC-3‘ C03-rev: 5‘-GTCGCCGTGCGGAATAATG-3‘
C06[1]	$(GCTT)_{17}$	294 - 315	C06-fwd: 5‘-TTCCGCGCAACAGAAATCC-3‘ C06-rev: 5‘-TAGGCACGTAACGGGAGTG-3‘
D11[4]	$(TCG)_{30}$	204 - 243	D11-fwd: 5‘-CTGCGTTATACACCATCCGC-3‘ D11-rev: 5‘-ACGAAGGCATTACATACTTGTC-3‘
F05[2]	$(GTGA)_{13}$	205 - 222	F05-fwd: 5‘-ATGCCCGTGTTTCATGCAG-3‘ F05-rev: 5‘-GCATATATTTCGAGGGCGGTC-3‘
F09[2]	$(GTCGA)_{8}$	174 - 200	F09-fwd: 5‘-TCGATGAGGTGATCTCGGG-3‘ F09-rev: 5‘-TCTGCTTCGGATTACGGAAAG-3‘
F11[1]	$(GTCA)_{16}$	169 - 190	F11-fwd: 5‘-TCCTTCGCCCTCGATAGTG-3‘ F11-rev: 5‘-TTCCCGATGAGTTTCACGC-3‘
G06[4]	$(GAGCC)_{10}$	245 - 270	G06-fwd: 5‘-GGGATGCGCACCATAAACC-3‘ G06-rev: 5‘-GAACGAGGGAAACGGGATG-3‘
H08[3]	$(GCGAT)_{9}$	180 - 202	H08-fwd: 5‘-ATCGTCCTCGCTCTGGAAG-3‘ H08-rev: 5‘-TCGATTCGCTCCGAAATGC-3‘

CB2 (Jermiin & Crozier 1994), which produced a 750 bp fragment of *cytb.* These sequences were also compared to those obtained by the other primer pairs. All multiply covered sequences were identical.

PCR was carried out in 25 µl volumes containing PCR-Buffer with a final $MgCl_2$ concentration of 3.1 mM, 0.09 mM dNTPs, 0.5 µM of each primer and 0.5 U *Taq* (Peqlab, Erlangen, Germany). DNA amplification was performed with a Mastercycler EP S (Eppendorf, Hamburg, Germany) and consisted of 3 min initial denaturation at 94 °C, followed by 35 cycles with 30 s denaturation at 92 °C, 30 s annealing at 46 °C – 47 °C, 1 min elongation time at 68 °C and a final 10 min elongation step at 72 °C. From elongation step 11 onwards, the elongation time was expanded stepwise from 1 min to 2 min.

PCR products were loaded on an agarose gel to check for correct product size and potential unintended byproducts. Afterwards, products were cleaned using ExoSap (ThermoFisher Scientific) and sequenced on a capillary sequencer (ABI3730; Life Technology, Darmstadt, Germany) at the Senckenberg Biodiversity and Climate Research Centre (BIK-F) in Frankfurt / Main (Germany).

Sequences were aligned using ClustalW and checked manually. The mitochondrial genome of *Solenopsis richteri* Forel 1909 with annotated CDS was used to align all coding regions. The mtDNA genetic code in insects differs slightly from the standard genetic code. Since most phylogeny programs cannot account for it, the respective codons had to be changed. In detail, the *Drosophila* code codes differently for Ser and Met and, more importantly, has one stop codon less (“UGA” codes for Trp instead of *). Codons at twelve positions had to be changed from “UGA” to “UGG” across *ND6* and *cytb* to run codon models. This reduced the variance at four out of these twelve positions (because the third codon position was already “G” in some individuals), which is considered to be minimal compared to the high variance within the alignment. The final alignment of the 32 ingroup individuals comprises 2280 bp with 118 variable sites (129 mutations) and consists of 23 haplotypes.

Phylogenetics models were run in MrBayes 3.2 (Ronquist & al. 2012). Three different runs with three chains were started for each analysis and the likelihood values checked for convergence to identify the minimum number of burn-in generations. The final burn-in period was set at twice the number of steps at which no further improvement in the likelihood could be observed, and the analyses were run for 300,000 to 5,000,000 generations. Phylogenies were calculated for *ND6* and *Cytochrome b* separately, as well as for the combined data set. To find the model fitting the data best, we applied nucleotide substitution models (identified by MrModeltest, Nylander 2004) as well as codon models. Those two models were run with equal parameters for *ND6* and *cytb* and compared to models which allowed the parameters to vary between the two partitions. The partitioned codon model was additionally compared to a strict molecular clock model, which forced the substitution rate to be equal along all branches. In a last step we applied the multi-species coalescence model (Edwards & al. 2007, Liu & Pearl 2007) to account for the found incongruence between the morphology-based species tree and the gene trees.

Estimates of population differentiations were calculated with DnaSP (Rozas & Rozas 1999) using the distance measure of Nei (1987) with Jukes and Cantor correction.

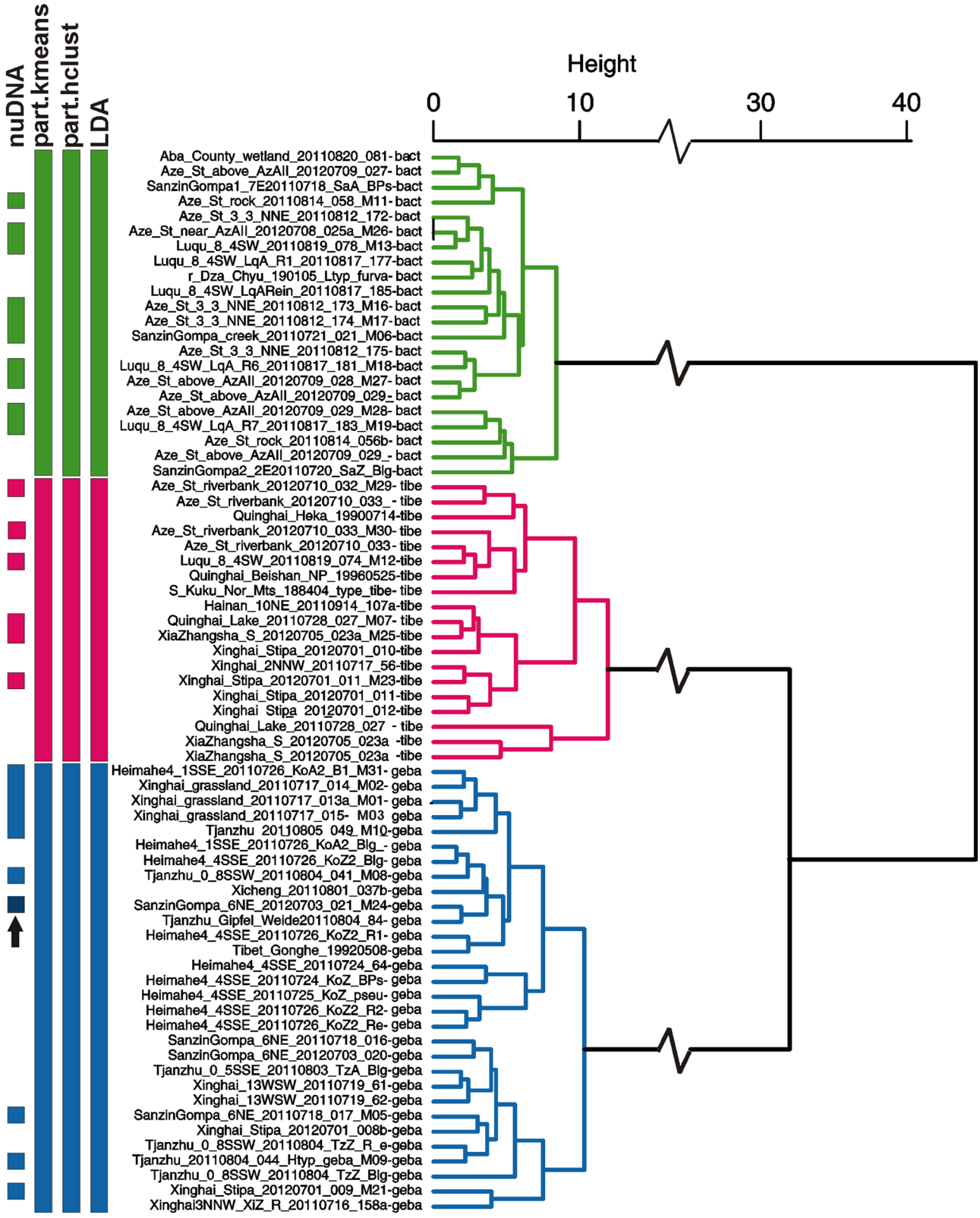

Fig. 1: NC-Ward clustering of worker nest samples of *Myrmica bactriana* Ruzsky, 1915 (upper, green branch), *M. tibetana* Mayr, 1889 (middle, red branch) and of *M. gebaueri* sp.n. (lower blue branch), considering nine morphometric characters. Bars indicate the classification by nuDNA (microsatellites), the partitioning algorithms part.kmeans and part.hclust based on recursive thresholding, and linear discriminant analysis. The arrow points to the sample placed in the microsatelite analysis intermediate between *M. gebaueri* and *M. tibetana*. A direct sample-by sample comparison with the microsatellite topology given in Figs. 3 and 4 is possible with the "M01"…"M30" strings at the end of the labels. The tree topology of mtDNA (Fig. 6) differs very strongly. For comments on the taxonomic value of mtDNA barcoding see the main text.

Microsatellite analyses

Microsatellite markers were developed by the company Starseq (Mainz, Germany) based on an Illumina-Miseq DNA library. After shearing, end-repairing, A-tailing and ligating to TruSeq adapters 100 ng genomic DNA of *Myrmica tibetana*, the library was amplified in eight cycles. Then the library was selected for a mean of 650 bp corresponding to 530 bp internal sequence length and then sequenced for 300 bp in "paired-end" module in one Illumina Miseq run. This resulted in 42 million "paired-end-reads" (12.7 Gb) in total. The overlapping "paired-end-reads" were assembled using FLASH (Magoc & Salzberg 2011) and screened for microsatellite motifs. Initially, 96 loci were selected, for which primers were designed. The 25 µl reaction mixture for amplifications contained 1 µl template DNA (10 ng), 1 µl of each primer (10 µM), 2 µl dNTPs (2.5 mM), 5 µl 5x PCR buffer (Promega, Mannheim, Germany), 2 µl $MgCl_2$ (25 mM), and 0.1 µl G2GoTaq-HotStart polymerase (1.25 U; Promega) and 12.9 µl aqua bidest, and PCR was performed under the following temperature cycles: initial denaturation for 3 min at 95 °C followed by 34 cycles each consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. PCR products were analysed using QIAxcel by Qiagen (Hilden, Germany) and 48 out of 96 reactions yielded distinct products of the respective expected size. Variability of the loci was tested by comparing PCR products of several individuals of *Myrmica tibetana* resulting in 11 suitable loci (Tab. 1). These loci were cross-amplified in *M. gebaueri* and *M. bactriana* and yielded also suitable results.

We performed the following multiplex PCR reactions (primer combinations see Tab. 1) based on the method of Schuelke (2000) in a total volume of 11.5 µl: 1 µl template DNA (10 ng), 1.25 µl of fwd primer (2 µM), 1.25 µl of rev primer (8 µM), 0.3 µl labeled M13 primer (2 µM), 2 µl dNTPs (2 mM), 1.25 µl S PCR buffer (Peqlab), 0.63 µl Enhancer (Peqlab) and 0.25 µl *Taq* polymerase (1.0 U; Peqlab). The PCRs were performed in an Eppendorf Mastercycler EP S programmed for 15 min at 94 °C followed by 34 cycles of 30 s at 94 °C, 30 s at 59 – 63 °C (see Tab. 1) and 60 s at 72 °C and a final extension for 30 min at 72 °C. Fragments were analysed on an ABI3730 sequencer using the size standard LIZ-500 (Life Technology) and scored with the software Peak Scanner v. 1.0 (Thermo Fisher Scientific).

We detected 5 - 10 alleles per locus and the final data set contained 26 samples and 89 alleles (5.1% missing values; supplementary information SI1, as digital supplementary material to this article, at the journal's web pages). The genotype data were transformed into Bruvo-distances, which incorporate mutational distances between alleles by including repeat motifs (Bruvo & al. 2004) using the package POLYSAT (Clark & Jaseniuk 2011) running under R environment (R Core Team 2016). Based on Bruvo distances we performed Principal Coordinate Analysis (PCoA; based on square-rooted distances) in R using the VEGAN v. 2.3.5 (Oksanen & al. 2015) and Neighbor-Net analysis using Splitstree v. 4 (Huson & Bryant 2006). Bayesian clustering was computed with the program Structure v. 2.3.4 (Pritchard & al. 2000; Falush & al. 2007) for co-dominant markers applying admixture model with correlated allele frequencies among populations. All runs of Structure were done without including geographic or morphological information. We analysed 1,000,000 generations after burn-in

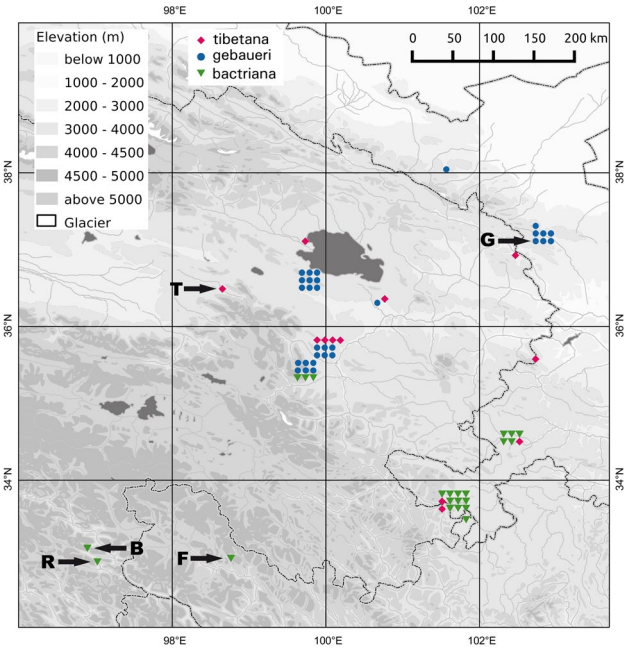

Fig. 2: Sampling sites of *Myrmica bactriana* Ruzsky, 1915 (green triangles), *M. tibetana* Mayr, 1889 (red rhombs), and of *M. gebaueri* sp.n. (blue dots) on the Tibetan Plateau. Coordinates are slightly manipulated to visualize the number of samples per site. Arrows point to type localities; G = *M. gebaueri* sp.n., T = *M. tibetana*; R = *M. ruzskyana* Radchenko & Elmes, 2010; B = *M. bactriana*, F = *M. furva* Ruzsky, 1915.

(500,000) for 10 replicates of models consisting of 1 to 6 clusters (K value). According to the method described by Evanno & al. (2005) the optimal number of clusters for the data set was three clusters (K = 3). The result of this model (K = 3) was graphically displayed using Distruct v.1.1 (Rosenberg 2004).

Results and discussion

Phenotypic clustering is convincing in explorative and supervised approaches

Myrmica tibetana Mayr, 1889, its cryptic sibling *M. gebaueri* sp.n. (formally described below) and *M. bactriana* Ruzsky, 1915 were convincingly demonstrable by NC clustering – the argumentation for the taxonomic naming of these clusters is provided below. Considering all 18 characters unselectively – the size indicator CS, the shape characters CL / CW_{950}, SL / CS_{950}, EYE / CS_{950}, FL / CS_{950}, FR / CS_{950}, PEW / CS_{950}, PPW / CS_{950}, PEH / CS_{950}, PEL / CS_{950}, SPBA / CS_{950}, SPTI / CS_{950}, SP / CS_{950}, MetL / CS_{950}, MetSp / CS_{950}, PoOc / CL_{950}, FL / FR_{950} and the setae character PPHL / CS_{950} – all three methods of exploratory data analyses showed three clear clusters with only two samples disagreeing in classification. If the latter were run as wild-cards in a three-class LDA to determine their final species hypothesis, they were allocated with posterior probabilities of p = 0.961 and p = 1.000. As result, NC-part.kmeans showed 0% deviation from the final species hypothesis whereas NC-Ward and NC-part.hclust misplaced one sample each, meaning 1.6% error (dendrogram not shown). These data show that the phenotypic classification was very strong already in the first run of exploratory data analyses considering all characters

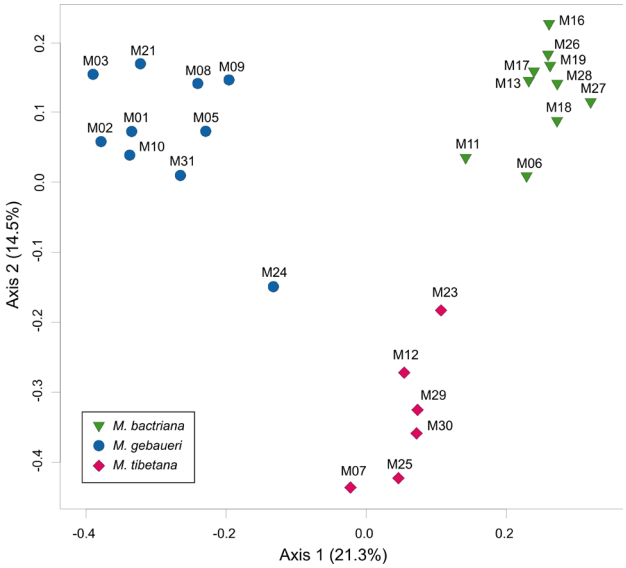

Fig. 3: Principal Coordinate Analysis of microsatellite data of *Myrmica bactriana* Ruzsky, 1915, *M. tibetana* Mayr, 1889, and of *M. gebaueri* sp.n. based on Bruvo distances presenting the first two axes.

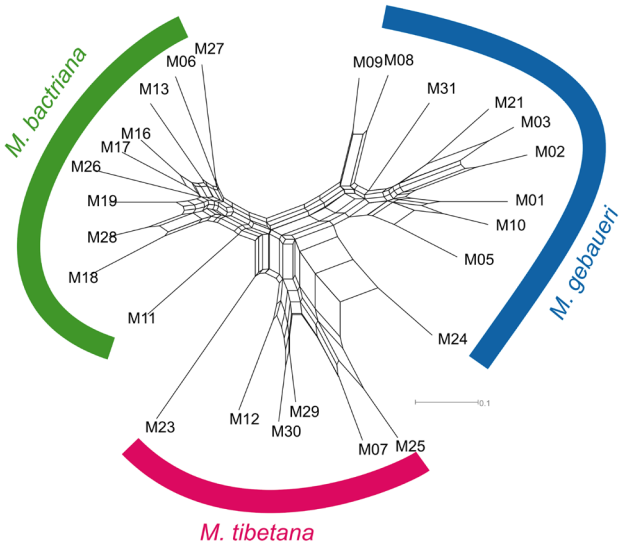

Fig. 4: Neighbor-Net analysis of microsatellite data of *Myrmica bactriana* Ruzsky, 1915, *M. tibetana* Mayr, 1889, and of *M. gebaueri* sp.n. based on Bruvo distances.

unselectively. The power of the applied NC-clustering methods becomes obvious if one considers that 54% of individuals are placed in the interspecific overlap range of the most discriminative character to separate the cryptic species *M. gebaueri* sp.n. and *M. tibetana* (EYE / CS).

To improve the separation, we ran a stepwise LDA reducing the number of characters to nine: EYE / CS_{950}, FL / CS_{950}, PPW / CS_{950}, PEL / CS_{950}, SPBA / CS_{950}, SPTI / CS_{950}, SP / CS_{950}, FL / FR_{950} and PPHL / CS_{950}. Using these characters, the classification of the 62 samples by all three data analyses was 100% coincident and no sample was misplaced (Fig. 1). The character reduction provided the favorable situation that the number of individuals in the smallest class (n = 45 in *Myrmica tibetana*) was 5fold larger than the

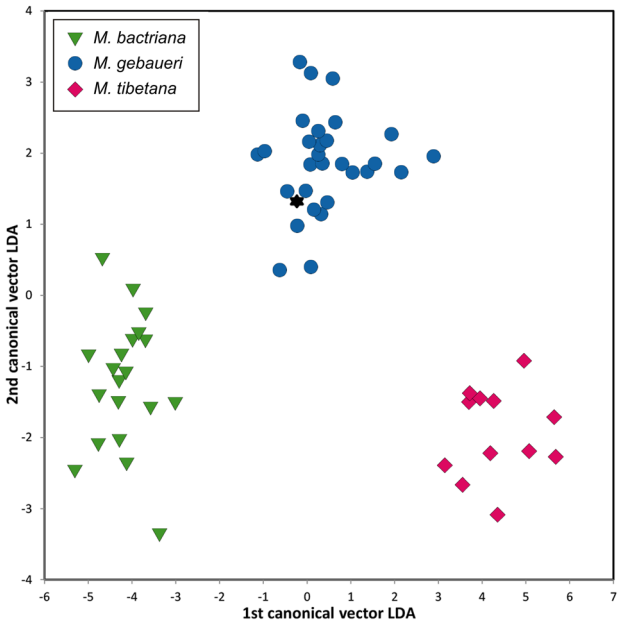

Fig. 5: Linear discriminant analysis (LDA) of 18 morphometric characters of *Myrmica bactriana* Ruzsky, 1915, *M. tibetana* Mayr, 1889 and of *M. gebaueri* sp.n. The black star represents sample M24 which was placed in the microsatelite analysis intermediate between *M. gebaueri* and *M. tibetana* and was run in the LDA as wild-card.

number of considered characters. Under this condition, the LDA and the leave-one-out-cross-validation LDA (LOOCV-LDA) confirmed the three-species classification in 178 individuals with an error of 0% and 0.6% respectively. If all individuals of the type samples were run as wild-cards in a LDA, the posterior probabilities were 1.000 in the lectotype of *M. tibetana*, 0.996 in the whole type series of *M. tibetana*, 1.000 in the holotype of *M. gebaueri* sp.n., 0.998 in the whole type series of *M. gebaueri* sp.n. and 1.000 in both type specimens of *M. furva* which were allocated to the *M. bactriana* cluster.

This very clear phenotypic clustering, the sharing of sympatric areas by all three entities and their syntopic occurrence at several sites (Fig. 2) clearly indicate separate species with significant reproductive barriers. There is also no indication for phenotypically mixed nests which excludes to explain *Myrmica gebaueri* sp.n. as intraspecific polymorphism of *M. tibetana* (Seifert 2016).

Microsatellite analysis confirms morphological classification

Analyses based on microsatellite data support the morphological discrimination into three species. The first two axes of the Principal Coordinate Analysis explain 35.8% of the variance in the data set and separate clearly between *Myrmica bactriana, M. gebaueri* and *M. tibetana* (Fig. 3). However, the sample M24 of *M. gebaueri* is placed intermediate between the remaining samples of *M. gebaueri* and *M. tibetana*. The situation in the Neighbor-Net analyses (Fig. 4) is likewise: A basically clear clustering into three species in agreement with morphology but an intermediate position of M24. We consider this result to be caused by the low sample size of *M. tibetana* causing instability in microsatellite analyses rather than to indicate a hybrid or

introgression of gene material. Morphological data clearly contradict a hybrid identity of M24. It has been repeatedly shown that NUMOBAT data indicate ant hybrids when the parental species are sufficiently separated in the vectorial space (Seifert 1984, 2006, Kulmuni & al. 2010, Seifert & al. 2010, Steiner & al. 2011, Bagherian Yazdi & al. 2012). In cases when the parental species are less clearly separable, the hybrid cluster may be close to one parental cluster or may merge with it (Seifert 2006). Yet, there is no case known where a hybrid sample is placed close to the centroid of one parent. If the three workers of sample M24 are run as wild-card in a three-class LDA considering all 18 phenotypical characters both individuals and sample mean are placed very near to the centroid of the *M. gebaueri* cluster (Fig. 5) with posterior probabilities of 1.000 in any case. A further argument for the species identity of M24 is provided by analysis of mtDNA: M24 is placed in the (paraphyletic) mtDNA tree within a branch only composed of *M. gebaueri* samples (Fig. 6).

Indication by mtDNA barcoding is in strong conflict with true species identities

The use of mitochondrial DNA as a leading tool in alpha-taxonomy is most problematic as it was already shown in the classic meta-analysis of 323 genera of Eumetazoa presented by Funk & Omland (2003). Given that other sources of error such as NUMTs (Bensasson & al. 2001) are excluded, the high frequency of paraphyly remains the biggest problem (e.g., Nichols 2001, Sota & Vogler 2001, Besansky & al. 2003, Funk & Omland 2003, Shaw 2003, Ballard & Whitlock 2004, Kocher 2004, Heinze & al. 2005, Hurst & Jiggins 2005, Lorenz & al. 2005, Mendelson & Shaw 2005, Meier & al. 2006, Wells & al. 2007).

The situation is similar in ants. Considering studies where mtDNA indication is controlled by reproducible and testable data sets of NUMOBAT and / or nuDNA, the error of barcoding on the alpha-taxonomic level ranges from 6% in *Tapinoma* (Seifert & al. 2017a) and 7% in *Cardiocondyla* (Seifert & al. 2017b) to 15% in the *Formica rufa* group (Seifert & Goropashnaya 2004), 17% in *Tetramorium* (Wagner & al. 2017), 19% in Neotropical *Linepithema* species (Wild 2009), and 23% in African *Cataglyphis* (Knaden & al. 2005). Considering studies with idiosyncratic morphology-based taxonomy as supervising system, mtDNA barcoding errors appear to be in the same range – e.g. in the genera *Anochoetus* and *Odontomachus* (Fisher & Smith 2008) and *Solenopsis* (Shoemaker & al. 2006). There is no integrative study known for ants, in which all disciplines were run in a controlled and testable mode, where mtDNA barcoding errors were below the range delimited above.

Taking mtDNA indication as final truth for the alpha-taxonomic structure of the Tibetan *Myrmica* studied here and accepting only nodes with bootstrap supports > 0.99, we would suppose six (or perhaps eight) instead of three species (Fig. 6). Relating a 6-species hypothesis based on mtDNA to the 3-species hypothesis achieved above by integrative taxonomy of NUMOBAT and nuDNA data, we have a minimum barcoding error of 24% if only the smaller of the deviant branches are considered as wrong and a bigger error if the larger deviant branches were wrong. The resulting average error of 16% of now seven studies in ants where mtDNA barcoding was controlled by reproducible and testable data sets of NUMOBAT and / or nuDNA shows the magnitude of the problem. These ant data add to the burden of evidence against the application of mtDNA barcoding as a leading tool in alpha-taxonomy.

In the particular case of the *Myrmica tibetana* species complex, we have no really strong data to conclude on the possible reasons for the mismatch between mtDNA barcoding and true species identities. A check, if different species shared mtDNA clades attributable to geographic spots in Tibet, was fully negative. From this point of view, incomplete lineage sorting appears as a less likely explanation for mismatches than occasional ancient hybridization with introgression of mtDNA. The latter issue has recently got a new, extreme component revealed by observations in *Formica* ants: there is virtually a selection favouring a mismatch of mitochondrial and nuclear DNA after a hybridization event and this selection acts in both directions instead of following the usual unidirectional pattern (Beresford & al. 2017). This situation may basically occur in any organisms with haplo-diploid sex determination.

Estimation of divergence times by mtDNA

There are strong methodological problems with datings of divergence times (see Takahata 2007 and references therein, Wilke & al. 2009). Furthermore, there is no fossil-based dating in *Myrmica* covering the range of the last 30 million years – Jansen & al. (2010) calibrated their *Myrmica* topology by fossil records dating back to 92 and 44 Ma. In the absence of ant-specific datings we used 1.2% nucleotide substitutions per Ma estimated for protostomians with the GTR model (Wilke & al. 2009). This certainly is under risk of a considerable error.

Based on the mean log likelihood values, variation in the data set was best explained by a codon model (GTR) with different parameters for the *ND6* and *Cytochrome b* partition, respectively. The partitioned codon model was clearly better than the same model with a strict molecular clock (delta log likelihood = 25) which is most likely due to a considerably lower evolutionary rate of the *Myrmica tibetana*-clade. The two phylogenies based on *ND6* and *cytb* separately showed the same topology as the data set combining the two genes and there was an identical tree topology when the analysis was run in BEAST (Drummond & al. 2012).

In contrast to NUMOBAT and nuDNA indication, Fig. 6 suggests a clear separation of a *Myrmica tibetana* clade (Tibe clade) from a *M. bactriana* / *M. gebaueri* sp.n. clade (BactGeba clade). The average number of nucleotide substitutions per site between the Tibe clade and the BactGeba clade was 10% in the GTR model which would translate into a divergence time of about 8 Ma when the 1.2% per Ma estimate should apply. The BEAST analysis suggested a range of 4.5 to 10% nucleotide substitutions corresponding to about 4 to 8 Ma. The Tibe clade and the four subclades of the BactGeba clade (Bact1, Bact2, Geba1 and Geba2) are "clean" – i.e., each of the five clades always contains only a single species classified by NUMOBAT and microsatellites. As most likely explanations for this type of paraphyly appear both ancient hybridization and incomplete lineage sorting when *M. bactriana* and *M. gebaueri* sp.n. split off. The fact that each of the four paraphyletic subclades contains only a single species may indicate that there was no crossbreeding or introgression event between *M. bactriana* and *M. gebaueri* sp.n. for a rather long evolutionary period.

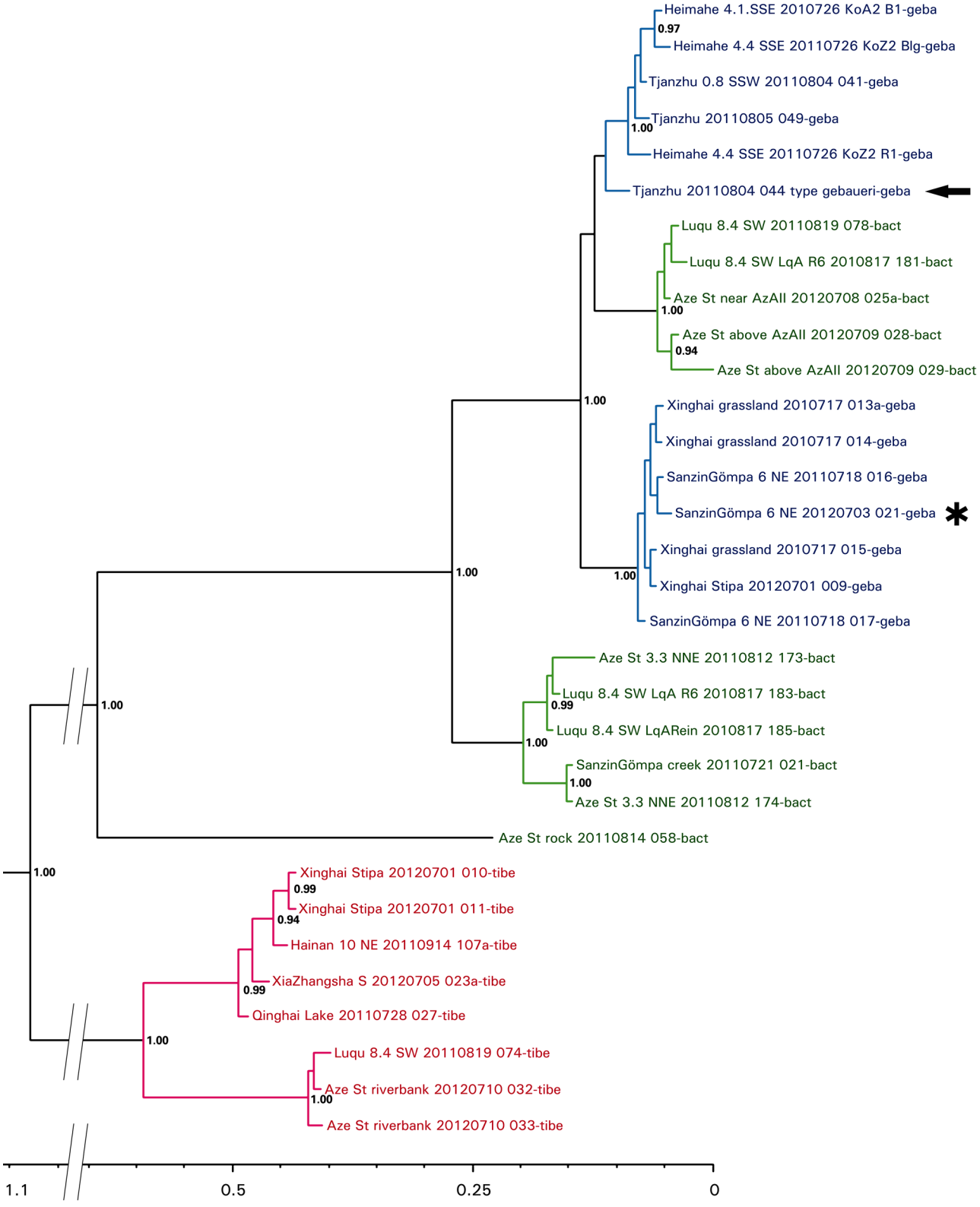

Fig. 6: mtDNA phylogeny of the *Myrmica tibetana* species complex based on the *cytb* and parts of *ND6* mitochondrial genes. Samples of *M. bactriana* Ruzsky, 1915 (in green), *M. gebaueri* sp.n. (in blue) and of *M. tibetana* Mayr, 1889 (in red). Support values (Bayesian posterior probabilities) < 0.90 are not shown. The arrow points to the type sample of *M. gebaueri* sp.n. and the asterisk marks the sample M24 which was placed in microsatellite analysis intermediate between *M. tibetana* and *M. gebaueri*.

Rapid speciation and development of cryptic species in the geographic region dealt with here has often been considered to be a consequence of a rapid rather recent uplift of the Tibetan Plateau (e.g., Liang & al. 2015, Liu & al. 2013, Lu & al. 2014, Wu & al. 2011). The strongest uplifts, generating the present shape of the Qinghai-Tibetan Plateau, were supposed to have occurred between 3.6 and 0.15 Ma Before Present (BP) and to have increased the mean altitude of the Plateau from 1000 to 4400 m (Li & Fang 1999). Yet, strongly opposing this extreme view, multiple arguments (reviewed in Renner 2016) rather indicate that a height of 4000 m has already been achieved by 40 Ma. Renner described a situation based on few outdated geological papers (such as Li & Fang 1999) as follows: "Biogeography of the Tibetan Plateau thus currently appears to be in a self-created bubble that encloses hundreds of authors and referees". As much further research has to be done on the issue, it is highly speculative, if not decisively wrong, to discuss speciation in the *Myrmica tibetana* complex within the context of a rapid Pliocene uplift scenario.

One sample of *Myrmica bactriana* from Aze Station, 2011.08.14, field number No 058 (= M11 in nuDNA data) showed a mtDNA sequence clearly different from the other conspecific samples (Fig. 6). We can offer no *a priori* explanation for this outlier. The sequence contains no frame shifts and no stop codons. We got identical sequences over 1370 bp across three genes (*ND6*, tRNASer, *cytb*) with three primer pairs. All workers from this nest sample showed no morphological abnormalities. This refers both to the complete nest series checked by subjective assessment and to the three workers investigated in the multivariate analyses. They were positioned near to the species' centroid.

How do our time estimates fit to the concept on *Myrmica* evolution of Jansen & al. (2010) in which two nodes were fixed by very ancient palaeochronological data? In their molecular phylogeny of Holarctic species, these authors identified a distinct monophyletic clade which they called the *M. rubra* group. Their material of this group contained samples of *Myrmica rubra* (Linnaeus, 1758), *M. ruginodis*, *M. kotokui* Forel, 1911 and *M. arisana* Wheeler, 1930, but they did not sample a species of the *M. tibetana* complex. The relatedness of the four species of the *M. rubra* group sensu Jansen & al. (2010) is confirmed by morphology: in the worker caste they share a produced, angular-convex clypeus without a median notch and a slender, moderately bent scape base without edges or carinae. Their males resemble in having a long scape with a slender, moderately bent base without edges or carinae (see also Radchenko & Elmes 2010). These are just the characters found in *M. tibetana, M. bactriana* and *M. gebaueri* sp.n. indicating a close relatedness with the *M. rubra* group sensu Jansen & al. (2010). Considering the findings of Radchenko & al. (2007) who dated the first *Myrmica* from Baltic and Saxonian amber back to 44.1 Ma BP, Jansen & al. (2010) estimated the beginning of radiation in the genus *Myrmica* back to the Eocene (41 Ma) and that of the *M. rubra* group to the Miocene (11 Ma). Our estimates of a divergence time of the *M. tibetana* complex from other members of the *M. rubra* group of about 16 Ma would indicate an earlier splitting.

Assessment of phylogenetic relatedness by the three indicators – morphology, nuDNA and mtDNA appears controversial. NC-Ward-clustering of morphology (Fig. 1) and NC-UPGMA clustering (not shown) suggest a sibling species relation between *Myrmica gebaueri* sp.n. and *M. tibetana* but mtDNA suggests a higher relatedness of *M. gebaueri* sp.n. and *M. bactriana*. In the morphological data set, a single character is responsible that *M. tibetana* and *M. gebaueri* sp.n. emerge from a common node in the dendrograms: They share a strong extension of frontal lobes (Fig. 7) and just this character is the most powerful discriminator of the two from *M. bactriana*. Yet, a summaric comparison over all characters (Tab. 2) shows that *M. gebaueri* sp.n. and *M. bactriana* show no significant differences in 47% of the characters whereas this figure is only 24% when *M. gebaueri* sp.n. and *M. tibetana* are compared. Thus it seems possible that metric characters as they are used here may lead to wrong genealogies in dendrograms of a group of cryptic species – the more as some of these characters are adaptive and subject to convergent evolution.

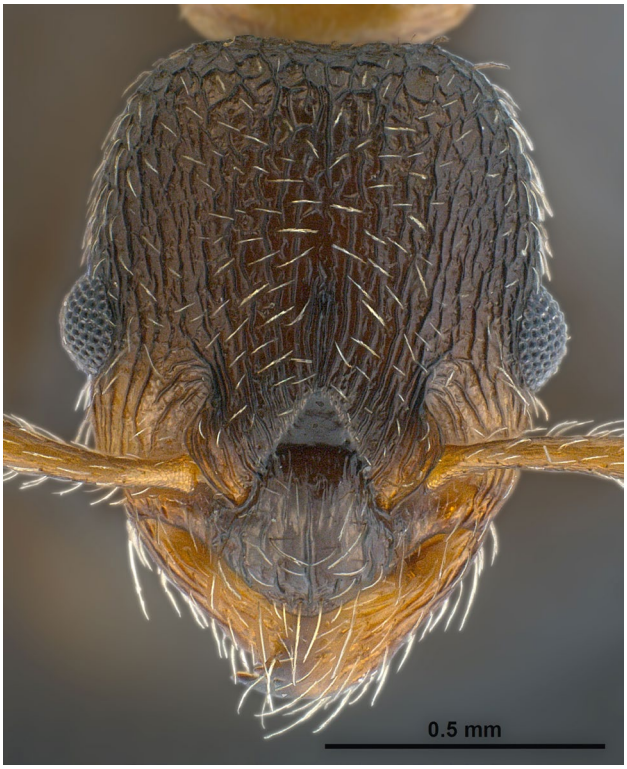

Fig. 7: Head of holotype of *Myrmica gebaueri* sp.n. in dorsal view.

Consideration of synonymies

Type samples of three taxa were available for the multivariate analyses and each of these was allocated to a different cluster. Because we introduce here a new species it must be asked if there are possible synonyms among described Palaearctic taxa of which types were not available. We found six candidates. The first two are *Myrmica bactriana* Ruzsky, 1915 [determined by Radchenko & Elmes (2010) as senior synonym of *M. furva* Ruzsky, 1915] and *M. ruzskyana* Radchenko & Elmes, 2010. The drawings of the lectotypes of *M. bactriana* and *M. ruzskyana* show only very slightly diverging frontal lobes: The ratio FL / FR is 1.070 and 1.080 respectively, both have a wedge shaped anterior clypeal margin, rather short scapes with a slender moderately curved basal part and numerous semierect setae, short and acute propodeal spines, a rather low petiole showing in profile a rounded node and no angular elements. The characters of

Fig. 8: Mesosoma of holotype of *Myrmica gebaueri* sp.n. in dorsal view.

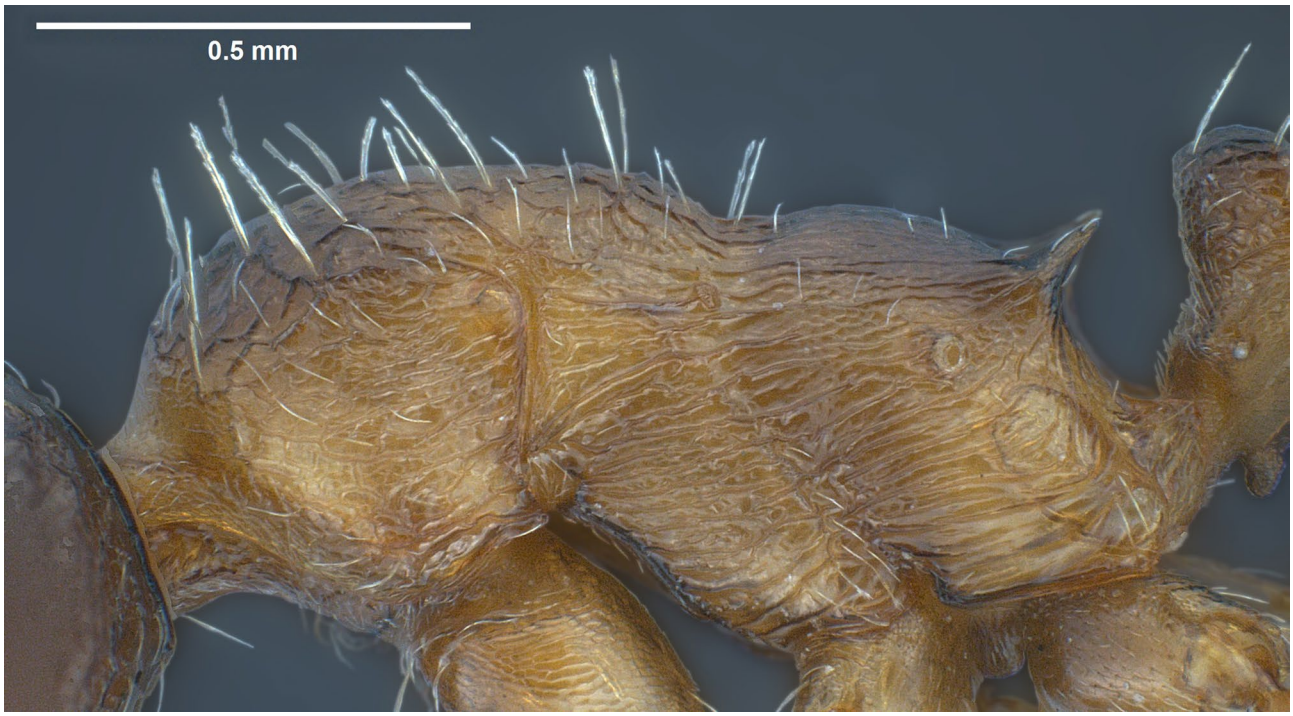

Fig. 9: Mesosoma of holotype of *Myrmica gebaueri* sp.n. in lateral view.

the investigated type sample of *M. furva* coincide with this diagnosis and FL / FR is similarly low: 1.083 and 1.115. There is no significant difference visible between these three taxa and their type localities are found in just the same region (Fig. 2): The basin of the river Yangtse around the present town of Yushu. The type localities of *M. bactriana* and *M. ruzskyana* are nearly syntopic and that of *M. furva* is perhaps some 170 km east. Radchenko & Elmes (2010), in separating *M. bactriana* and *M. ruzskyana,* presented the following arguments. "*M. bactriana* is very similar to *M. ruzskyana*, differing only by its distinctly longer scape ($SI_2 \geq 0.93$ vs. ≤ 0.91) with more abundant suberect hairs, and it is quite possible this represents different populations of the same species." We found that scape pilosity and length differed considerably within our *M. bactriana* material and do not have taxonomic significance. Arithmetic

mean, standard deviation, minimum and maximum of the scape length index SI_2 were 0.928 ± 0.23 [0.875, 0.992] in 52 measured workers of *M. bactriana* and several nest samples contained workers with both $SI_2 \geq 0.93$ and ≤ 0.91. Furthermore the NC-Ward dendrogram (Fig. 1) does not indicate a clear morphology-based substructuring with the *M. bactriana* clade and NC-part.hclust and NC-part.kmeans could not resolve subclusters. These data multiply to a high probability that *M. bactriana, M. furva* and *M. ruzskyana* belong to the same species and we follow Radchenko & Elmes (2010) in determining *M. bactriana* as the senior synonym.

The ratio FL / FR varied in the types of *Myrmica bactriana*, *M. furva* and *M. ruzskyana* in the narrow span between 1.070 and 1.115 whereas it ranged 1.162 - 1.319 in 45 workers of *M. tibetana* and 1.151 - 1.275 in 81 workers of *M. gebaueri* sp.n. This argument alone excludes a synonymy of *M. tibetana* and *M. gebaueri* sp.n. with *M. bactriana* and its synonyms.

The angularity and much stronger divergence of the frontal lobes also excludes a synonymy of the Himalayan *Myrmica smythiesii* Forel, 1902 [FL / FR of the lectotype 1.085 according to drawing in Radchenko & Elmes (2010)], *M. fortior* Forel, 1904 [FL / FR of a paralectotype 1.046 according to a photo from www.AntWeb.org, specimen CASENT0904090] and *M. wittmeri* Radchenko & Elmes, 1999 [FL / FR of the lectotype 1.060 according to drawing in Radchenko & Elmes (2010)]. Furthermore, the Himalayan species live in a very different climatic context compared to the Tibetan Plateau: mean annual air temperatures are by 10 – 15 °C higher and annual precipitations 2 - 3fold larger.

The 6th possible synonym of *Myrmica gebaueri* sp.n. – *M. tenuispina* Ruzsky, 1905 – was described from Western Tian Shan. It is similar to the species of the *M. tibetana* complex in overall body size, sculpture, shape of scape, clypeus and petiole and it inhabits a comparable montane to subalpine grassland habitat. A synonymy can be clearly excluded because the three measured worker specimens of the lectotype series of *M. tenuispina* showed a scape and spine length much larger than known for any specimen of the *M. tibetana* complex. The index SL*SP / FR was

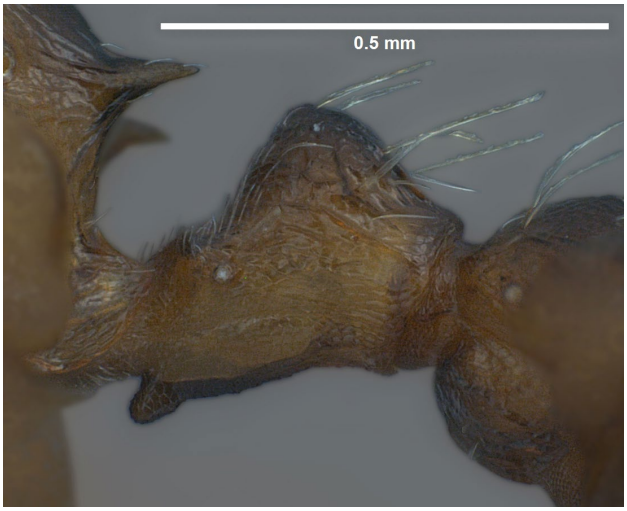

Fig. 10: Petiole of holotype of *Myrmica gebaueri* sp.n. in lateral view.

0.345 ± 0.038 [0.234, 0.440] in 178 workers of the *M. tibetana* complex but 0.679 ± 0.022 [0.653, 0.693] in the three *M. tenuispina* types and 0.610 ± 0.067 [0.526, 0.708] in 19 workers of *M. tenuispina* from Tian Shan.

***Myrmica gebaueri* sp.n.**

Etymology: Named after the German naturalist Axel Gebauer who made several expeditions to the Tibetan Plateau and was the first to collect this species in 1992.

Type material: Holotype labeled "CHI: 37.1852° N, 102.7844° E, Tianshu station-1.2 S, 2939 m moist pasture, under stone R.Schultz 2011.08.04-044" and "Holotype *Myrmica gebaueri* Seifert, Ritz & Schultz"; two worker paratypes on a different pin and 27 worker paratypes stored in ethanol – all from the same nest sample and with equal collecting data label as the holotype; all material stored in SMN Görlitz.

Description: Worker (Figs. 7 - 11, Tabs. 2 - 3, all morphometric ratios given in the following description are

Fig. 11: Left scape of holotype of *Myrmica gebaueri* sp.n. in caudodorsal view.

Tab. 2: Data of worker individuals of the three species of the *Myrmica tibetana* complex given as arithmetic mean ± standard deviation [lower extreme, upper extreme]; i = number of individuals. F values and significance levels p are from an univariate ANOVA and placed between the columns of the species pair compared; the F values of the characters best separating the species are given in heavy type.

	M. bactriana (i = 52)	**ANOVA F, p**	***M. gebaueri* sp.n.** (i = 81)	**ANOVA F, p**	***M. tibetana*** (i = 45)
CS [µm]	920 ± 37 [845, 1000]	0.00 n.s.	919 ± 37 [809, 1012]	33.16 0.000	960 ± 42 [886, 1059]
CL/CW	1.136 ± 0.017 [1.101, 1.172]	3.86 n.s.	1.129 ± 0.021 [1.070, 1.179]	3.34 n.s.	1.122 ± 0.019 [1.090, 1.169]
SL/CS	0.789 ± 0.017 [0.749, 0.834]	3.12 n.s.	0.794 ± 0.014 [0.750, 0.824]	**21.71** 0.000	0.782 ± 0.012 [0.761, 0.809]
PoOc/CL	0.419 ± 0.007 [0.398, 0.434]	**53.81** 0.000	0.409 ± 0.007 [0.394, 0.433]	3.58 n.s.	0.406 ± 0.010 [0.382, 0.422]
EYE	0.193 ± 0.007 [0.179, 0.209]	3.15 n.s.	0.191 ± 0.005 [0.177, 0.206]	**138.96** 0.000	0.204 ± 0.007 [0.191, 0.216]
FL/CS	0.437 ± 0.009 [0.412, 0.451]	**511.08** 0.000	0.481 ± 0.013 [0.462, 0.507]	**86.95** 0.000	0.502 ± 0.011 [0.478, 0.526]
FR/CS	0.397 ± 0.009 [0.377, 0.415]	4.87 0.029	0.401 ± 0.011 [0.380, 0.430]	0.52 n.s.	0.400 ± 0.011 [0.371, 0.425]
SPBA/CS	0.284 ± 0.012 [0.258, 0.308]	3.21 n.s.	0.281 ± 0.010 [0.262, 0.316]	**47.64** 0.000	0.294 ± 0.012 [0.269, 0.320]
SPTI/CS	0.309 ± 0.022 [0.266, 0.365]	**21.02** 0.000	0.325 ± 0.017 [0.288, 0.369]	**41.01** 0.000	0.302 ± 0.021 [0.269, 0.341]
PEW/CS	0.251 ± 0.009 [0.232, 0.273]	4.43 0.037	0.254 ± 0.011 [0.232, 0.281]	14.40 0.000	0.246 ± 0.012 [0.213, 0.270]
PPW/CS	0.379 ± 0.012 [0.348, 0.403]	**20.42** 0.000	0.390 ± 0.014 [0.359, 0.424]	**50.54** 0.000	0.371 ± 0.015 [0.342, 0.401]
PEH/CS	0.323 ± 0.008 [0.303, 0.348]	0.27 n.s.	0.324 ± 0.010 [0.305, 0.349]	**42.13** 0.000	0.312 ± 0.009 [0.290, 0.326]
PEL/CS	0.456 ± 0.014 [0.424, 0.485]	9.33 0.003	0.463 ± 0.013 [0.437, 0.499]	**50.47** 0.000	0.447 ± 0.012 [0.415, 0.470]
PPHL/CS	0.201 ± 0.010 [0.176, 0.222]	3.53 n.s.	0.206 ± 0.015 [0.121, 0.234]	**33.94** 0.000	0.190 ± 0.013 [0.154, 0.201]
SP/CS	0.180 0.011 [0.158, 0.206]	0.11 n.s.	0.179 ± 0.018 [0.135, 0.223]	**40.33** 0.000	0.159 ± 0.013 [0.125, 0.186]
MetL/CS	0.222 ± 0.009 [0.208, 0.244]	5.98 0.016	0.226 ± 0.010 [0.208, 0.249]	16.46 0.000	0.220 ± 0.007 [0.205, 0.234]
MetSp/CS	0.201 ± 0.013 [0.177, 0.232]	0.26 n.s.	0.200 ± 0.013 [0.171, 0.228]	1.82 n.s.	0.196 ± 0.016 [0.168, 0.227]

arithmetic nest sample means without removal of allometric variance): Most similar to *Myrmica tibetana*. One of the smallest species of the genus (CS 918 µm). Head with a weakly concave to straight posterior margin and strongly convex sides (Fig. 7) and rather elongated (CL / CW 1.129). Postocular distance rather low (PoOc / CL 0.410). Frontal lobes broad and significantly diverging (FL / CS 0.482, FL / FR 1.190), their lateral outline more angulate than convex, usually forming an angle ± 110°, frontal carinae merging with the rugae that surround antennal sockets. Eyes with few microsetae and rather small (EYE / CS 0.191), distinctly smaller than in *M. tibetana* (EYE / CS 0.205). Clypeus in dorsal view of head produced, its anterior margin more angulate than curved, forming an angle of 115 - 125°. Scape moderately long (SL / CS 0.794), with a slender, evenly curved basal part which performs a total bend of ±35° when viewed in the frontal or caudal standard viewing positions (SVP f or c in Seifert & al. 2014; Fig. 11 does not show a fully caudal aspect). Dorsal profile of mesosoma with a strongly convex promesonotal part, a strong metanotal depression and a convex dorsal part of propodeum (Fig. 9). Propodeal spines acute and short but on average longer than in *M. tibetana* (SP / CS 0.179 but 0.155 in the latter), spine axis in lateral view deviating from longitudinal mesosomal axis by 35 - 45°. Spines slightly diverging (Fig. 8): distance of spine base usually smaller than distance of tips (SPBA / CS 0.271, SPTI / CS 0.284) – in *M. tibetana* there is usually no divergence of spines (SPBA / CS 0.282, SPTI / CS 0.263). Central height of propodeal lobe only slightly larger than equal-level height of subspinal excavation (MetL / CS 0.226, MetSp 0.200). Petiole rather low (PEH / CS 0.323) and in lateral view with a concave anterior profile, a rounded dorsum of node and a slightly concave to almost straight caudodorsal profile (Fig. 10). Petiole in dorsal view with rather straight sides, slightly diverging caudad, its width about 65% of postpetiolar width. Setae are present

Tab. 3: Worker nest sample means of RAV-corrected morphometric data in three species of the *Myrmica tibetana* complex given as arithmetic mean ± standard deviation [lower extreme, upper extreme]; n = number of nest sample, i = number of individuals. F values and significance levels p are from an univariate ANOVA; the F values of the characters best separating *M. gebaueri* sp.n. and *M. tibetana* are given in heavy type.

	M. bactriana (n = 20)	**ANOVA F, p**	***M. gebaueri* sp.n.** (n = 30)	**ANOVA F, p**	***M. tibetana*** (n = 12)
CS [µm]	921 ± 32 [855, 977]	1.56 n.s.	918 ± 31 [847, 973]	12.93 0.001	958 ± 38 [907, 1045]
CL/CW (950)	1.134 ± 0.012 [1.108, 1.157]	2.26 n.s.	1.127 ± 0.016 [1.100, 1.153]	1.525 n.s.	1.121 ± 0.012 [1.094, 1.142]
SL/CS (950)	0.786 ± 0.012 [0.761, 0.810]	3.19 n.s.	0.793 ± 0.012 [0.769, 0.822]	11.81 0.001	0.779 ± 0.009 [0.762, 0.796]
PoOc/CL (950)	0.416 ± 0.005 [0.405, 0.423]	21.95 0.000	0.407 ± 0.005 [0.397, 0.422]	0.45 n.s.	0.406 ± 0.008 [0.392, 0.418]
EYE (950)	0.196 ± 0.005 [0.187, 0.204]	3.88 n.s.	0.193 ± 0.004 [0.186, 0.203]	**72.95** 0.000	0.206 ± 0.005 [0.200, 0.215]
FL/CS (950)	0.437 ± 0.006 [0.416, 0.447]	**328.70** 0.000	0.482 ± 0.010 [0.464, 0.502]	**55.08** 0.000	0.505 ± 0.008 [0.494, 0.518]
FR/CS (950)	0.395 ± 0.007 [0.380, 0.407]	4.12 0.048	0.400 ± 0.008 [0.387, 0.417]	0.58 n.s.	0.402 ± 0.007 [0.388, 0.412]
SPBA/CS (950)	0.283 ± 0.010 [0.267, 0.299]	1.20 n.s.	0.280 ± 0.008 [0.261, 0.300]	13.24 0.001	0.291 ± 0.009 [0.271, 0.305]
SPTI/CS (950)	0.303 ± 0.015 [0.279, 0.334]	13.80 0.001	0.318 ± 0.014 [0.289, 0.346]	**24.35** 0.000	0.294 ± 0.017 [0.264, 0.330]
PEW/CS (950)	0.249 ± 0.007 [0.238, 0.263]	3.31 n.s.	0.253 ± 0.006 [0.241, 0.266]	11.38 0.002	0.244 ± 0.011 [0.225, 0.255]
PPW/CS (950)	0.378 ± 0.011 [0.358, 0.404]	11.32 0.002	0.388 ± 0.010 [0.366, 0.405]	18.56 0.000	0.372 ± 0.013 [0.349, 0.392]
PEH/CS (950)	0.322 ± 0.007 [0.309, 0.336]	0.15 n.s.	0.323 ± 0.007 [0.307, 0.334]	**25.91** 0.000	0.311 ± 0.007 [0.299, 0.320]
PEL/CS (950)	0.455 ± 0.011 [0.435, 0.474]	5.16 0.028	0.461 ± 0.009 [0.442, 0.485]	16.60 0.000	0.448 ± 0.011 [0.430, 0.466]
PPHL/CS (950)	0.200 ± 0.008 [0.187, 0.218]	2.82 n.s.	0.205 ± 0.010 [0.170, 0.221]	19.70 0.000	0.190 ± 0.010 [0.175, 0.206]
SP/CS (950)	0.179 0.009 [0.158, 0.194]	0.07 n.s.	0.179 ± 0.015 [0.152, 0.216]	**23.66** 0.000	0.155 ± 0.012 [0.125, 0.167]
MetL/CS (950)	0.223 ± 0.008 [0.212, 0.242]	2.76 n.s.	0.227 ± 0.008 [0.208, 0.243]	8.64 0.005	0.219 ± 0.005 [0.207, 0.226]
MetSp/CS (950)	0.201 ± 0.010 [0.189, 0.220]	0.11 n.s.	0.200 ± 0.010 [0.181, 0.221]	3.40 n.s.	0.194 ± 0.012 [0.174, 0.212]

on all dorsal parts of body and rather long (PPHL / CS 0.206). Vertex moderately strong longitudinally rugose, posterior vertex reticulate; about 16 - 23 rather linear rugae are found between the most approximated parts of frontal carinae. Mesosoma and waist with a weak sculpture in terms of genus *Myrmica*, larger surface areas may be smooth. Dorsum of promesonotum as a rule reticulate-rugose, meso- and metapleuron longitudinally carinulate. Dorsal propodeum weakly carinulate-rugulose, substantial parts of its surface often completely smooth. Dorsum of petiole reticulate-rugulose, central dorsum of postpetiole at lower magnification always appearing smooth and shining, a delicate microreticulum becomes visible at larger magnifications. Whole body usually rather uniformly medium brown with a weak yellowish component and sometimes with a lighter mesosoma.

Distribution and biology: NE Tibet between 35.5 and 38.0° N and 99.8 and 102.8° E (Fig. 2), in altitudes of 2900 - 3500 m. Found on montane to subalpine grassland, usually pastures. Nests in soil, under stones or in grass tussocks. Polygynous.

Systematic position: Based on morphological and genetic arguments, we stated above a close relatedness of the three species of the *Myrmica tibetana* complex to the *M. rubra* group sensu Jansen & al. (2010). Yet, the closest relatives from a morphological point of view most probably are the Himalayan species *M. smythiesii* Forel, 1902, *M. fortior* Forel, 1904 and *M. wittmeri* Radchenko & Elmes, 1999. They resemble the members of the *M. tibetana* complex in the following characters:

a) a produced, angular-convex clypeus without a median notch,
b) a slender, moderately bent scape base without edges or carinae,
c) absence of any angularity in frontal, dorsal and caudodorsal parts of petiole profile,

d) rather reduced sculpture and weakly developed propodeal spines.

In our opinion the three Tibetan and three Himalayan species can be combined in a *Myrmica tibetana* group which divides into a *M. tibetana* and a *M. smythiesii* complex. There are no genetic data available for the Himalayan species.

Differential diagnosis against the next similar species: The separation of *Myrmica gebaueri* sp.n. and *M. tibetana* is most difficult and a safe discrimination on the worker individual level is only possible by the multivariate analyses described above. Compared to *M. tibetana*, *M. gebaueri* sp.n. shows smaller eyes and longer and more diverging propodeal spines. A parsimonious morphometric method allows a determination on nest sample level if two or three workers per sample are measured. We simplified the species delimitation procedure by using absolute linear measurements and by reducing the number of characters for the condition that the error at nest sample level was zero. We emphasize at this point that the measuring instructions for each character have to be considered. The extracted morphometric method requires five minutes working time in a mounted specimen. With all measurements recorded in mm, a linear discriminant function

D(3) = 62.835 EYE + 44.41 SPBA - 50.213 SP - 15.713

resulted in an error of 0% on the nest sample level. Samples with D(3) < 0 are classified as *Myrmica gebaueri* sp.n., those above this threshold as *M. tibetana*. The error on the worker individual level was 5.6% in 126 specimens. The easy separation from *M. bactriana*, the three species of the *M. smythiesii* complex and from *M. tenuispina* has been treated in the section considering possible synonymies.

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A new extinct ant genus (Hymenoptera: Formicidae: Myrmicinae) from the Late Eocene Rovno amber – a putative ancestor of the *Leptothorax* genus group

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Abstract

A new genus and a new species of ant, *Proleptothorax primitivus*, are described based on two males from the Late Eocene Rovno amber of Ukraine. This genus is characterized by the antennae 13-segmented, with the funiculus filiform without an apical club, by the very short antennal scape, by the short and narrow, bidentate mandibles, and by the forewing with cell 3r closed. We consider these features as obvious plesiomorphies compared with *Leptothorax* Mayr, 1855, *Formicoxenus* Mayr, 1855, and *Harpagoxenus* Forel, 1893 and assume that *Proleptothorax* could be regarded as a putative ancestor of the extant genera of the *Leptothorax* genus group.

Key words: Ants, palaeontology, *Proleptothorax primitivus* gen.n. et sp.n., new genus, new species, Late Eocene, Rovno amber, evolution.

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Introduction

The ant fauna of the Late Eocene European ambers (Priabonian stage, 33.9 - 37.2 million years ago, mya) is the best studied among all fossil myrmecofaunas worldwide. The subfamily Myrmicinae is particularly well represented in these ambers, showing a high diversity: Currently, there are an estimated 78 myrmecine species from 27 genera, including several new, yet undescribed taxa (Radchenko & Dlussky 2017a), which is about 40% of both genera and species of the total number of the Late Eocene European amber ants.

Ten out of eleven previously described extinct myrmicine genera from the Late Eocene European ambers are highly specialized morphologically and could not be considered as the ancestors of any extant ant genus (Dlussky & Radchenko 2006a, b, 2009, Radchenko & Dlussky 2012, 2013, 2016, 2017a, b). The sole exception to this is *Parameranoplus* Wheeler, 1915, which might be a putative ancestor of the modern Old World tropical genus *Meranoplus* F. Smith, 1853. *Parameranoplus* workers possess several plesiomorphies comparable with those of workers of *Meranoplus*, such as 11-segmented antennae, and, perhaps most importantly, a promesonotum that does not form a developed shield overhanging the pleurae laterally and has no long promesonotal spines posteriorly. In contrast, the antennae in *Meranoplus* are 9-segmented, and the promesonotum forms a shield that overhangs the pleurae laterally and has long promesonotal spines posteriorly (see Wheeler 1915, Bolton 2003). On the other hand, *Parameranoplus* itself possesses a set of apomorphies compared with many less specialized morphologically genera, such as 11-segmented antennae *vs.* 12-segmented, developed antennal scrobes that are above the eyes, pointed humeral angles, flattened promesonotum, short and clavate femora. Consequently, even if this genus were the putative ancestor of *Meranoplus*, it forms itself a derived lineage relative to a yet unknown precursor.

Mayr (1868) then Wheeler (1915) described six fossil species, initially attributed to the genera *Leptothorax* Mayr, 1855, *Macromischa* Roger, 1863, and *Nothomyrmica* Wheeler, 1915. However, all these were later transferred to the genus *Temnothorax* Mayr, 1861 (Bolton 2003, Dlussky & Radchenko 2006b).

The taxonomic history of the generic names *Leptothorax* and *Temnothorax* is quite complicated. Mayr (1855) described *Leptothorax*, based on 12 species. A few years later, he described the closely related genus *Temnothorax* (Mayr 1861) with a single species – *T. recedens* (the type species of this genus by monotypy). For many years, *Temnothorax* was treated by various authors either as a genuine genus or as a subgenus of *Leptothorax*, or even as a junior synonym of *Leptothorax* (see Bolton 2003).

Bingham (1903) designated *Formica acervorum* Fabricius, 1793 as the type species of the genus *Leptothorax*. Almost at the same time, Ruzsky (1904) established the genus *Mychothorax*, to which *F. acervorum* was also assigned as the type species (by original designation). Emery (1912) later designated *Myrmica clypeata* Mayr, 1853 as the type species of *Leptothorax*. Despite the fact that this designation was unjustified, all subsequent authors considered *Mychothorax* as a subgenus of *Leptothorax* and attributed species with 11-segmented antennae in workers

Figs. 1 - 2: Photographs of the holotype male of *Proleptothorax primitivus* gen.n. et sp.n. (1) Body in dorsal view; (2) head and mesosoma in dorsal view.

and queens to *Mychothorax*, and those with 12-segmented antennae to *Leptothorax* s.str.

Eventually, M.R. Smith (1950) synonymised *Mychothorax* with *Leptothorax* as they have the same type species (hence, an absolute synonymy) and established *Myrafant* as a new subgenus of *Leptothorax*, with the type species *L. curvispinosus* Mayr, 1866. Then, species from the former subgenus *Leptothorax* s.str. (sensu Emery 1912) were transferred to the subgenus *Myrafant*, and those that were in *Mychothorax* were considered *Leptothorax* s.str. (sensu Bingham 1903).

Essentially, there are many differences between *Leptothorax* s.str. and the subgenus *Myrafant* and the possibility of separating them into two genera was discussed by many myrmecologists. Consequently, Bolton (2003) formally divided them as different genera, revived several generic names from synonymy, and provided new synonyms. He proposed the following arrangement of the former *Leptothorax* (s.l.) (only Holarctic taxa are given here; for more details, see Bolton 2003, Prebus 2017): *Leptothorax* Mayr, 1855 (= *Mychothorax* Ruzsky, 1904; = *Doronomyrmex* Kutter, 1945); *Temnothorax* Mayr, 1861 (= *Myrafant* M. R. Smith, 1950).

Lastly, Prebus (2017) established an informal *Leptothorax* genus group within the tribe Crematogastrini (sensu Ward & al. 2015), including the genera *Leptothorax*, *Formicoxenus* Mayr, 1855, and *Harpagoxenus* Forel, 1893, and considered this group as a sister clade of *Temnothorax*.

Below, we describe a new fossil genus, *Proleptothorax* gen.n. and assume that it can be the putative ancestor for the genera *Leptothorax*, *Formicoxenus*, and *Harpagoxenus*.

Material and methods

Two males were investigated of the herein described genus and species, *Proleptothorax primitivus* gen.n. et sp.n., from the Rovno amber (Ukraine, Late Eocene, Priabonian stage, 33.9 - 37.2 mya; see Perkovsky & al. 2010 for details on the age and deposit). The holotype and paratype specimens were deposited in the Schmalhausen Institute of Zoology of the National Academy of Sciences of Ukraine, Kiev (SIZK). For comparison, we also studied 80 males of 24 extant species of the genera *Leptothorax*, *Temnothorax*, and *Harpagoxenus*, as well as 41 fossil *Temnothorax* specimens from the Late Eocene European ambers.

The figures are based on the original drawings of the specimens and photographs made using an Olympus Camedia C-3030 digital camera fitted to an Olympus SZX9 microscope in conjunction with the computer program CorelDraw 13.

The nomenclature of the wing venation follows Dlussky & Perfilieva (2014; see also Fig. 3). Two indices that characterize important features of venation of the forewings were used:

Icu = [1*Cu* + (2*M*+*Cu*)] / 1*Cu*

Icua = [(1*M*+*Cu*) + (2*M*+*Cu*)] / (1*M*+*Cu*) (for details, see Dlussky & Perfilieva 2014).

Morphometrics: The holotype specimen of *P. primitivus* was measured (accurate to 0.01 mm), and the measurements were used to calculate the various ratios defined below. Additionally, we measured head length, scape length, and length of the first funicular segment of males of modern *Leptothorax*, *Harpagoxenus*, and *Temnothorax* species.

2FSL length of second funicular segment
GL length of genae, measured from anterior margin of eyes to mandibular insertion
HTL maximum length of hind tibia
HL maximum length of head in dorsal view, measured in a straight line from anteriormost point of clypeus to mid-point of occipital margin
HW maximum width of head in dorsal view behind (above) the eyes
ML length of mesosoma in dorsal view from anterior end of scutum to the point of articulation with petiole
OL maximum diameter of eye
PL maximum length of petiole in dorsal view, measured from posterodorsal margin of petiole to articulation with propodeum
PH maximum height of petiole in profile, measured from uppermost point of petiolar node perpendicularly to a virtual line between tip of subpetiolar process and posteroventral points of petiole
PPH maximal height of postpetiole in profile
PPL maximum length of postpetiole in dorsal view between its visible anterior and posterior margins
PPW maximum width of postpetiole in dorsal view
PW maximum width of petiole in dorsal view
ScL length of the scutum + scutellum in dorsal view
ScW maximum width of scutum in dorsal view
SL maximum straight-line length of scape from its apex to the articulation with condylar bulb

For simplicity, in this paper ratios of various measurements are used (e.g., HL / HW) rather than indices and their abbreviations (e.g., CI) as done elsewhere previously.

Systematic palaeontology

Family Formicidae Latreille, 1809

Subfamily Myrmicinae Lepeletier de saint-fargeau, 1835

Genus *Proleptothorax* gen.n.

Type species: *Proleptothorax primitivus* sp.n.

Derivation of name: from Greek "*pro*" – before, prior to, and the ant genus *Leptothorax*.

Diagnosis:

- antennae 13-segmented
- antennal scape very short (SL / HL 0.16, SL / HW 0.18)
- antennal funiculus filiform, without apical club
- length of the second funicular segment subequal to the third one, while distinctly longer than scape, the longest segments are 5th, 6th and 7th
- mandibles short and narrow, bidentate
- forewing with cell 3r closed by vein 5RS
- maxillary palps 5-segmented, labial palps 3-segmented
- scutum with deeply impressed and crenulated notauli

Proleptothorax primitivus sp.n. (Figures 1 - 8)

Derivation of name: from Latin "*primitivus*" – primitive, primal, which means presence of the many primitive morphological features in this species.

Material examined: holotype: SIZK No. K-26591, male, complete specimen; **paratype:** SIZK No. K-18699, male, complete specimen.

Type locality: Ukraine, Rovno Prov., vicinity of Klesov.

Type horizon: Rovno amber, Late Eocene (Priabonian stage).

Diagnosis: As for genus.

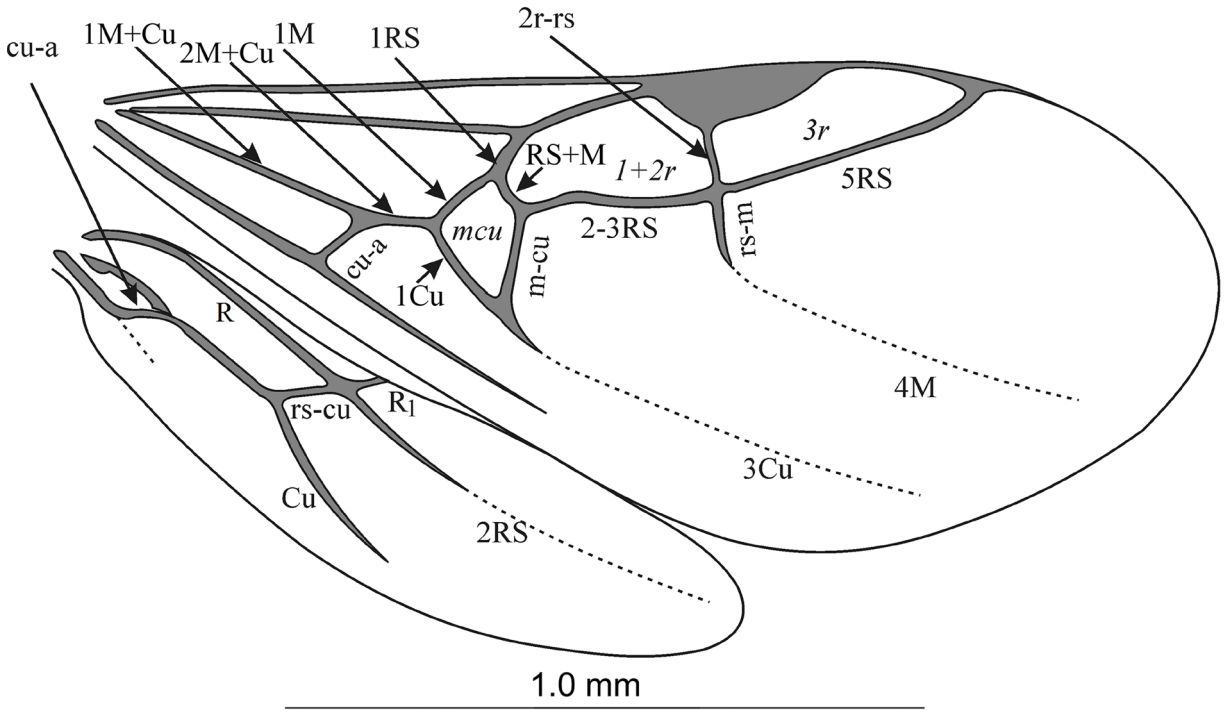


Fig. 3: Forewing venation and hind wing of *Proleptothorax primitivus* gen.n. et sp.n. with designation of cells and veins.

Description: Total length ca. 2.2 - 2.3 mm. Head slightly longer than wide, widely and gradually rounded above eyes and with strongly convex occipital margin as seen dorsally. Frons slightly longitudinally depressed; frontal carinae and frontal lobes not developed. Distance between antennal insertions 0.08 mm. Torulus subvertical. Clypeal surface gradually convex along its width, without longitudinal carinae, anterior margin of clypeus slightly convex, without medial notch. Eyes large, bulging, their maximal diameter about half of head length, situated distinctly in front of midlength of sides of head, so that temples longer than maximum diameter of eye. Genae very short, four times shorter that maximum diameter of eyes. Ocelli well developed and large, diameter of central ocellus 0.06 mm, distance between posterior ocelli 0.09 mm; lower edge of central ocellus lays slightly posteriorly of imaginary line connecting posterior margins of eyes. Length of mandibles 0.12 mm.

Mesosoma relatively long and rather narrow, scutum quite strongly convex as seen laterally, scutellum much less convex, lays distinctly lower than surface of scutum, scutoscutellar sulcus wide, deep and crenulated. Propodeum angulate, without teeth, its dorsal surface subequal to posterior one. Petiole with quite long peduncle, twice longer than high, anterior surface of petiolar node very weakly concave, posterior one slightly convex, node dorsum widely rounded; postpetiole subglobular. Legs long and slender, femora not swollen. Hind coxae widely separated in ventral view, when coxae directed outward, their inner margins far apart. Pretarsal claws simple. Middle and hind tibiae with short simple spur.

Forewing with closed cells *mcu, 1+2r* and *3r*, cells *cua* and *rm* are absent. Pterostigma quite big, somewhat rounded. Cell *3r* relatively short, its length subequal to length of cell *1+2r*, apex of cell *3r* touches wing margin. Cell *mcu* small, trapezoid, high, its height equal to length of midline, section 2-3RS more than 3 times longer than RS+M. Vein 5RS very feebly curved, almost straight, vein section 2-3RS S-shaped. Cross-vein rs-m merging with vein section 4M at a blunt angle, it diverges with cross-vein 2r-rs and vein 5RS from same knot so that vein section 4RS is absent. Vein 3Cu feebly marked, not sclerotized. Wing length 1.87 mm, distance from wing base to pterostigma 0.82 mm. Icu = 1.57, Icua = 1.27.

Hind wing without free section of medial vein, free section of cubital vein (Cu) very slightly curved, almost straight. Cross-vein rs-cu not curved. Veins R, rs-cu, 2RS and R_1 diverge from same knot. Cross-vein cu-a located approximately at the midlength between base of wing and branching of rs-cu and Cu. Wing length 1.21 mm.

Since wings of the specimen were somewhat deformed (curved down) during fossilization, the shape of the cells *mcu* and *1+2r* appear slightly distorted.

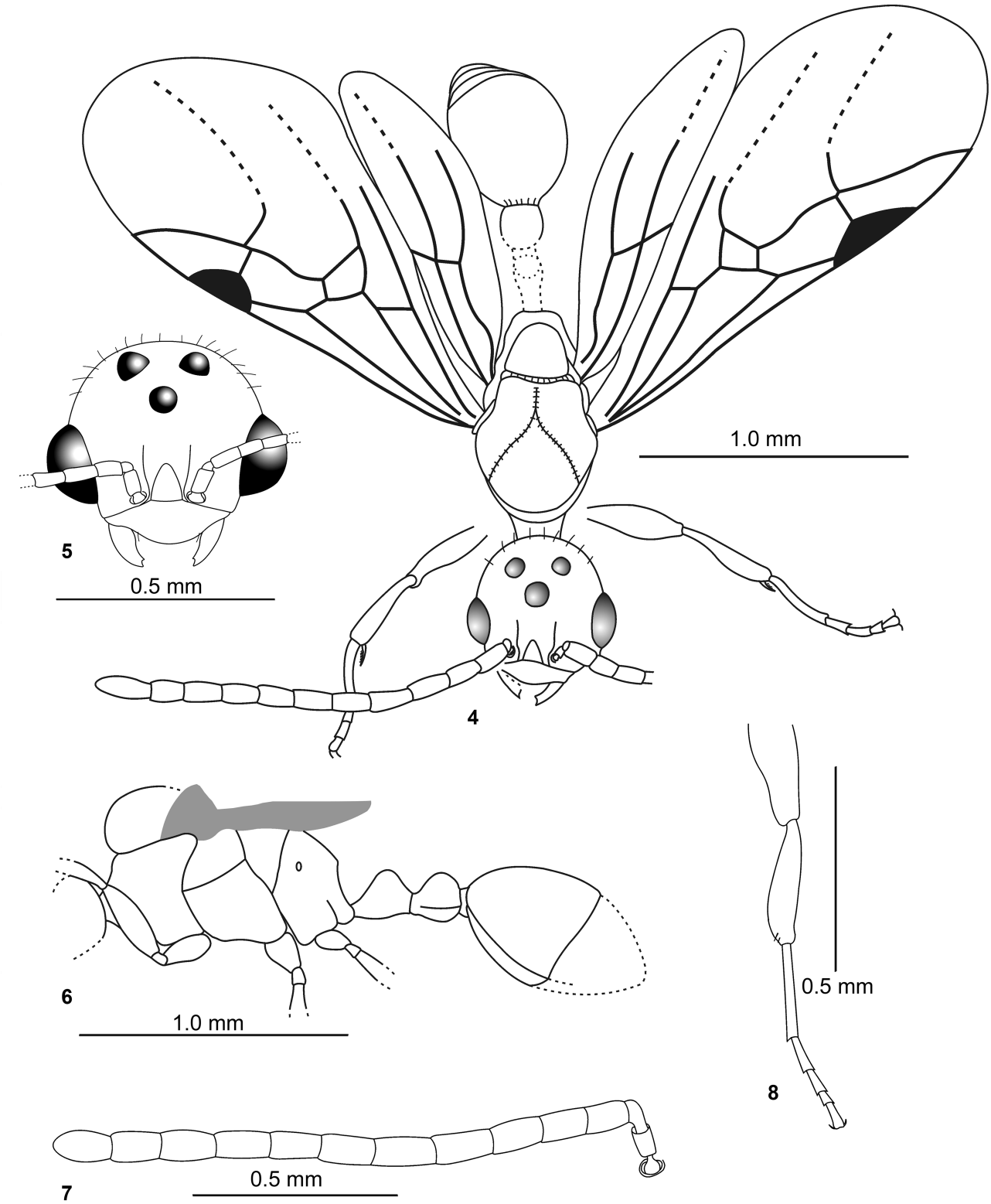
Head dorsum quite coarsely and densely punctated, frons laterally also with longitudinal striation. Scutum, scutellum, propodeal dorsum waist and gaster smooth and shiny; pronotum, mesopleura and sides of propodeum finely superficially punctated, appears dull. Whole body with quite abundant, thin erect to suberect hairs, scapes and legs with dense decumbent pubescence.

Measurements (in mm): GL 0.06, HL 0.51, HTL 0.27, HW 0.46, ML 0.67, OL 0.24, PH 0.16, PL 0.32, PPH 0.16, PPL 0.16, PPW 0.13, PW 0.11, ScL 0.57, ScW 0.43, SL 0.08.

Ratios: GL/OL 0.25, HL/HW 1.12, OL/HL 0.47, PL/HL 0.63, PL/PH 2.00, PPL/PPH 1.20, ScL/ScW 1.34, SL/HL 0.16, SL/HW 0.18.

Workers and queens unknown.

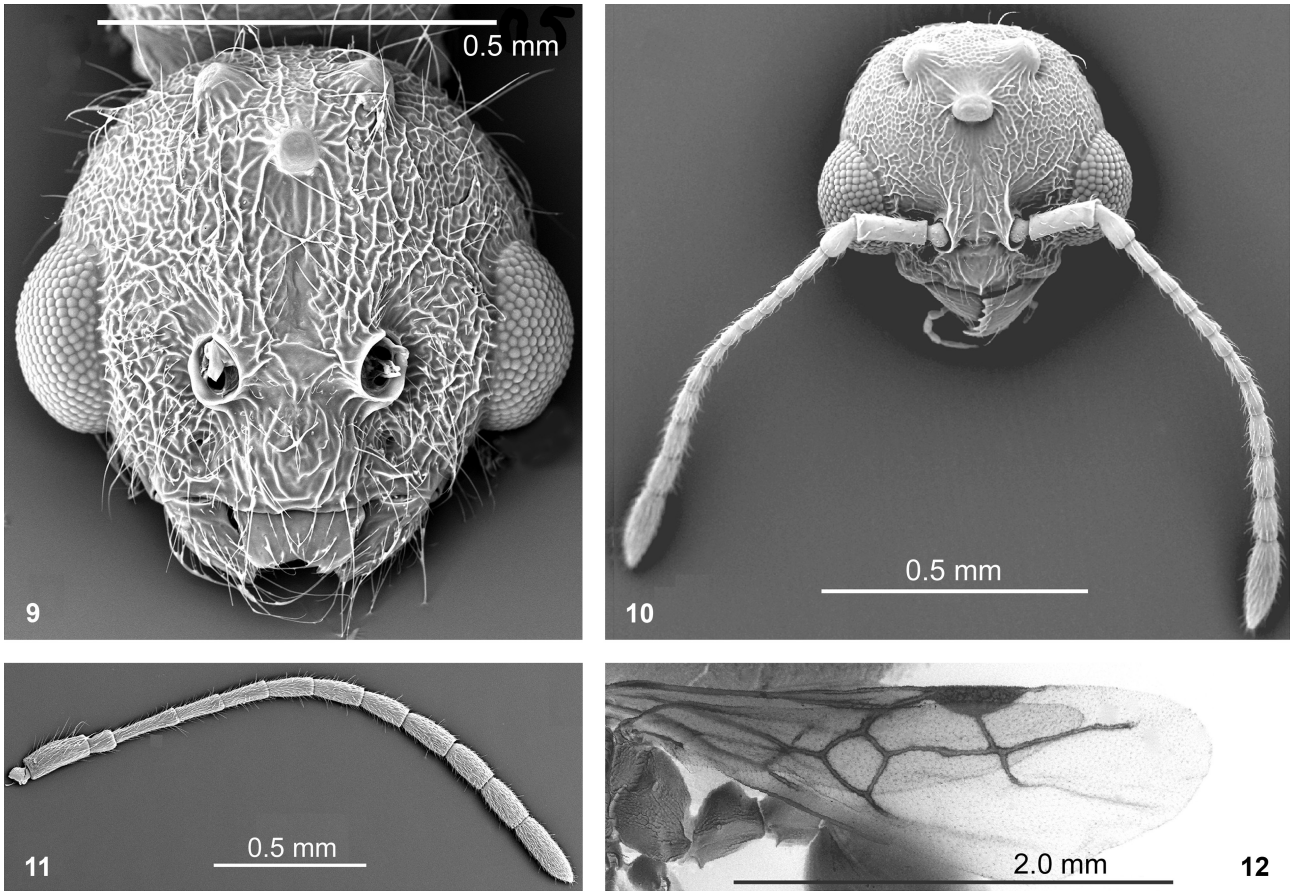
Note: The paratype specimen is mostly concealed by turbid film and hardly measurable, though the forewing venation, antennal structure and general shape of the body (in profile) are visible. Its body length is ca. 2.2 mm.



Figs. 4 - 8: Line drawings of the holotype male of *Proleptothorax primitivus* gen.n. et sp.n. (4) Body in dorsal view; (5) head in dorsal view; (6) mesosoma, waist, and gaster in lateral view; (7) antenna; (8) hind tibia and tarsus.

Discussion

Males of the genera of the *Leptothorax* genus group (sensu Prebus 2017) (i.e., *Leptothorax, Harpagoxenus*, and non-ergatoid *Formicoxenus*) have mandibles with the short, blunt, edentate masticatory margin, 12-segmented antennae with the filiform funiculus without an apical club; the second funicular segment long, obviously longer than each of the remainder ones except for the apical one and nearly the same length as the antennal scape in *Leptothorax* and *Harpagoxenus* (ratio 2FSL / SL 0.857 – 1.056, mean 0.988 ± 0.044) (Figs. 9, 11), while it is somewhat longer in



Figs. 9 - 12: SEM photographs of the details of structure of *Leptothorax* and *Temnothorax* males. (9, 11) *Leptothorax muscorum*; (10) *Temnothorax unifasciatus*; (12) *Leptothorax kutteri*; (9) head in dorsal view; (10) head and antennae in dorsal view; (11) antenna; (12) forewing.

the non-ergatoid males of *Formicoxenus* and subequal to the total length of the first and second funicular segments (for more details, see Francoeur & al. 1985). In contrast, males of the genus *Temnothorax* have mandibles with a well developed masticatory margin and with 5 - 7 teeth, 13-segmented antennae (very rarely 12-segmented), the antennal funiculus with a distinct 4-segmented apical club, and a short second funicular segment, at least twice shorter than the length of scape (Fig. 10).

In general, the antennal scape is somewhat shorter in males of *Leptothorax* and *Harpagoxenus* species than in *Temnothorax* ones: means SL / HL are 0.342 ± 0.014 [n = 23, five species of *Leptothorax* and *H. sublaevis* (Nylander, 1849)] *vs*. 0.361 ± 0.037 (n = 57, 18 species of *Temnothorax*), while these ratios highly overlap between the mentioned genera: minimum - maximum are 0.310 - 0.383 in *Leptothorax* and *H. sublaevis vs*. 0.290 - 0.439 in *Temnothorax*.

Additionally, the cell *3r* on the forewing is open in the *Leptothorax* group genera (Fig. 12) as well as in *Temnothorax* males, though this cell occasionally might be closed in some *Temnothorax* species [e.g., *T. unifasciatus* (Latreille, 1798), *T. crassispinus* (Karawajew, 1926), *T. rottenbergii* (Emery, 1870)], sometimes even only on one forewing, but this feature varies within the same species, which have normally an open cell *3r* (A. Radchenko, G.M. Dlussky† & K. Perfilieva, unpubl.).

Ward & al. (2015) and then Prebus (2017) based on molecular analysis considered clades of (*Leptothorax* + *Harpagoxenus* + *Formicoxenus*) and *Temnothorax* (including synonymised generic names *Chalepoxenus* Menozzi, 1923 and *Myrmoxenus* Ruzsky, 1902) as sister groups, with the time of their divergence between 45 and 50 mya (Middle to Early Eocene). As for the genera of the *Leptothorax* genus group, separation of the subclades *Harpagoxenus* and (*Formicoxenus* + *Leptothorax*) from the common ancestor occurred ca. 10 mya (Late Miocene), and divergence of the genera *Formicoxenus* and *Leptothorax* took place even later, ca. 5 mya (Late Miocene or even Pliocene), however not many species were included in the analysis.

Males of *Proleptothorax* have 13-segmented antennae with a filiform funiculus without an apical club, a very short antennal scape, short and narrow, bidentate mandibles, and, which is very important, a closed cell *3r* on the forewing. Interestingly, the second funicular segment in *Proleptothorax* is not the longest and is subequal to the third one (although distinctly longer than the scape), while the longest segments are 5th, 6th and 7th. The differences in structure of the basal funicular segments between *Leptothorax* genus group and *Proleptothorax* can be explained by the fusion of the 2nd and 3rd segments in the genera of *Leptothorax* genus group, which makes the 2nd segment the longest. A similar fusion of the second and third funicular segments was noted in ants from different subfamilies, for example in males of some species of *Leptomyrmex* Mayr, 1862 (Dolichoderinae) and in males of *Tetramorium* Mayr, 1855 and *Strongylognathus*

Mayr, 1853 (Myrmicinae) (Dlussky & al. 2014, Radchenko 2016). On the other hand, *Proleptothorax* males have 5-segmented maxillary palps and 3-segmented labial palps. These features as well as the general shape and structure of the head, mesosoma and waist, and the presence of the notauli fully match the diagnostic characters of males of the *Leptothorax* genus group.

It should be noted that the short and narrow bidentate mandibles in all castes are a plesiomorphy in the family Formicidae (Wilson & al. 1967, Dlussky 1983, Dlussky & Rasnitsyn 2007), and a closed cell *3r* on the forewing is a plesiomorphy in the subfamily Myrmicinae (Dlussky & Radchenko 2009, Radchenko & Dlussky 2013).

Thus, we may state that the above-mentioned diagnostic characters of *Proleptothorax* are obvious plesiomorphies, and it has no apomorphic features compared with the genera of the *Leptothorax* genus group. Moreover, the time when *Proleptothorax* existed is much older than the estimated time of separation of the genera *Leptothorax*, *Harpagoxenus*, and *Formicoxenus* but younger than the divergence of the *Leptothorax* genus group and *Temnothorax*. Consequently, we assume that *Proleptothorax* might be considered a putative ancestor of the genera of the *Leptothorax* genus-group but not of *Temnothorax*.

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