Insect. Soc. 51 (2004) 24–29
0020-1812/04/010024-06
DOI 10.1007/s00040-003-0699-8

Insectes Sociaux

Research article

Phylogeny and bionomics of *Lasius austriacus* (Hymenoptera, Formicidae)

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Received 10 February 2003; revised 24 June 2003; accepted 23 July 2003.

Summary. Phylogenetic analysis based on sequence data of the mitochondrial COI gene confirms the species status of the recently described *Lasius austriacus*. The five haplotypes of *L. austriacus* do not cluster according to their geographic origin, indicating a recent gene flow among the populations. The molecular data corroborate the morphology based hypothesis that *L. austriacus* belongs to the *Lasius* (*Lasius* s.str.) *brunneus* group. The invasive species *Lasius neglectus* forms a sister taxon with *L. turcicus*, both next related to *L. austriacus.* Other phylogenetic relationships within the genus *Lasius* are in accordance with morphological data.

First data on the bionomics of *L. austriacus* are discussed in context with its phylogenetic position. Based on gyne and male morphology, excavations of nests, pitfall trapping and observations in formicaries, we hypothesize that *L. austriacus* is a mainly hypogaeic, monogynous species with nuptial flight. These are characters of the *Lasius brunneus* group in general, except the polygynous-polycalic, intranidally copulating *L. neglectus*. Aggression tests, however, revealed non-aggressive behaviour (antennation) between separated *L. austriacus* populations, but pronounced interspecific aggression against *L. neglectus*. This confirms the species status of *L. austriacus* and indicates a reduced level of intraspecific aggression, similar to *L. neglectus*.

The status of *L. austriacus* as a native species in Central Europe is confirmed.

Key words: Ants, *Lasius austriacus*, *Lasius neglectus*, aggression test, mtDNA.

Introduction

Lasius austriacus Schlick-Steiner, Steiner, Schödl and Seifert 2003 was recently discovered. At present four populations are known, three in Austria and one in the Czech Republic. The latter had formerly been referred to as *L. neglectus* Van Loon, Boomsma and Andrásfalvy 1990 by Seifert (2000), an invasive ant with a putative centre of radiation in Asia Minor, of which presently no population in natural habitats is known. Based on morphology, *L. austriacus* is attributed to the *Lasius* (*Lasius* s.str.) *brunneus* group, that includes, in Europe, *L. neglectus*, *L. turcicus*, *L. lasioides* and *L. brunneus*.

This study aims to test the species status of *Lasius austriacus* and to determine its phylogenetic position within the genus. First data on ecology and social biology of *L. austriacus* are presented and discussed in context with the phylogenetic findings.

Material and methods

Fieldwork

Lasius austriacus was collected in 2002 at Feldberg (type locality, 15°51′E/48°41′N, 360 m a.s.l.), in Retz (15°56′E/48°45′N, 280 m a.s.l.), and at Braunsberg near Hainburg (16°57′E/48°09′N, 340 m a.s.l.). Most nests were found by lifting the poor vegetation (frequently *Calluna* sp. or moss) at the side of emerging rocks. Often the plant carpet could be gently rolled aside, uncovering the hypogaeic rock surface and *L. austriacus* nests.

In order to find nest queens, more than 50 nests were inspected at the type locality on 9 occasions between 10 July and 1 October 2002 at varying daytimes and under various weather conditions.

On 13 occasions at Feldberg and on 5 occasions at Braunsberg, from May to October 2002, soil surface and vegetation were inspected for foraging *L. austriacus* workers.

Fifty pitfall traps (diameter 18 mm; ethanol : glycerol = 5 : 1 plus several drops of detergent) were exposed continuously from 10 July to

1 October 2002 at Feldberg to obtain information on the ant assemblage, to capture gynes or males outside nests and to estimate the surface activity of the workers. The design was adapted from Majer (1978) with two lines of 25 traps and 1 m distance between the traps. They were emptied four times in intervals of 15 to 27 d.

Within a radius of 2.5 km around Feldberg and Braunsberg, 16 sites each were searched for *L. austriacus* to obtain information on the spatial size of the populations and on the habitat. All types of open habitats were examined.

Phylogenetic analysis

A total of 14 species were investigated (Table 1). DNA of single individuals was extracted using the Sigma Genelute Extraction kit (Saint Louis, MO, USA). PCR reactions were done in reaction volumes of 50 µl : 4 µl template DNA; 1 × reaction buffer; 0.2 mM dNTPs; 0.2 µM forward and reverse primers; 2 U Taq DNA polymerase (Biotherme, VA, USA) and H_2Odd. PCR was run with a touchdown programme under following conditions: initial step 94 °C 1 min, 3 cycles 94 °C 1 min, 55 °C 30 s, 72 °C 2 min, 5 cycles 94 °C 2 min, 53 °C 30 s, 72 °C 2 min, 7 cycles 94 °C 1 min, 51 °C 30 s, 72 °C 1 min, 7 cycles 94 °C 1 min, 49 °C 30 s, 72 °C 2 min, 9 cycles 94 °C 1 min, 47 °C 30 s, 72 °C 1 min, final step 72 °C 2 min. Extension time was shortened to 1 min for sequences shorter than 800 bp. For PCR a MJ thermocycler (MJ Research, MD, USA) was used. Primers used for amplification of the 1250 bp long Cytochrome Oxidase I (COI) gene stretch were RON-C1-J-1751f and PAT L2-N-3014r described by Simon et al. (1994). As the DNA of the 23-year-old sample of *L. turcicus* was degraded, the forward primer Neg2f 5′-gatgagcaataggatttatcttc-3′ (position 2803 in COI genome of *Apis mellifera*, Crozier and Crozier, 1993) was designed to amplify a short PCR product within that region.

Usually 1202 bp of COI were used for the phylogenetic analysis (*L. turcicus*: 424 bp). PCR products were purified using the QUiaquick PCR purification kit (Quiagen, Hilden, Germany), subsequently sequenced in both directions using the Big Dye termination reaction chemistry (Applied Biosystems, Foster City, CA, USA) and analysed with an ABI 377 automatic sequencer (Applied Biosystems). Sequence alignment was achieved with Clustal X (Thompson et al., 1997). To infer phylogenetic relationships, both distance (neighbour-joining algorithm, NJ) and maximum parsimony (MP) analysis were performed with the software package PAUP (test version 4.0b3a; Swofford, 1998). Tamura-Nei distance (Tamura and Nei, 1993) was used for the NJ trees. MP trees were generated with heuristic search using the tree bisection reconnection (TBR) algorithm and a random taxon addition sequence. For MP analysis, gaps were treated as missing data. In addition to unweighted MP analysis, successive weighting was performed by reweighting characters by the maximum value of the rescaled consistency index. Bootstrapping (Felsenstein, 1985) with 1000 replicates was applied for all trees.

Aggression tests

Workers from five *L. austriacus* nests (minimum distance 10 m) from Feldberg and Braunsberg and *L. neglectus* from Budapest were chosen for aggression tests in the lab following Holway et al. (1998). All tests were performed within 10 days after taking the colonies to the lab. Additionally, one nest of *L. alienus* from Feldberg was used to test interspecific aggression of *L. austriacus* when confronted with a member of the same ant assemblage. For each replicate a pair of randomly selected workers from the same or two different nests were put in a fluon-coated glass vial (diameter 2 cm). Five pairings of each population were run to test the intra-nest aggression, five pairings each were run to test aggression among nests of the same population and 10 pairings each were run to test aggression among populations. Five replicates of all pairings were run (5 min each), yielding a total of 300 tests. The temperature was 23 ± 1 °C. The behaviour was scored 0 = ignore, 1 = touch, 2 = avoid, 3 = aggression, 4 = fight. Levels 0–2 are referred to as non-aggressive behaviour, 3–4 as aggressive behaviour.

Test for intranidal copulation

A total of 20 nest samples from Feldberg and Braunsberg collected between early July and mid-August were tested. For each test about 100 workers and up to 50 alate gynes and 50 males, together with larvae and pupae, were kept in the laboratory in a 1 l plastic box for 3 weeks. Every second day the diet – crushed frozen grasshoppers – and water were renewed. The formicaries were inspected 5–10 × a day at varying daytimes for copulation or dealation of gynes.

Results

Phylogeny

Partial regions of the mitochondrial COI gene (1202 bp) of 14 palaearctic *Lasius* species were deposited in GenBank (for accession numbers see Table 1). Topologies of the NJ and MP trees were congruent. Within the Feldberg population, five different haplotypes of *L. austriacus* were detected, with a sequence divergence range of 0.08–0.8% within the Feldberg population. One of the Feldberg haplotypes occurs at Braunsberg, another one at Retz (in the cladograms in Fig. 1. all investigated samples of *L. austriacus* are included, reflecting haplotype–frequency). All mutations except one were transitions. Nine mutations were on the third codon position, one on the first and one on the second. Only the second codon mutation led to a change of the amino acid from valine to isoleucine.

Phylogenetically, *Lasius austriacus* forms a sister clade to *L. neglectus* with a sequence divergence of 6.7–6.9%. When *L. turcicus* is included (Fig. 1b) *L. neglectus* forms a sister clade to *L. turcicus* with a sequence divergence of 3.1%. This result is supported by high bootstrap values.

Next related to the three species is *L. lasioides,* with a sequence divergence of 6.8 to 9.1%. *L. brunneus* is in a similar genetic distance (Fig. 1a). These five species form a monophyletic cluster (A in Fig. 1b) supported by high bootstrap values. *Lasius psammophilus*, *L. sakagamii*, *L. alienus*, *L. niger*, *L. platythorax* and *L. emarginatus* form the cluster (B), supported by a bootstrap value of 100%. *Lasius flavus* (D) and *L. mixtus* (E) are separated by 5.1% sequence divergence and form a distinct lineage (bootstrap value 100%).

With values between 11.3 and 13.0%, *Lasius fuliginosus* shows the highest sequence divergence.

Ecology and life habits

Pitfall trapping and hand collecting yielded 31 ant species at Feldberg including *Anergates atratulus* (Schenck 1852), *Messor structor* (Latreille 1798), *Polyergus rufescens* (Latreille 1798) and *Tetramorium moravicum* Kratochvil 1941. The search for *L. austriacus* near the known populations resulted in records at two sites around Feldberg (maximum distance 2.0 km) and at three sites around Braunsberg (maximum distance 0.5 km). All these habitats were natural xerothermous sites with interspersed rocks.

Table 1. *Lasius* spp. included in the phylogenetic analyses. Geographic origin (A = Austria, E = Spain, H = Hungary, J = Japan, T = Turkey), collectors (AT = András Tartally, BCS & FMS = Birgit C. Schlick-Steiner & Florian M. Steiner, HMS = Hans M. Steiner, JH = Jürgen Heinze, NL = Niyazi Lodos, XE = Xavier Espadaler) and accession number of COI sequences in GenBank are given

Species	Locality	Collector	GenBank
L. alienus (Förster 1850)	A: Braunsberg	BCS & FMS	AY225865
L. austriacus Schlick-Steiner, Steiner, Schödl and Seifert 2003	A: Braunsberg	BCS & FMS	AY225869
	A: Retz	HMS	AY225873
	A: Feldberg	BCS & FMS	AY225870
	A: Feldberg	BCS & FMS	AY225871
	A: Feldberg	BCS & FMS	AY225872
L. brunneus (Latreille 1798)	A: Hof	BCS & FMS	AY225877
L. emarginatus (Olivier 1791)	A: Vienna	BCS & FMS	AY225868
L. flavus (Fabricius 1781)	A: St. Egyden/Steinfeld	BCS & FMS	AY225878
L. fuliginosus (Latreille 1798)	A: Urschendorf	BCS & FMS	AY225880
L. lasioides (Emery 1869)	E: Sant Cugat	XE	AY225874
L. mixtus (Nylander 1846)	A: Göpfritz	BCS & FMS	AY225879
L. neglectus Van Loon, Boomsma and Andrásfalvy 1990	H: Budapest	BCS & FMS	AY225875
	H: Debrecen	AT	AY225876
L. niger (Linnaeus 1758)	A: Vienna	BCS & FMS	AY225866
L. platythorax Seifert 1991	A: Moosbrunn	BCS & FMS	AY225876
L. psammophilus Seifert 1992	A: Gföhl	BCS & FMS	AY225863
L. sakagamii Yamauchi and Hayashida 1970	J: Gifu	JH	AY225864
L. turcicus Santschi 1921	T: Bornova-Izmir	NL	AY225881

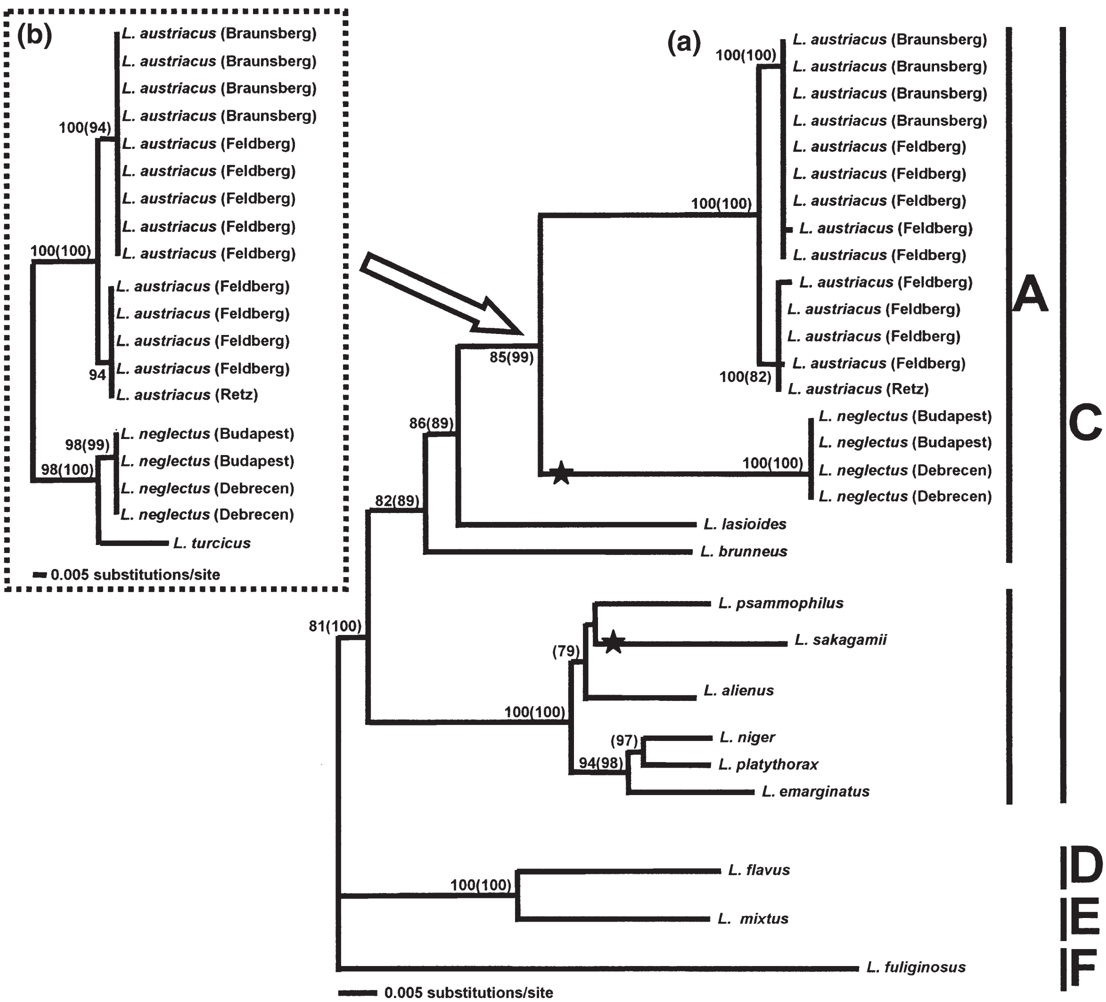

Figure 1. Phylogenetic trees of some species of the genus *Lasius* based on Neighbour Joining calculated with the Tamura-Nei algorithm of (a) 1202 bp and (b) 424 bp of the COI gene. Bootstrap values > 75 are given at nodes, bootstrap values of the MP branches by successive weighting are given in parentheses. A = *L. brunneus* group, B = *L. niger* group + *L. alienus* group C = *Lasius* s.str., D = *Cautolasius*, E = *Chthonolasius*, F = *Dendrolasius*. Asterisks indicate the acquisition of polygyny-polycaly

Lasius austriacus nests contain c. 1,000–10,000 workers. During daytime, workers were never observed on the soil surface, in the herb layer or on trees. Sexuals were found in the nests from early July to mid September.

The pitfall catches yielded a total of 5192 ants, among these 82 *L. austriacus* workers (1.6%) and 1 dealate *L. austriacus* gyne. Dissection showed that the gyne had not been inseminated. No inseminated gynes were found inside nests. Copulation was never observed among sexuals in the 20 nests in plastic boxes, and dealate gynes were never found. As nothing suggested that gynes had copulated, they were not dissected.

Aggression tests

All intra-nest pairings of *L. austriacus* from Feldberg or Braunsberg and of *L. neglectus* from Budapest resulted in non-aggressive behaviour: exclusively touch (1). The same was observed in the various intraspecific inter-nest pairings: *L. austriacus* from nests of the same population; *L. austriacus* from Feldberg vs. Braunsberg; *L. neglectus* from Budapest (Fig. 2).

The observed behaviour between *L. austriacus* and *L. neglectus* ranged from touch (1) to fight (4). Between *L. austriacus* from Feldberg and *L. neglectus* from Budapest, the ten pairings resulted in an average value of 2.94 ± 0.68, between Braunsberg and Budapest in 2.46 ± 1.04. Additionally, one nest of *L. alienus* from Feldberg was paired with five *L. austriacus* nests from Feldberg (five replicates each). The result in all repeats was fight (4). In most pairings the *L. alienus* workers were killed.

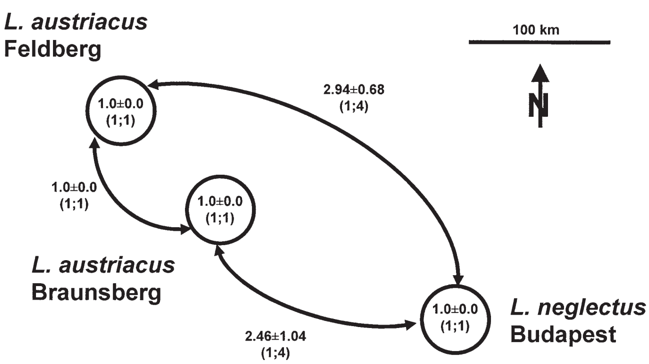

Figure 2. Aggression tests following Holway et al. (1998) performed with 5 nests each of *Lasius austriacus* from Feldberg and Braunsberg and *L. neglectus* from Budapest. In circles results of intra-population pairings (n = 10 × 5 replicates each), alongside of arrows results of inter-population pairings (n = 10 × 5 replicates each): Average ± standard deviation, (minimum; maximum). 0 = ignore, 1 = touch, 2 = avoid, 3 = aggression, 4 = fight

Discussion

Species status and phylogeny

The presented phylogenetic relationships based on mtDNA are the first molecular phylogeny of the genus *Lasius*. Existing morphological, ecological and sociobiological data are used for evaluation and interpretation.

The molecular analyses (Fig. 1) confirm the species status of *L. austriacus.* Despite the existence of several haplotypes, all of them strongly diverged from *L. neglectus* and *L. turcicus,* which seem to be sister species. The geographic distribution of the different haplotypes suggests a rather recent gene flow, after the intraspecific diversification.

Lasius austriacus forms a monophyletic group with *L. neglectus*, *L. turcicus*, *L. lasioides* and *L. brunneus* (A in Fig. 1a). This agrees with the morphologically based concept of a *L. brunneus* group within the subgenus *Lasius* s.str. (Seifert, 1992; Schlick-Steiner et al., 2003b).

Lasius sakagamii is not closely related to *L. neglectus*. The shared character of polygyny-polycaly ('polygyny-polycaly' is used in the same sense here as 'unicoloniality' by Bourke and Franks, 1995) – not known from any other *Lasius* s.str. species (Seifert, 1992) – seems to be an adaptive trait. As suggested by Espadaler and Rey (2001) on the basis of morphological data, its acquisition occurred independently (Fig. 1a). *Lasius sakagamii* clusters with *L. psammophilus*, *L. alienus*, *L. emarginatus*, *L. niger* and *L. platythorax* forming another monophyletic group within the subgenus *Lasius* s.str. (B). Within this group, *L. emarginatus*, *L. niger* and *L. platythorax* are closely related and *L. niger* and *L. platythorax* are sister species. This is again in accordance with morphological results (Seifert, 1992 and unpubl.). The maximum sequence divergence within the whole subgenus *Lasius* s.str. (C) is high. The clear separation of *L. flavus* and *L. mixtus* (D, E) from *Lasius* s.str. goes well with current taxonomy, which separates them from *Lasius* s.str. Whether the genetic difference justifies a classification into two different subgenera (*Cautolasius* and *Chthonolasius*) should be investigated using further genetic markers.

Bionomics of Lasius austriacus

All *L. austriacus* nest sites around Feldberg and Braunsberg are situated in undisturbed warm and dry habitats with interspersed rocks. Within the species assemblage at Feldberg, *Polyergus rufescens*, *Messor structur* and *Tetramorium moravicum* are restricted to natural xerothermous habitats, thus indicating a good habitat quality (Schlick-Steiner et al., in press). *L. austriacus* is a rare species in Central Europe (Schlick-Steiner et al., 2003b). Among more than 60,000 voucher specimens from the past 150 years from about 1,200 sites in Lower Austria, Schlick-Steiner et al. (2003a) detected not a single *L. austriacus* specimen from any other site. *L. austriacus* appears tolerant with respect to soil chemistry (silicate bed rock at Feldberg, limestone at Braunsberg). This contrasts with the preference of several other ant species which occur on or avoid certain soil types (Seifert, 1996; Fisher, 1997; Way et al., 1997).

The negative results of extensive hand collecting at Feldberg and Braunsberg during daytime support the opinion of Schlick-Steiner et al. (2003b) that *L. austriacus* is a hypogaeic species. Pitfall catches (1.6% of the ant total), however, suggest that workers leave the soil, at least occasionally and probably during nighttime. The life habits of *Lasius austriacus* are unique within the *Lasius brunneus* group and rather resemble those of *Chthonolasius* species in xerothermous grassland (Seifert, 1996). Thus, ecology provides another argument in favour of the species status. Striking morphological adaptations, such as small eye size which is unique in the *L. brunneus* group, go well with the relatively big phylogenetic distance based on mtDNA.

A voluminous mesosoma and large wings (Schlick-Steiner et al., 2003b) suggest that *L. austriacus* gynes are capable of nuptial flight (cf. Peeters and Ito, 2001; Seifert and Buschinger, 2001). In addition, the absence of copulations and gyne dealations in the formicaries make intranidal copulation and subsequent colony budding improbable. The unseminated alate gyne in a pitfall trap supports the hypothesis that nuptial flight is the common mating and dispersal strategy of *L. austriacus*.

Presently, only *L. neglectus* and *L. sakagamii* are known as polygynous-polycalic *Lasius* s.str. species (Seifert, 1992). Two observations suggest monogyny of *L. austriacus*: (a) no inseminated gyne, i.e. no nest queen was found inside a nest, despite repeated inspections; (b) the large volume of the gyne's mesosoma (cf. Stille, 1996; Seifert, 2000; Espadaler and Rey, 2001).

Hence, *Lasius austriacus* exhibits the colony organisation, mating and dispersal strategy common in the *Lasius brunneus* group. The outstanding situation in *L. neglectus* is further pronounced.

Aggression tests, originally developed as a taxonomic tool (Le Moli and Mori, 1986), are mainly used to gather information on the social structure of ant populations (Giraud et al., 2002). In our aggression tests, *L. austriacus* and *L. neglectus* interacted aggressively, which may indicate separate species. *L. austriacus* workers from different colonies of the same population showed no aggressive behaviour. This feature is typical of polygynous-polycalic species (e.g., Holway et al., 1998; Steiner et al., 2003) and was never observed in a monogynous species so far. We postpone an interpretation and raise the following questions: Must the concept of polygyny-polycaly be revised? Are aggression tests as currently performed a proper means to determine the coloniality type of a species? Other inconsistencies (Espadaler, unpubl.) cast some doubt on the latter.

Native or invasive species?

Phylogenetic vicinity to the invasive *L. neglectus* questions the autochthony of *L. austriacus* in Central Europe. *L. austriacus* was found to coexist with a high number of autochthonous ant species. This is not the case with invasive ants which drive native ants away from their habitat (Majer, 1994). The habitats of *L. austriacus* are of high quality, as shown by the presence of species like *Messor structor*, *Polyergus rufescens* and *Tetramorium moravicum*, indicating little to no disturbance. This is in sharp contrast with the degraded habitats of invasive species (Passera, 1994) which only exceptionally colonize near-natural sites (Ward, 1987; Vega and Rust, 2001).

Among the characters of tramp species listed by Passera (1994), only two were observed with *L. austriacus*: the high interspecific aggression against *L. neglectus* and *L. alienus* and the small size of the monomorphic workers. Several other characters – occurrence in human environment, migration, polygyny-polycaly (listed as 'unicoloniality'), intranidal copulation, colony budding, small gyne size – were not observed with *L. austriacus.* The remaining traits – lifespan of queen and worker sterility – were not considered in this study.

Furthermore, the occurrence of five different haplotypes in a narrow geographic region provides evidence of genetic diversification on the spot.

Finally, the native character of *L. austriacus* is supported by a sample from Feldberg (leg. H. Franz, in coll. NHM Vienna) that dates back to the years before 1965 (C.A. Collingwood, pers. comm.).

Acknowledgements

For donating samples of *L. sakagamii,* to J. Heinze, of *L. neglectus,* to L. Passera and S. Csösz; for help with the phylogenetic analyses, to E. Haring; for help with field work, to J.S. Pedersen, S. Cremer and D. Nash; for revealing the approximate sampling date of the oldest known *L. austriacus* sample, to C.A. Collingwood; for stimulating discussions, to M. Sanetra, A. Buschinger, H.M. Steiner, E. Haring, H. Zettel, K. Ross, J.J. Boomsma, J.S. Pedersen, S. Cremer; for valuable information, to N. Aktac, A. Beckenbach, G. Bracko, R.H. Crozier, W. Czechowski, K. Kiran, T. Kondo, D. Matile-Ferrero, R. Schultz, A. Schulz, A. Tartally, P. Werner (alphabetic order); for linguistic improvements, to M. Stachowitsch. We wish to thank two anonymous reviewers for their comments improving this paper.

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