

Quantitative morphology and mtDNA reveal that *Lasius maltaeus* is not endemic to the Maltese Islands (Hymenoptera, Formicidae)

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Abstract

Lasius maltaeus Seifert, 2020 was recently described as a Maltese endemic ant based on quantitative morphology, after decades of uncertainties over the identity of the local population, which has a phenotype resembling *L. emarginatus* (Olivier, 1791). At the same time, Sicilian *L. emarginatus* populations were discovered to diverge in their mitochondrial DNA to a degree that suggested heterospecificity. Considering the biogeographic similarity of Malta and Sicily, with land bridges connecting them repeatedly until the last glacial maximum, we questioned the assumption that *L. maltaeus* was endemic to Malta. We integrated quantitative morphology and mtDNA in the study of the Maltese and southern Italian populations phenotypically close to *L. emarginatus*. We discovered that the range of *L. maltaeus* extends over most of Sicily, while the true *L. emarginatus* replace it in the north-eastern sector of the island, the nearby Aeolian Islands, and the Italian peninsula. The distributions of *L. emarginatus* and *L. maltaeus* in Sicily follow biogeographic patterns recalling the island's complex paleogeographic history. Further investigations should verify the existence of truly Maltese endemic ants, since the status of other allegedly endemic species is not strongly supported.

Keywords

ants, biogeography, Formicinae, Mediterranean islands, Sicily

* These authors contributed equally to this study.

Introduction

The ant genus *Lasius* Fabricius, 1804 is one of the most ecologically important ant genera in the Holarctic realm (Hölldobler and Wilson 1990; Seifert 2018, 2020). It currently counts 125 extant species (Bolton 2022), which belong to two major clades and at least 10 species groups, many of which independently evolved social parasitism (Maruyama et al. 2008; Boudinot et al. 2022).

The taxonomy of *Lasius* ants is considered among the most challenging among Holarctic ants (Seifert 2018). In particular, the former subgenus *Lasius* s. str., which is now known to represent two distinct lineages (the *brunneus* group and the *niger* group, see Boudinot et al. 2022), has witnessed significant taxonomic changes. It was once thought to count only seven species (Wilson 1955), but since then this number increased several times, especially in the last three decades (Van Loon et al. 1990; Seifert 1991; Seifert 1992, 2020; Schlick-Steiner et al. 2003; Borowiec and Salata 2013; Seifert and Galkowski 2016; Salata and Borowiec 2018; Schär et al. 2022).

The West-Palaearctic *Lasius emarginatus* (Olivier, 1791) is an iconic European species characterized by a large geographic range and remarkable bicoloured appearance (Seifert 2018). However, at least four additional cryptic species with a superficially similar appearance were revealed during recent years (Seifert 2018, 2020): *L. tebessae* Seifert, 1992 from the Maghreb (Seifert 1992, 2020); *L. illyricus* Zimmermann, 1935 ranging from the Balkans to the Caspian sea, in a large area sympatric with *L. emarginatus* (Borowiec and Salata 2013; Schifani and Massa 2020; Seifert 2020); the Iranian *L. persicus* Seifert, 2020, partly sympatric with *L. illyricus* (Seifert 2020); and *L. maltaeus* Seifert, 2020 described from Malta.

The recent description of *L. maltaeus* came after a long debate on the identity of this Maltese morphotype. Baroni Urbani (1968) attributed the local bicolored *Lasius* ants with hairy scapes to *L. emarginatus*. However, Schembri and Collingwood (1981) considered the workers' head pilosity to be too dense, their frontal triangle too shiny and the scapi of males to be too hairy compared to samples of *L. emarginatus* from continental Europe, and instead proposed to classify the Maltese population as a reddish phenotype of *L. niger* (Linnaeus, 1758). Later on, the same authors changed their opinion, defining the Maltese ants as representatives of a morphospecies different from both *L. niger* and *L. emarginatus* (Schembri and Collingwood 1995). The issue remained uninvestigated until Seifert (2020) included Maltese specimens in a large West-Palaearctic taxonomic revision based on quantitative morphology. He demonstrated their distinctiveness from both *L. niger* and *L. emarginatus*, and described the morphospecies as *L. maltaeus*, included within the *L. emarginatus* complex, emphasizing differences in head pilosity in agreement to the comments by Schembri and Collingwood (1981).

At the same time, an inventory of the Sicilian ant fauna highlighted a remarkable genetic distance for the mitochondrial COI of *L. emarginatus* samples from Sicily compared to those of the nearby Aeolian Islands and of peninsular Italy (Schär et al. 2020).

Similar results were also published from Corsica, yet the local population resembling *L. emarginatus* was attributed to *L. grandis* Forel, 1909 based on morphology (Blatrix et al. 2020; Seifert 2020). Sicily and the Maltese Islands are well-known for their strong biogeographic similarity (e.g., Thake 1985; Fattorini 2011; Salvi et al. 2014; Médail 2022), and were connected repeatedly by land bridges until the last glacial maximum (Foglini et al. 2016) (light blue line Fig. 1). These observations led us to investigate the relationships between the Sicilian populations historically attributed to *L. emarginatus* and *L. maltaeus*, questioning the endemic status of the latter.

Materials and methods

Ant specimens were manually collected and stored in 70–96% ethanol (Suppl. material 1: table S1, Fig. 1). Vouchers are deposited in the authors' personal collections (see Suppl. material 1: table S1):

- MMBS** M. Menchetti pers. coll., Barcelona, Spain;
ESPI E. Schifani pers. coll., Palermo, Italy;
AACI A. Alicata pers. coll., Catania, Italy;
BDEL R. Vila, Butterfly Diversity and Evolution Lab coll.

Morphological analysis

The morphological study was performed using stereoscopic microscopes at 45–80× magnification, in addition to photography-based morphometry. Species were identified using the keys provided by Seifert (2020).

Morphological measurements were obtained by taking pictures with a Carl Zeiss Stemi 2000-C stereomicroscope at magnification 2.25× equipped with a CMEX PRO-5 DC.5000p digital camera and ImageFocus 4 software (M. Menchetti) and at 5× magnification using Canon MP-E 65mm f/2.8 1–5× macro lens analysed with the software ImageJ (Schneider et al. 2012) (E. Schifani).

We measured a total of 22 workers of *L. maltaeus* from Malta and Italy (Sicily and Calabria) and 13 workers of *L. emarginatus* from Italy (Sicily, Aeolian Islands, Calabria and Emilia-Romagna). We recorded six characters, including one chaetotaxonomic and four morphometric characters needed to distinguish *L. emarginatus* from *L. maltaeus* according to Seifert (2020), and the cephalic size. The acronyms and character definitions follow Seifert (2020):

- CL:** maximum cephalic length in median line; the head must be carefully tilted to the position with the true maximum. Excavations of posterior head and/or clypeus reduce CL.
CW: maximum cephalic width; this is either across, behind, or before the eyes.

- PoOc:** postocular distance. Use a cross-scaled ocular micrometer and adjust the head to the measuring position of CL. Caudal measuring point: median occipital margin; frontal measuring point: median head at the level of the posterior eye margin. Note that many heads are asymmetric and average the left and right postocular distance.
- MP6:** length of the sixth (terminal) segment of maxillary palps.
- nGen:** with head in full face view, number of setae on head sides frontal of anterior eye margin (gena). The bilateral sum is halved.
- CS:** arithmetic mean of CL and CW as less variable indicator of body size.

For all specimens we also calculated ratios (CL/CW, PoOC/CL and MP6/CL) and the linear discriminant distinguishing *L. maltaeus* ($D > 0$) from *L. cinereus* Seifert, 1992, *L. emarginatus*, *L. illyricus* and *L. grandis* ($D < 0$) at step 22a of the dichotomous key by Seifert (2020): $45.42 * \text{PoOc/CL} - 0.183 * \text{CL/CW} + 55.63 * \text{MP6/CL} + 0.312 * \text{nGen} - 25.59$. *Lasius emarginatus* was distinguished from the other above-mentioned species based on cuticle microsculpture, clypeal pubescence and setosity of the scapi following Seifert (2020). All measurements are presented in mm.

Genetic analysis

A total of seven *L. maltaeus* and three *L. emarginatus* specimens belonging to different nests were selected for the genetic analysis. A few legs per specimens were used. DNA-barcoding (mitochondrial gene cytochrome c oxidase I, COI, 658 bp) data was generated at two institutes: the Centre for Biodiversity Genomics, University of Guelph, Canada, using the primers LepF1 and LepR1 (deWaard et al. 2008); the Butterfly Diversity and Evolution Lab (BDEL), following the protocol by Schär et al. (2020) and using the primers LCO1490/HC02198 (Folmer et al. 1994). In the latter case, PCR products were visualized by gel electrophoresis and sent to Macrogen Europe for Sanger sequencing. Raw sequences were edited and aligned in Geneious Prime 2020.2.4 (Kearse et al. 2012). Chromatograms and sequences have been inspected for the presence of, respectively, double peaks and stop codons.

We also retrieved from GenBank a total of ten sequences of 658 bp identified as *L. emarginatus*: one from Schär et al. (2018) (accession number [LT977448](https://doi.org/10.26434/chemrxiv-2018-07-11-LT977448)), four from Schär et al. (2020) (accession numbers [MT606324](https://doi.org/10.26434/chemrxiv-2020-07-11-MT606324), [MT606325](https://doi.org/10.26434/chemrxiv-2020-07-11-MT606325), [MT606326](https://doi.org/10.26434/chemrxiv-2020-07-11-MT606326), and [MT606327](https://doi.org/10.26434/chemrxiv-2020-07-11-MT606327)) and five from Blatrix et al. (2020) (accession numbers [MH138380](https://doi.org/10.26434/chemrxiv-2020-07-11-MH138380), [MH138381](https://doi.org/10.26434/chemrxiv-2020-07-11-MH138381), [MH138384](https://doi.org/10.26434/chemrxiv-2020-07-11-MH138384), [MH138385](https://doi.org/10.26434/chemrxiv-2020-07-11-MH138385), and [MH138386](https://doi.org/10.26434/chemrxiv-2020-07-11-MH138386)). The haplotype network was created using the program TCS 1.21 (Clement et al. 2000) and then graphically edited with tcsBU (dos Santos et al. 2016) and Adobe Illustrator CC 2019. All the newly generated sequences were submitted to GenBank (accession numbers [OQ025622](https://doi.org/10.26434/chemrxiv-2022-07-11-OQ025622)-[OQ025631](https://doi.org/10.26434/chemrxiv-2022-07-11-OQ025631), see also the Suppl. material 1: table S1) and to BOLD (dataset DS-ANT-LMAL, doi: [http://dx.doi.org/10.5883/DS-ANTLMAL](https://dx.doi.org/10.5883/DS-ANTLMAL)).

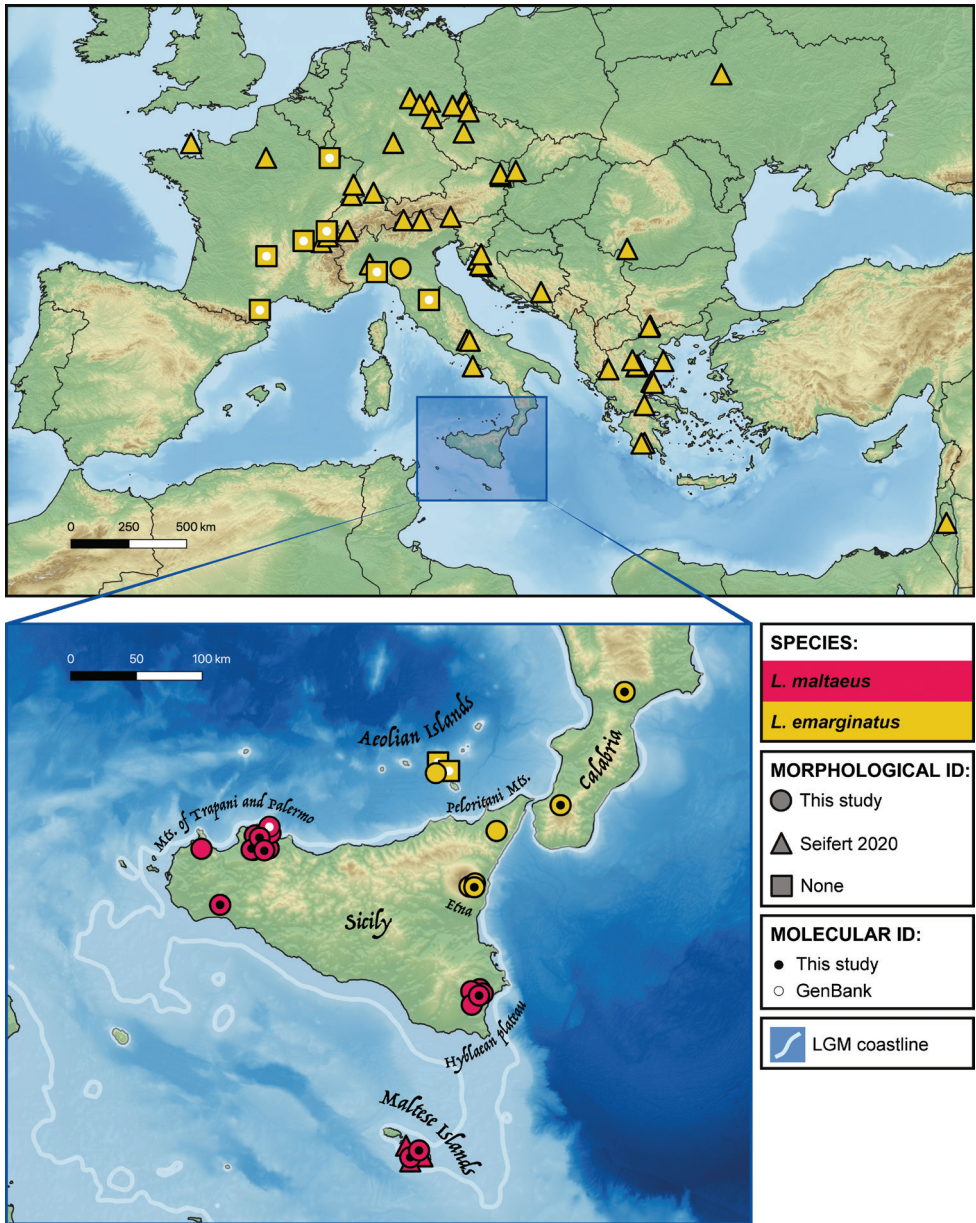


Figure 1. Distribution of the samples of *L. maltaeus* (red) and *L. emarginatus* (yellow) investigated in this study with quantitative morphology and/or molecular analysis. The shape of the points represents whether the samples were identified with the linear discriminant (circles: ID in this study; triangles: ID by Seifert 2020; squares: no linear discriminant was used). Inner circles indicate whether molecular data (COI) has been used (black circles: sequences generated in this study; white circles: sequences retrieved from GenBank). Light blue lines in the map below indicate the coastline during the Last Glacial Maximum (LGM). Main toponyms discussed in the text are highlighted in the map.

Results

Samples identified by quantitative morphology and/or molecular analysis as *L. maltaeus* and *L. emarginatus* were distributed allopatrically (Fig. 1). All samples from the Italian peninsula, the Aeolian Islands and from north-eastern Sicily (Peloritani mountains and Etna) belonged to *L. emarginatus*, while all samples from Malta and all other samples from Sicily (coming from the north-western and south-eastern sectors) were identified as *L. maltaeus* (Fig. 1).

Morphological analysis

Quantitative morphological data are summarized in Table 1 and raw data is available in the Suppl. material 1: table S2. All specimens were unambiguously classified as either *L. maltaeus* or *L. emarginatus* according to the linear discriminant scores even without considering nest means of multiple workers, while all individual characters overlapped between the two species (Table 1, Figs 2, 3). For what concerns *L. maltaeus*, our data slightly extend the minimum and maximum range of all investigated characters except for the maximum range of PoOC and CW.

Table 1. Summary of the morphological differences between *L. maltaeus* (n=22) and *L. emarginatus* (n=13) specimens measured in this study. All morphometric characters are reported in mm as mean \pm standard deviation (minimum, maximum). The raw data is available in Suppl. material 1: table S2.

	<i>L. maltaeus</i>	<i>L. emarginatus</i>
CL	0.96 \pm 0.07 (0.79, 1.11)	0.99 \pm 0.05 (0.91, 1.05)
CW	0.9 \pm 0.07 (0.73, 1.03)	0.93 \pm 0.07 (0.82, 1.02)
PoOC	0.22 \pm 0.02 (0.17, 0.25)	0.22 \pm 0.02 (0.19, 0.25)
MP6	0.22 \pm 0.02 (0.18, 0.26)	0.21 \pm 0.01 (0.18, 0.23)
nGen	13.36 \pm 2.45 (8, 18)	7.04 \pm 1.83 (4, 10)
CS	0.93 \pm 0.07 (0.76, 1.07)	0.96 \pm 0.05 (0.87, 1.03)
CL/CW	1.07 \pm 0.03 (1.01, 1.14)	1.07 \pm 0.07 (1.01, 1.25)
PoOC/CL	0.23 \pm 0.01 (0.20, 0.25)	0.22 \pm 0.01 (0.21, 0.24)
MP6/CL	0.23 \pm 0.02 (0.21, 0.26)	0.21 \pm 0.01 (0.2, 0.22)
D	1.91 \pm 0.83 (0.76, 3.92)	-1.35 \pm 0.84 (-3.17, -0.17)



Figure 2. Frontal view of the head and lateral view of a *L. maltaeus* worker from Sicily (voucher ES16A036).

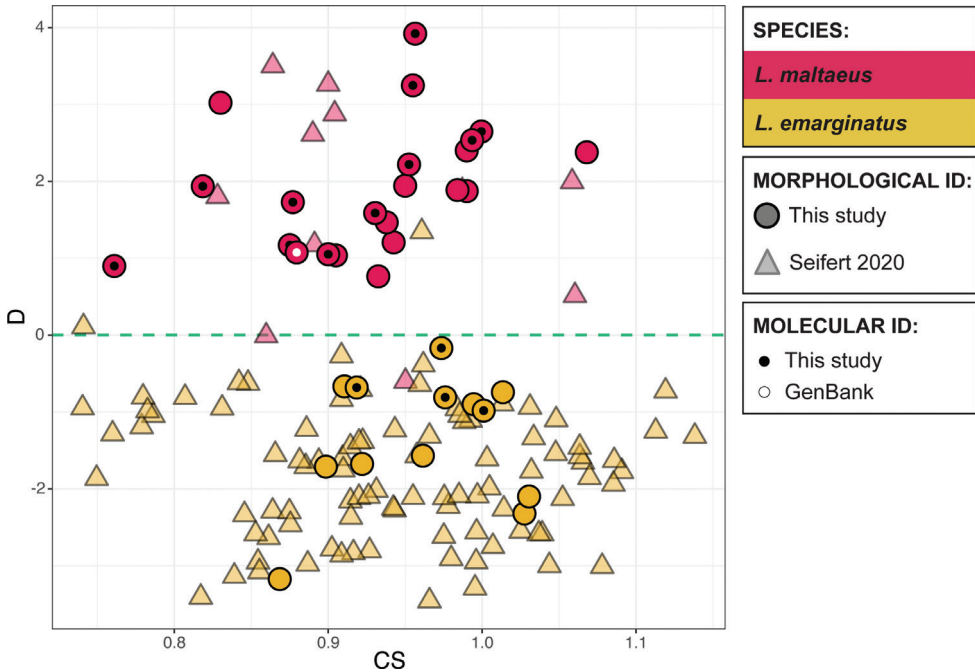


Figure 3. Morphological differences between *L. maltaeus* (red) and *L. emarginatus* (yellow) specimens according to cephalic size (CS) and the linear discriminant D from Seifert (2020) (green dashed line: < 0 *L. emarginatus*, > 0 *L. maltaeus*). Circles represent the specimens measured in this study, while triangles indicate the specimens from Seifert (2020). Sequenced specimens present an inner circle, which is black if generated in this study or white if obtained from GenBank (i.e. from Schär et al. 2020). Note that individual specimens may be misidentified by the discriminant scores, but not nest means of multiple workers according to the data provided by Seifert (2020).

Genetic analysis

We generated ten COI sequences with a length of 658 bp. The haplotype network analysis (Fig. 4) based on a total of 20 COI sequences (8 for *L. maltaeus* and 12 for *L. emarginatus*) show two distinct haplogroups that match the morphological species identification. Within the *L. maltaeus* haplogroup, the two *L. maltaeus* colonies analysed from Malta had distinct haplotypes, both shared with the Sicilian populations: one shares the haplotype with a colony from the Hyblean Plateau (voucher MM21B061a1 from Cavagrande del Cassibile, Siracusa) and the other with samples from the mountains in the area of Palermo (voucher MM18A053a1 from Monte Moarda and sequence [MT606327](#) from Monte Pellegrino). A total of five haplotypes were found in *L. maltaeus*, with a maximum intraspecific mtDNA divergence of 0.6%, while in *L. emarginatus* we found six haplotypes and a maximum intraspecific mtDNA divergence of 1.1%. The minimum and maximum interspecific mtDNA divergence found were, respectively, 2.9% and 3.8%.

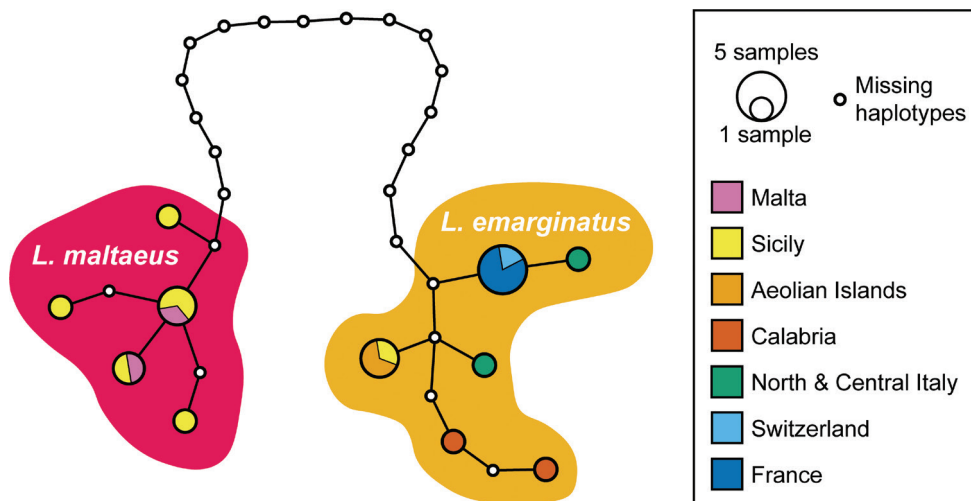


Figure 4. Haplotype network of *L. maltaeus* and *L. emarginatus* mitochondrial COI sequences. Colour and size of the circles indicate geographical origin and number of the samples, respectively.

Discussion

Quantitative morphology and mtDNA data agree and allow for the unambiguous distinction of *L. emarginatus* from *L. maltaeus*. Our results demonstrate that the range of the latter mostly resides in Sicily (where it shows the larger haplotype diversity), making it a Siculo-Maltese endemic rather than a Maltese endemic species. The long history of doubts over the identity of the Maltese populations, culminating with the description of *L. maltaeus* (Schembri and Collingwood 1981,1995; Seifert 2020), perhaps reflects the great interest of naturalists for the biodiversity of smaller islands, while the Sicilian populations were long overlooked. As a consequence of this discovery, at least two species of the *L. emarginatus* complex inhabit Italy, while further investigation should check for the possible presence of *L. illyricus* (Schifani 2022). As a result, *L. balearicus* Talavera, Espadaler & Vila, 2015 from Mallorca (Balearic Islands), *L. cypereus* Seifert, 2020 from Cyprus, and *L. kritikos* Seifert, 2020 and *L. tapinomoides* Salata & Borowiec, 2018 from Crete are the only *Lasius* species known to occur in a single island (Talavera et al. 2015; Salata and Borowiec 2018; Seifert 2020).

The vicariance between *L. emarginatus* and *L. maltaeus* in the broader context of the Siculo-Maltese archipelago and Italian peninsula follows a fascinating biogeographic pattern that reflects the complex paleogeographic history of the region. The region of Trapani and Palermo Mountains in north-western Sicily, and the Hyblaean plateau in south-eastern Sicily (recurrently linked to Malta by land bridges), represent the two most ancient sectors of the island to have emerged from the sea perhaps even before the upper Pliocene (Masini and Sarà 1998; Guarino and Pasta 2018). Both regions are recognized as well-defined biogeographic provinces hosting a significant number of endemic fauna and flora (Brullo et al. 1995, 2011; Guarino and Pasta 2018; Schifani

et al. 2020; Schmitt et al. 2021). On the other hand, the Etna and Peloritani regions of north-eastern Sicily, have a distinct geology and paleogeographic history and appear biogeographically more similar to the Italian peninsula or other Mediterranean regions: the Etna is fairly recent, emerging only about 570 thousand years ago, while the Peloritani alongside Calabria rotated counterclockwise from the Sardinian-Corsican microplate into their current position during the Alpine Orogeny (Stöck et al. 2008; Sciandrello et al. 2015; Scalercio et al. 2020; Schmitt et al. 2021).

The fauna and flora of north-western and south-eastern Sicily are more influenced by colonization from the Africa's Maghreb region compared to the north-east, which hosts more species from continental Europe (Masini and Sarà 1998; Stöck et al. 2008; Sciandrello et al. 2015; Alicata and Schifani 2019; Schifani et al. 2020, 2022a, b). The case of the green toads *Bufo boulengeri siculus* (Stöck et al., 2008) and *Bufo viridis balearicus* (Boettger, 1880) is particularly striking as the distribution of these two subspecies (the first of Maghrebian origin, the second one European) mirrors those of *L. maltaeus* and *L. emarginatus* respectively (Stöck et al. 2008; Dufresnes et al. 2019). Among other ants, the distribution of *Aphaenogaster trinacriae* Alicata & Schifani, 2019 resembles that of *L. maltaeus* within Sicily, while *Formica clara* Forel, 1886 and *Solenopsis orbula* Emery, 1875 may be restricted to the north-east of the island (Alicata and Schifani 2019; Schifani et al. 2021, 2022b). However, western and southern Sicily may also have acted as a refugium for relict European lineages, as recently shown among butterflies (Scalercio et al. 2020). Based on the available morphological and molecular data, it is possible that the sister species of *L. maltaeus* is the European *L. emarginatus*, but phylogenetic evidence for a broader number of species (e.g., including the Maghrebian *L. tebessae*) is needed to confirm this.

The ant faunas of Malta and Sicily share most species and the Siculo-Maltese archipelago may be considered as a single bioregion (Wang et al. 2022). The observation that the Maltese and Sicilian populations of *L. maltaeus* are not genetically differentiated and share two different COI haplotypes, suggest recent exchanges between the two (involving multiple queens), which is congruent with the hypothesis that the species exploited land bridges during the last glacial period. Beyond the case of *L. maltaeus*, our results question the existence of Maltese endemic ants. While *Lasius maltaeus* becomes the fourth Siculo-Maltese endemic species after *Aphaenogaster fiorii* Emery, 1915, *Temnothorax lagrecai* (Baroni Urbani, 1964), and *Temnothorax marae* Alicata, Prebus & Schifani, 2022 (Alicata and Schifani 2019; Schifani et al. 2022c), there are three remaining species which are currently considered Maltese endemics: *Aphaenogaster melitensis* Santschi, 1933, *Strongylognathus insularis* Baroni Urbani, 1968, and *Temnothorax splendidiceps* (Baroni Urbani, 1968) (Baroni Urbani 1968; Boer 2013). Each of them belongs to a taxonomically unresolved species group and their validity should be reassessed. In particular, the characters allegedly distinguishing *S. insularis* from its southern Italian counterpart *S. destefanii* Emery, 1915 were never quantified (Baroni Urbani 1968; Sanetra et al. 1999). A similar situation occurs with *T. splendidiceps* and the Sicilian endemic *T. laestrygon* (Santschi, 1931), with the difference that the species status of both is poorly supported because no character has been described to distinguish them from *T. exilis* in its current concept

(Baroni Urbani 1968; Salata et al. 2018). Finally, concerning *A. melitensis*, some morphological data are available, but the identity of the Sicilian morphotypes corresponding to the old *A. ionia* concept requires further investigation (authors' unpublished data) and the whole group awaits a comprehensive revision (Schifani et al. 2022d).

Estimated speciation times for ants (and especially Formicinae, see Schär et al. 2018, 2020) are on average longer than in many other insect groups and the short time of separation between Malta and Sicily suggests allopatric speciation of isolated Maltese populations to be unlikely.

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Supplementary material I

The collecting data and voucher identifiers of the specimens

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Data type: morphological data, specimen collection data, GenBank accession numbers

Explanation note: The Suppl. material presents the collecting data and voucher identifiers of the specimens we analysed as well as the morphological data and the GenBank accession numbers of the sequences produced/analysed in this study.

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