ORIGINAL ARTICLE

Integrative biodiversity inventory of ants from a Sicilian archipelago reveals high diversity on young volcanic islands (Hymenoptera: Formicidae)

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Abstract

Islands are fascinating study systems for biogeography, allowing researchers to investigate patterns across organisms on a comparable geographical scale. They are also often important for conservation. Here, we present the first bio-inventory of the ant fauna of the Aeolian Islands, a Sicilian volcanic archipelago formed within the last million years. We documented a total of 40 species, including one first record for Italy (*Lasius casevitzi*). Mitochondrial DNA barcodes were obtained for all 40 taxa sampled on the islands, 13 of which were studied genetically for the first time. Mitochondrial DNA sequences of island specimens were compared with those of conspecific samples from other Aeolian Islands, Sicily and mainland Italy. Standardized photographical documentation of all sequenced specimens is provided. All but one currently recognized species (97.5%) were recovered as monophyletic. Genetic divergence within species ranged up to 12.4% in *Pheidole pallidula*, although most species had much lower levels of intraspecific divergence. At the scale of the Aeolian Islands, intraspecific genetic divergence varied significantly between subfamilies, with species of the subfamily Myrmicinae showing higher intraspecific divergences than the Formicinae. Comparison of specimens from the Aeolian Islands with conspecific ones from the putative source populations (Sicily and mainland Italy) suggested that the island of Panarea has the genetically most derived myrmeco-fauna among the seven focal Islands. Overall, DNA barcoding is a useful-albeit not perfect-method for classifying poorly studied groups of organisms and ants in particular.

Keywords Aeolian Islands · DNA barcoding · Check list · Genetics · Images · Molecular · Taxonomy

Introduction

Islands account for nearly one-sixth of the Earth's total area and are among the most fascinating study systems in biogeography

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(Paulay 1994). Islands play a special role in our understanding of evolutionary processes since isolation triggers a wide set of phenomena, as increased rates of speciation and extinction, founder effects and small population sizes can lead to accelerated evolution. As a result, organisms found on islands are often unique (Whittaker 1998) and about one-third of global biodiversity hotspots are islands (Myers et al. 2000). Due to their confined and often manageable areas, islands serve as natural "laboratories" to address research questions across organisms on a comparable geographical scale (Warren et al. 2015; Whittaker et al. 2017). Insights resulting from such research contributed to classical studies such as the theory of evolution itself or the equilibrium theory of insular biogeography (Darwin 1859; MacArthur and Wilson 1967; Wallace 1869; Warren et al. 2015; Whittaker 1998; Whittaker et al. 2017). Mediterranean islands have been largely investigated for animal and plant diversification, dispersal, colonization history, species interactions, occurrence of relics, evolution of insular

gigantism and dwarfism, and the impact of introduced on native species (Ketmaier and Caccone 2013; Ostende et al. 2017; Pretto et al. 2012) including taxonomically complex insect groups (Allegrucci et al. 2017; Dapporto and Dennis 2009; Fattorini et al. 2017; Papadopoulou et al. 2009). In addition, due to their unique taxa and high ecological vulnerability, Mediterranean islands are often of importance for conservation (Dapporto and Dennis 2008; Gippoliti and Amori 2006; Médail and Quézel 1999; Vodă et al. 2015, 2016). Investigating the biology of island organisms is especially informative if the geological history is well documented. For example, organisms' dispersal abilities or diversification rates can be associated with the age of the island they inhabit. This is often the case for islands dating back to known volcanic eruptions (e.g. Emerson 2003). The Aeolian Islands are a volcanic archipelago in the Tyrrhenian Sea, located close to both the much larger island of Sicily and the Southern tip of mainland Italy (Calabria). All of the seven main islands, Alicudi, Filicudi, Lipari, Panarea, Salina, Stromboli and Vulcano, are thought to have formed within the last million of years (< 1MA) (Rosa et al. 2003). They still undergo strong volcanic activity in the form of fumaroles, hot springs and eruptions. The nearest distance between one of the islands (Vulcano) and Sicily is about 20 km, while the closest distance between Stromboli and Calabria is ~ 55 km (Fig. 1). The altitude reaches a maximum of 968 m above sea level on Salina. Unlike most circum-Sicilian islands, the Aeolian Islands have never been connected with the mainland during glaciations because the sea straits separating these

islands are much deeper than the maximum isostatic variations of sea level of about 110 m occurred during the glacial maxima (Fattorini 2011). The natural landscape consists of lava deserts, volcanic shrub land and scarce maquis or rocky grasslands in dryer regions, and Mediterranean bush forests in more humid zones. Native forests are dominated by *Arbutus* spp. and other shrubs. However, humans have inhabited the islands for at least 8000 years and strongly changed the original natural landscape (Lo Cascio 2017). For example, on some of the larger islands (e.g. Salina), pine or eucalyptus forests have been planted at higher altitudes.

Ants (Hymenoptera: Formicidae) are an abundant and dominant insect group throughout most terrestrial ecosystems. Perhaps due to their abundance and relatively high diversity, ants are often used as model organisms in ecological studies. Species richness of ants is generally low in ecosystems with temperate or boreal climate, but steeply increases towards the equator (Economo et al. 2018). The ant fauna of Europe has been subject to a long history of intense study effort and is perhaps the most studied of any continent. However, within Europe, there are pronounced inter-regional differences in the number of studies conducted on this family of insects. Due to the strong latitudinal diversity gradient, ant species richness is highest in southern Europe. Two European hotspots of species richness are known: the southern Balkan Peninsula and adjacent islands (247 species) and Iberia (279 species) (Lebas et al. 2016). The documented ant diversity in Italy is somewhat lower (196 species) (AntWeb 2020). However, it is possible
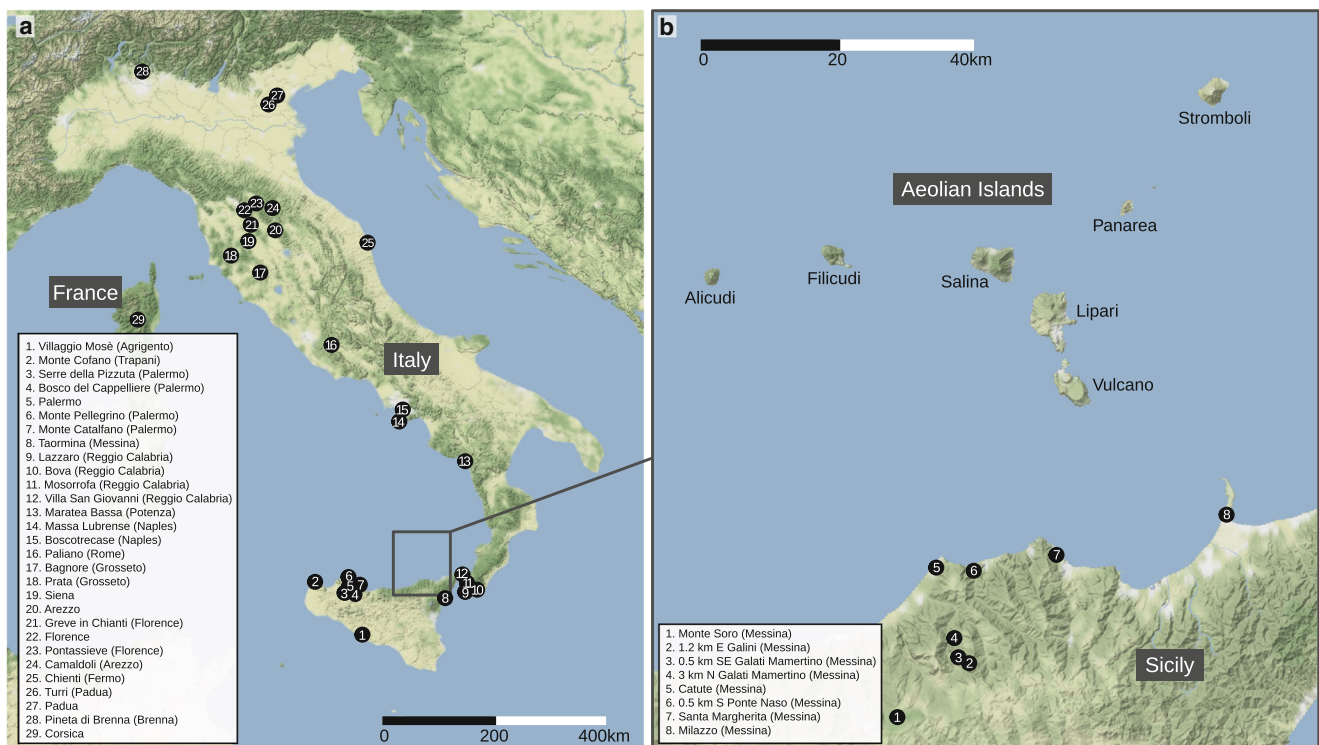

Fig. 1 Map of the sampling locations for the specimens used in this study.

that this may reflect a lower study effort. For example, a recent update of the list of ants occurring on Sicily reported no less than 32 new records for this island (Schifani and Alicata 2018). This steep increase of knowledge suggests that the ant diversity in Italy may currently be underestimated.

So far, the ant fauna of the Aeolian archipelago has been relatively rarely studied. Existing records are mostly from Lipari (Kutter 1927), although a number of other contributions to the knowledge of the archipelago were published (Baroni Urbani 1964; Jucker et al. 2008; Lo Cascio et al. 2006; Lo Cascio and Navarra 2003; Mei 1998; Sanetra et al. 1999; Santschi 1934; Seifert 2016; Scupola 2019). In total, these studies reported 20 ant taxa. No more than ten species have previously been recorded on each of the individual islands, and virtually no genetic data have been provided.

DNA barcoding has become an important, widely applied tool in different areas of biology. Its various applications include the identification of samples, molecular taxonomy including the detection of diverged mitochondrial lineages that potentially represent cryptic species, classification of poorly known biota, phylogeography and studies of ecological and evolutionary patterns (Hebert et al. 2016). Up to date, more than 6.7 million individual specimens belonging to >290,000 species have been sequenced and published on the public "Barcoding Of Life Data System" database (Ratnasingham and Hebert 2007), providing an unprecedented amount of reference material. The convergence of DNA barcoding with morphological taxonomy varies only slightly between and within insect orders, with relatively high performance in Hymenoptera and Orthoptera and a lower one found in Diptera (Virgilio et al. 2010). For ants, a study has observed that about 85% of Palearctic species are monophyletic, while the remaining 15% were non-monophyletic or shared DNA barcodes with members of another species (Schär et al. 2018). The COI gene containing the "barcode" region has also become important for the detection and distinction of cryptic species in ants (e.g., Wagner et al. 2017).

The aim of this study is to conduct a regional bio-inventory of the ant fauna of the Aeolian Islands and to study patterns of genetic diversity using DNA barcoding. In addition, we provided reference sequences for the study species from the Aeolian Islands, Sicily and the Italian Peninsula, as well as photographic documentation of all individual ant specimens investigated (Electronic Supplementary Material ESM 1).

Materials and methods

Collection and identification of specimens

A survey of the ant fauna on the Aeolian Islands (Alicudi, Filicudi, Lipari, Salina, Stromboli and Vulcano, Fig. 1) was conducted during the course of the "Butterfly Week 2016" between April 30 and May 5, 2016. At least one collection trip was performed to each island and all encountered morphospecies of ants were collected by hand and stored in >95% ethanol. Winkler traps and sieving were used to detect soil-inhabiting species. All specimens were deposited in the collection of the Institute of Evolutionary Biology in Barcelona. On the larger islands, sampling locations were recorded using GPS tracking devices. Environmental data were collected by observation or retrieved retrospectively from satellite images using Google Earth. To allow a genetic comparison of ants from the Aeolian Islands with conspecific populations from Sicily and mainland Italy, the collection was complemented with specimens from these two regions in the period between 2015 and 2016. All ants were identified using the most recent identification key available for the study region. In many cases this was the key by Agosti and Collingwood (1987). For particular groups, more recent keys were available: genus *Ponera* (Csösz and Seifert 2003), *Aphaenogaster testaceopilosa* group (Boer 2013), genus *Oxyopomyrmex* (Salata and Borowiec 2015) and *Tetramorium punctatum* (Sanetra et al. 1999). The encountered cryptic species, *Aphaenogaster ichnusa* (*A. subterranea* complex), *Lasius casevitzi*, *Messor ibericus*, *Tapinoma magnum* (*T. nigerrimum* complex) and *Tetramorium immigrans* were partially identified by a comparison with reference mtDNA sequences (published or identified by the authors of the publications describing or raising those taxa to species level) and visual or morphometric examination of diagnostic characters (Seifert and Galkowski 2016; Steiner et al. 2018; Wagner et al. 2017; Galkowski et al. 2019). To estimate the completeness of our sampling, we calculated the Chao species richness estimator (Chao 1987) on the level of collection tubes using the R package "vegan" (Oksanen et al. 2019).

DNA barcoding

If available, four specimens per taxon were sequenced: two from the geographically most distant Aeolian Islands, one from Sicily and one from mainland Italy. Tissue material was non-destructively isolated from specimens by removing one to several legs. Total genomic DNA was extracted using Chelex 100 resin, 100–200 mesh, sodium form (Biorad), under the following protocol: one leg was removed and put into 100 μl of Chelex 10% and 5 μl of Proteinase K (20 mg/ml) were added. The samples were incubated overnight at 55 °C in the shaker VorTemp 56 (Labnet International). Subsequently, they were incubated at 100 °C for 15 min. All specimens from which DNA was extracted were isolated into separate tubes, used for subsequent photography and stored in >95% ethanol thereafter. PCR reactions were carried out according to a published protocol (Talavera et al. 2015). Depending on the species, either the primer pairs LCO1490/HC02198 (Folmer et al. 1994), LCO1490/Nancy (Simon et al. 1994) or LEp1F/R (Hebert et al. 2003) yielded

better results and were used for PCR reactions. For problematic samples, we used the forward primer MLepF1 (Hajibabaei et al. 2006) in combination with HCO02198 (reverse) to obtain partial barcode sequences. The eventually successful primer combination is given for all specimens (ESM 1, Table 1). PCR products were visualized by gel electrophoresis and sent to Macrogen Europe for Sanger sequencing. Raw sequences were edited and aligned in Geneious v. 6 (Kearse et al. 2012).

Specimen images

After removal of tissue for DNA extraction, a set of focused stacked images were produced for each ant using a Carl Zeiss

Table 1 Checklist of the 40 ant species documented for the Aeolian Islands in this study and reference areas included for DNA comparison. Barcoded specimens are shown by the symbol "X" and not barcoded specimens by "O"

Species	Alicudi	Filicudi	Lipari	Panarea	Salina	Stromboli	Vulcano	Corsica	Sicily	Mainland Italy
Aphaenogaster pallida					X				X	O
Aphaenogaster semipolita		X	O	X	O				X	X
Aphaenogaster splendida						X			O	
Aphaenogaster subterranea complex	X	O	O	O	O	X			X	X
Camponotus aethiops			X						X	X
Camponotus lateralis		X	O	O	O	O	X		X	X
Camponotus micans		X	O		O		X		X	
Camponotus nylanderi		X	O	O	O	X	O		X	X
Camponotus piceus			X						X	X
Camponotus ruber			X	X					O	
Crematogaster scutellaris		X	O	O	O	X	O		X	X
Crematogaster sordidula			X	X					X	
Formica cunicularia			X						X	X
Lasius casevitzi	X		X		O			X	X	O
Lasius emarginatus			X		X				X	X
Lasius lasioides	X	O	X	O	O	X	O		X	X
Lasius myops			O		X		X		X	
Lepisiota frauenfeldi			X						X	X
Lepisiota nigra			X	X	X		O			X
Linepithema humile		X				O				
Messor bouvieri		X	O	O	O	X	O		X	X
Messor capitatus			X						X	X
Messor ibericus		X			X				X	X
Monomorium subopacum		X	O	O		X			X	X
Nylanderia sp.						X				
Oxyopomyrmex saulcyi			X							O
Pheidole pallidula		X	O	O	O	X	X		X	O
Plagiolepis pallescens	O		X	X	O	X	X		X	
Plagiolepis pygmaea		X	O						X	X
Ponera coarctata			X		X				X	O
Solenopsis latro		X	X		O	X			X	
Strongylognathus destefanii							X		X	X
Tapinoma nigerrimum complex		X	O	O	O	X	O		X	X
Temnothorax exilis	X	O	O	O	O	X	O			
Temnothorax lichtensteini					X				X	X
Temnothorax mediterraneus		X	O	O		X	O		X	
Tetramorium bicarinatum			O	X					O	
Tetramorium immigrans			X							X
Tetramorium punctatum		X	O	O	O	X	O		X	
Tetramorium semilaeve	X	X	X	X	O	X	X		X	X

Stemi 2000-C stereomicroscope equipped with a CMEX PRO-5 DC.5000p digital camera, at magnifications adjusted to the individual vouchers, and in standard angles of view (full-face view, lateral and dorsal view). The photographed specimens were always the very same individuals as used for DNA sequencing. Processing of the images was performed in the programs align_image_stack, Helicon Focus 6.7.1 and GIMP 2.8. All specimen images are available in ESM 1 of this article.

Genetic and statistical analyses

A maximum likelihood phylogenetic tree with integrated search for the ideal nucleotide substitution model was obtained in IQ-TREE (Nguyen et al. 2015), using the best nucleotide substitution model estimated by the program (GTR + I + G4), and a total of 1000 bootstrap pseudoreplicates (Fig. 2). Intraspecific uncorrected genetic distances were calculated using the "ape"-package (Paradis et al. 2004) and were
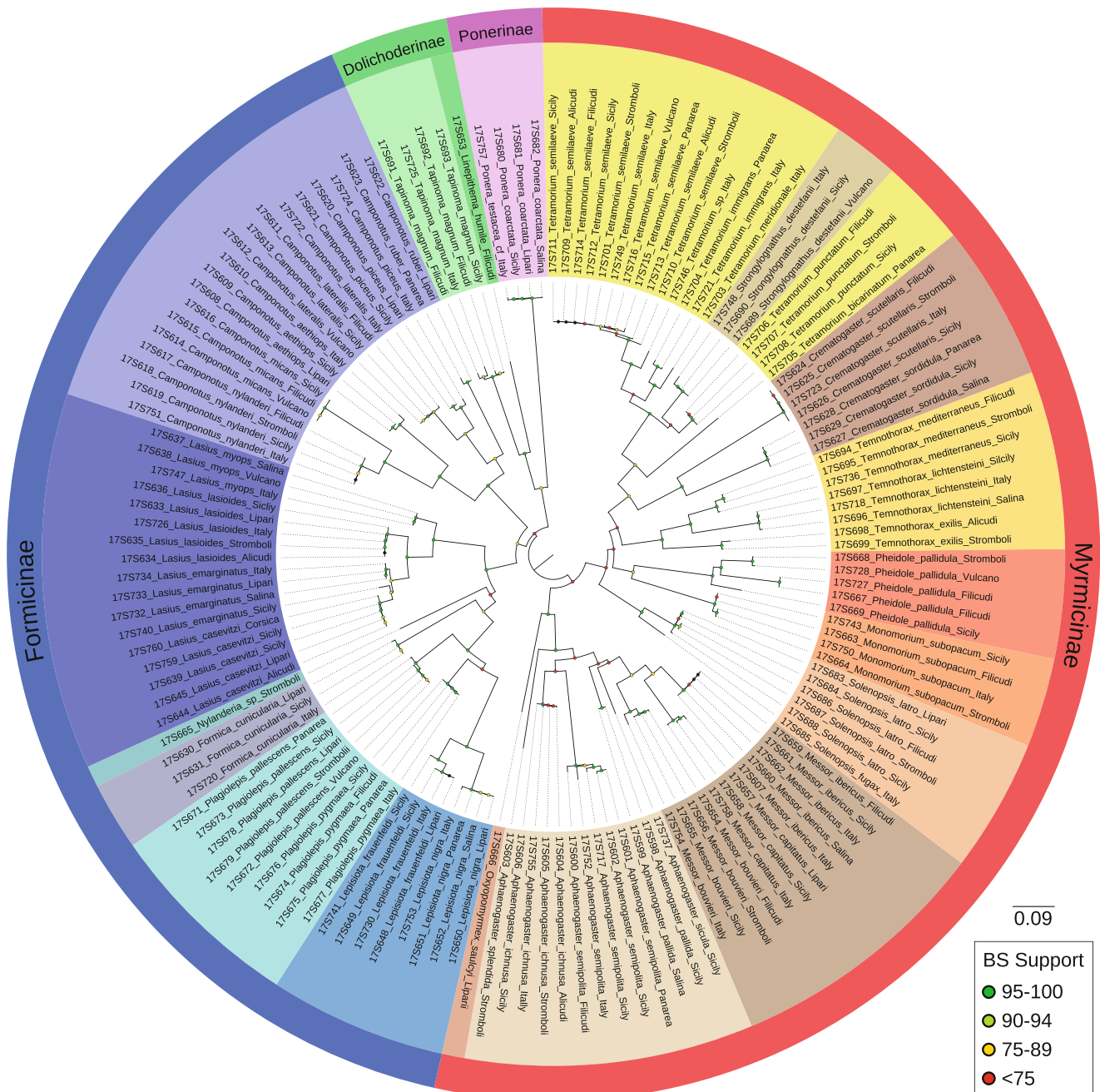
Fig. 2 Maximum likelihood tree of 143 DNA barcodes belonging to 44 ant taxa. The scale bar indicates the number of changes per site. The colours given at nodes show % node support from 1000 bootstrap replicates.

compared between subfamilies using Kruskal-Wallis tests in R v. 3.5 (R Core Team 2018). Medians and their corresponding confidence intervals were calculated using the R package "rcompanion" (Mangiafico 2019). No adjustment for multiple statistical testing was performed. The haplotype network was made with the program TCS 1.21 (Clement et al. 2000) and then edited with tcsBU (dos Santos et al. 2016) and Adobe Illustrator CC 2019.

Results

A total of 566 individual ant samples were collected from 52 locations spread across the Italian Peninsula and its Western adjacent islands (Fig. 1). A total of 40 ant species, belonging to 22 genera and four subfamilies, were identified among the samples from the Aeolian Islands (Table 1). According to the Chao species richness estimator, we estimate that our sampling efforts may have missed five species and that the true number of species may be 45 (ESM 1, Fig. 1). For subsequent DNA barcoding, we selected 143 representative samples belonging to 44 species. These include all 40 ant taxa occurring on the Aeolian Islands, plus four additional closely related species from Sicily or mainland Italy, included for identification purposes.

DNA barcoding eventually succeeded for all 44 investigated ant species, although the required effort varied strongly between individual samples and between species, both between and within genera. PCR reactions were often initially successful for samples belonging to the genera *Lasius*, *Tetramorium* and *Solenopsis*, but much less so for *Aphaenogaster*, *Camponotus* and *Lepisiota.* For two species (*Camponotus ruber* and *Lepisiota nigra*), DNA barcoding was found to be notoriously difficult with both commonly used primer pairs. Only partial barcodes could finally be obtained for the representatives of these two species, using the forward primer MLepF1 (ESM 1, Table 1).

Monophyly was recovered for all four subfamilies. Most genera were also found to be monophyletic, with the exception of three nodes of low bootstrap support (< 75%, Fig. 2), suggesting non-monophyly of the genera pairs *Aphaenogaster/Messor*, *Lepisiota/Plagiolepis* and *Tetramorium/Strongylognathus* (cases of paraphyly). Species as identified by morphology corresponded to monophyletic clades in all but one species (97.5%), because *Lasius emarginatus* was paraphyletic (Fig. 2). In addition, it remained somewhat unclear whether *Tetramorium punctatum* represents a monophyletic taxon: one sample from Calabria initially identified as this species appeared to be more closely related to *T. semilaeve* than to *T. punctatum* (specimen 149 in Fig. 2). However, for this sample, we could not clearly rule out nor confirm *T. diomedeum*, a taxon that appears to be difficult to identify by the worker caste alone (Csösz and Schulz 2010).

The maximum intraspecific mtDNA divergence was 12.4% in *Pheidole pallidula* (Table 2). At the scale of the Aeolian Islands, the maxima of intraspecific mtDNA divergence differed significantly between ant subfamilies (Kruskal-Wallis $\chi^2 = 7.83$, df = 3, $P = 0.0497$), with species belonging to the subfamily Myrmicinae generally showing higher divergences within currently defined species than those belonging to the Formicinae. However, this pattern was not significant at larger geographical scales, i.e. for Sicily + the Aeolian Islands (Kruskal-Wallis $\chi^2 = 2.41$, df = 2, $P = 0.299$) and for Italy + islands (Kruskal-Wallis $\chi^2 = 3.40$, df = 3, $P = 0.334$) (Fig. 4).

A total of 18 (45%) of the 40 species found on the Aeolian Islands in this study are widespread elements of the Mediterranean fauna, 4 (10%) are Western and 1 (2.5%) Eastern Mediterranean, 5 species (12.5%) have their focal distribution in North Africa, 5 species (12.5%) represent regional endemics, 3 species (7.5%) are part of the Eurosiberian fauna and 4 (10%) are introduced tramp species (Schifani and Alicata 2018) (Table 1). We managed to confirm the presence of all but four (*Messor minor*, *M. wasmanni*, *Solenopsis fugax* and *Strumigenys membranifera*) of the 20 species previously reported from the Aeolian archipelago. All but one species detected in our survey are free living. The exception is *Strongylognathus destefanii*, which is an obligate parasite (slave-maker) of mainly *Tetramorium semilaeve* (Sanetra et al. 1999). Most species are generalist foragers, while some *Aphaenogaster* spp., *Ponera* spp., *Solenopsis* spp. and *Lasius myops* forage primarily in the top ground layer, either in leaf litter or in soil. Despite dedicated searching, no specialized tree-living ants were found on the islands, which seem to harbour only a few native trees. Detailed ecological data on the habitats and microhabitats of most species are provided in Table 1.

When comparing genetic distances of specimens from individual Aeolian islands with conspecific samples from Sicily and mainland Italy, the Island of Panarea was found to have the genetically most diverged myrmeco-fauna from both putative source populations (median divergence from Sicilian samples: 0.9%; from mainland Italian samples: 1.1%). According to the island's geographical position, the specimens from the northernmost island Stromboli were also found to be rather genetically distinct from those from Sicily (median divergence 0.8%), while samples from the westernmost Alicudi had a median of 0.7% divergence from conspecific samples from mainland Italy. For the other Islands, median divergence from samples from both source populations was < 0.5% (Fig. 5).

Discussion

To our knowledge, this study represents the first comprehensive checklist of the ant fauna of the Aeolian Islands and the

Table 2 Maxima of intraspecific mtDNA divergence at three geographic scales. Left: Italy+ Islands; center: Sicily+Aeolian; right: within Aeolian

Species	n	Max divergence (%) Italy+Islands	n	Max divergence (%) Sicily+Aeolian	n	Max divergence (%) within Aeolian
Aphaenogaster ichnusa	4	0.9	3	0.4	2	0.3
Aphaenogaster pallida			2	0	1	
Aphaenogaster semipolita	5	4.7	4	3.4	2	3
Aphaenogaster splendida					1	
Camponotus aethiops	3	2.3	2	0.2	1	
Camponotus lateralis	4	0.6	3	0	2	0
Camponotus micans			3	0.2	2	0
Camponotus nylanderi	4	0.2	3	0.2	2	0.2
Camponotus piceus	3	2.3	2	0	1	
Camponotus ruber					2	1.4
Crematogaster scutellaris	4	0.3	3	0.2	2	0
Crematogaster sordidula			3	0.3	2	0.3
Formica cunicularia	3	0.8	2	0.5	1	
Lasius casevitzi	5	0.6	4	0.2	2	0
Lasius emarginatus	4	3	3	3	2	0
Lasius lasioides	5	0.3	4	0.3	3	0
Lasius myops	3	0.3			2	0.1
Lepisiota frauenfeldi	4	2.2	3	2.2	1	
Lepisiota nigra	4	0.7			3	0
Linepithema humile					1	
Messor bouvieri	4	3.5	3	0.6	2	0.3
Messor capitatus	3	1.5	2	1.2	1	
Messor ibericus	5	0	3	0	2	0
Monomorium subopacum	4	1.1	3	1.1	2	1.1
Nylanderia sp.					1	
Oxyopomyrmex saulcyi					1	
Pheidole pallidula			5	12.4	4	12.2
Plagiolepis pallescens			5	1.1	4	1.1
Plagiolepis pygmaea	4	0.5	3	0.5	1	
Ponera coarctata			3	0.5	2	0.3
Solenopsis latro			5	1.5	3	1.2
Strongylognathus destefanii	3	2	2	0.2	1	
Tapinoma magnum	4	2.9	3	2.3	2	2.3
Temnothorax exilis					2	2
Temnothorax lichtensteini	3	0.8	2	0.6	1	
Temnothorax mediterraneus			3	2.1	2	0.3
Tetramorium bicarinatum					1	
Tetramorium immigrans	2	1.2			1	
Tetramorium punctatum			3	0.5	2	0.2
Tetramorium semilaeve	10	1.7	9	1.7	7	1.7

total number of species recorded from this archipelago is now more than doubled. Moreover, genetic and ecological data was provided (Tables 1 and 2, ESM 1), and this work appears to be one of few holistic DNA barcoding studies of a local European myrmeco-fauna published so far. Given that all specimens were sampled during the course of a single week, it is well possible that the ant diversity on the Aeolian archipelago is even higher than the 40 species reported here and the additional four reported earlier. This is particularly likely given that several ant genera with hidden lifestyle (e.g. *Leptanilla, Stigmatomma, Stenamma* spp.) are known from the neighbouring island of Sicily (Schifani and Alicata 2018). In addition, rare species are commonly overlooked in short biodiversity inventories. In ants, this could be the case

with socially parasitic species, hiding in only a small percentage of the nests of their host species (Buschinger 2009).

Geographic origin of the islands' ant fauna

The ant fauna found on the Aeolian Islands represents an interesting mixture of widespread Mediterranean ants, several species of North African origin and a number of regionally endemic species (ESM 1, Table 2). Noteworthy, 10% of the encountered fauna consisted of introduced, widespread tramp species. Although the latter so far seem to remain restricted to anthropogenic habitats (ESM 1, Table 2), they may sooner or later affect the balance of the island's native ecosystems. Moreover, since the island long history of human activities, it remains unclear how many of the currently present taxa have been introduced in the past. The pattern of intraspecific divergence we recovered largely corresponded to the geographic location of the individual islands with respect to Sicily and Italian mainland (Fig. 5). Interestingly, the fauna of Panarea was found to be the most genetically distinct from both Sicily and mainland Italy, despite Stromboli being the geographically most isolated of the islands. This finding could possibly be explained by a higher age or less volcanic activity in Panarea compared with that in Stromboli, allowing populations to have existed for a longer period of time. Similar to this study, previous COI assessments of butterflies from Sicily and circum-Sicilian islands including the Aeolian Islands found that even these highly mobile insects show a high divergence in this area compared with surrounding regions (Italy and North Africa) (Vodă et al. 2016; Dapporto et al. 2019; Scalercio et al. 2020).

Using DNA barcoding for the classification of ants

In agreement with previous investigations employing DNA barcoding of Palaearctic ants (Jansen et al. 2009; Schär et al. 2018), morphologically defined taxonomy agreed with monophyletic mtDNA clades, in 39 of 40 species collected in Italy (97.5%). At the local level (Aeolian Islands), we even found full agreement between taxonomy and mtDNA clades (Fig. 2). We interpret these findings as (1) DNA barcoding being a relatively reliable, albeit not perfect, tool for classifying poorly known faunas and (2) high correspondence between taxa defined by morphology and evolutionary units in the investigated area, despite the fact that this region has never been intensively studied by myrmecologists and was regularly not included in the efforts of modern taxonomical revisions of European ants (Schifani and Alicata 2018).

At the intraspecific level, a wide range of divergence in mtDNA sequences has been detected, ranging up to 12.4% uncorrected genetic distance in Sicilian and 12.2% in Aeolian samples of the species *Pheidole pallidula*. Although intraspecific mtDNA divergence has been found to vary strongly between groups of organisms, divergence of about 3% has been

suggested as a threshold for the definition of sister species (e.g. 2.7% in birds) (Hebert et al. 2003). In this study, a 3% threshold has been reached or surpassed in four ant taxa: *Pheidole pallidula* (12.4%), *Aphaenogaster semipolita* (4.7%), *Messor bouvieri* (3.5%) and *Lasius emarginatus* (3%) (Table 2). However, the existence of such mitochondrial "deep lineages" is not always proof for the existence of cryptic species (Dincă et al. 2019; Hinojosa et al. 2019; Hundsdoerfer et al. 2019). Cases of high divergence in mitochondrial DNA which is not reflected by a similar variation in nuclear DNA can be produced by numts (Song et al. 2008), infection with endosymbiont bacteria (Xiao et al. 2012) and introgression with extant or extinct species (Wielstra and Arntzen 2014; Toews and Brelsford 2012). More suggestive of cryptic diversity is non-monophyly, here detected in *Lasius emarginatus* (Fig. 2). However, since more coverage of the genome would be needed to safely demonstrate non-monophyly and lack of recent gene flow, it remains unclear for now whether these taxa comprise cryptic species or not. Hence, testing for reproductive isolation of potential cryptic species within these taxa may represent an interesting area of future research.

Taxa of special interest

Lasius casevitzi

This taxon was so far thought to be endemic for Corsica and is here reported for the first time for the fauna of Italy. *Lasius casevitzi* was described based on morphometrics as one of three cryptic species formerly known as *L. paralienus*: *L. bombycina*, *L. casevitzi* and *L. paralienus s. str.* (Seifert and Galkowski 2016). Combining the results of this study with those of a previous investigation (Schär et al. 2018), we here found support for this hypothesis at least at the level of mitochondrial DNA-lineages: *L. paralienus* s.l. comprises three deep mitochondrial lineages separated from each other by relatively high levels of divergence, similar to the other presently recognized species within the European *Lasius alienus* species group (Fig. 3). Those lineages correspond to the morphologically separable groups characterized by Seifert and Galkowski (2016). However, the mitochondrial lineage and phenotype (terminal segment of maxillary palps longer than 0.2*cephalic size, genae with on average > 2.8 projecting hairs, see ESM 1 for specimen images) named *L. casevitzi* is apparently not endemic to Corsica, but at least also occurs on Sicily and the Aeolian Islands (Figs. 2 and 3, Table 2).

Lasius emarginatus

The mitochondrial DNA of representatives of this taxon showed an unexpected pattern of relationships. *Lasius emarginatus* was found to be paraphyletic by DNA barcoding, although with little support (Fig. 2). Samples from Italy and the
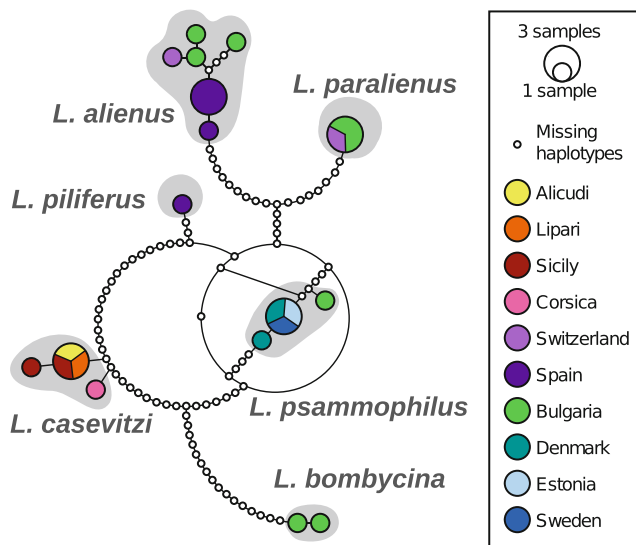
Fig. 3 Haplotype network of mtDNA barcode haplotypes found within the European members of the *Lasius alienus* species group sensu Seifert and Galkowski (2016). Note the placement of the Sicilian members of the group within the taxon *L. casevitzi*. Sequences other than those of *L. casevitzi* are from Schär et al. (2018).

Aeolian Islands cluster together, while a sample from western Sicily (Monte Pellegrino, specimen 143) appears as an outlier, with a divergence of 3% (Table 2). However, strong morphological differences between this and the other specimens were not observed. It appears to be somewhat hairier than other *L. emarginatus* and shows a more homogenous coloration (ESM 1, specimen 143 vs. 135–137). It is thus possible that this population represents a distinct lineage or even a cryptic species, which could be a subject of future investigations.

Pheidole pallidula

The maximal divergence of intraspecific mtDNA found within this taxon (12.4% within greater Sicily and 12.2% within the Aeolian archipelago) is unmatched by any other taxon investigated here (Table 2, Figs. 2 and 4). Three highly derived mitochondrial lineages were found, one of which only on Filicudi and another one on both Stromboli and Vulcano. The third deep lineage was collected in Milazzo (Fig. 2). Finding such a high divergence of mtDNA barcodes within a species is uncommon. Double peaks or stop codons, which are typical of numts, were not found in these sequences. Sometimes such findings are suggestive of cryptic species, hybridization/introgression or populations separated by large geographical distances (Hosoishi and Ogata 2019). Remarkably, the distinct mitochondrial lineages of *P. pallidula* identified here occur in a geographically very small area, situated as little as 30 km from each other (Fig. 1). Furthermore, we did not observe any particular morphological or ecological differences between them (ESM 1). The hypothesis that *P. pallidula* contains cryptic species has
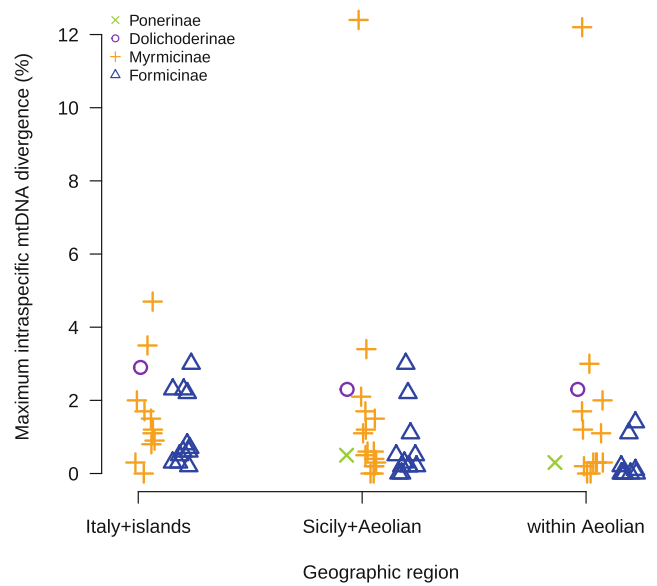
Fig. 4 Species' maxima of raw mtDNA divergence, grouped by subfamily and geographical scale.

already been raised by a very detailed morphometric study (Seifert 2016). However, the latter work suggests relatively clear geographic separation of the proposed four cryptic taxa, with the only geographical area of co-occurrence being the Eastern Mediterranean. In the reference area of Sicily and the Aeolian Islands, only the type form (type locality: Messina) should theoretically occur (Seifert 2016). Alternatively, the deep lineages may be explained by some of the processes already discussed above. Thus, the study of nuclear markers is required to assess the implications of our findings in mtDNA.
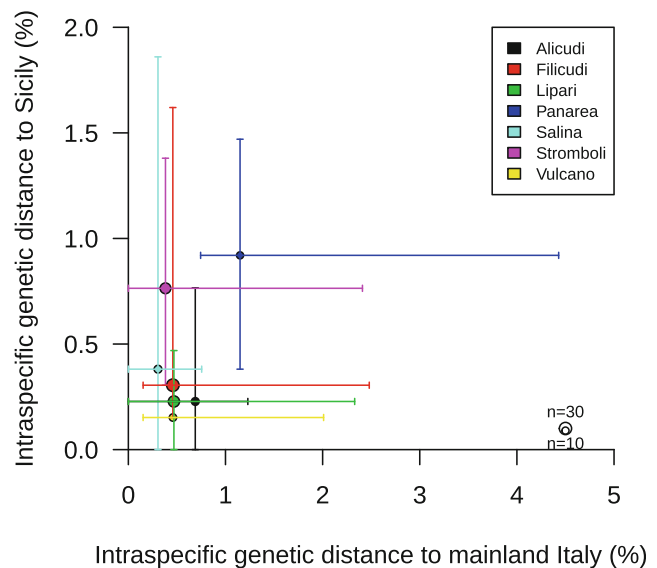
Fig. 5 Median (circles) and corresponding 95% confidence intervals (lines) of all intraspecific genetic distances between specimens from individual Aeolian islands and the two putative source populations Sicily and mainland Italy. The areas of the circles are proportional to the sample sizes available for each island.

Tetramorium sp.

A sample of the genus *Tetramorium* from a site in Calabria, mainland Italy (Bova, specimen 149, see ESM 1 for image), could not be identified with certainty. Visually, this worker resembles *T. punctatum* due to its small size, yellowish colour and smooth integument. However, it is genetically distinct (10% divergence) and, according to mtDNA, more closely related to *T. semilaeve* (Fig. 2). On the other hand, *T. diomedeum* cannot be safely excluded in this case, a taxon of somewhat uncertain identity thought to be related to *T. ferox* (Csösz and Schulz 2010). Thus, future improvement of the taxonomic knowledge of the genus *Tetramorium* may help to identify this sample or evaluate its status as a potentially undescribed species.

Divergence in different subfamilies

Within the comparable geographical scale of the Aeolian Islands, intraspecific divergences varied between ant subfamilies, with Myrmicinae showing relatively high and Formicinae low levels of intraspecific maximum genetic distances (Fig. 4). There are several possible scenarios that could explain this observation: different substitution rates, dispersal capabilities, strength of selection, degrees of ecological specialization, prevalence of *Wolbachia* or other endosymbiont bacteria or different frequencies of cryptic species, among other possibilities. However, this local trend of seemingly rapid molecular evolution in the ant subfamily Myrmicinae is in harmony with the high species richness and cryptic diversity observed in this hyperdiverse group (Ward et al. 2015).

Conclusions

At least 40 species, 22 genera and 4 subfamilies of ants occur on the Aeolian Islands. The taxon *Lasius casevitzi* was found for the first time in Italy. DNA barcoding is feasible for all species, although the required effort varies between taxa and individuals when using commonly employed primers. All but one species (97.5%) were found to represent monophyletic groups. Maxima of intraspecific divergence ranged from 0 to 12.4% (*Pheidole pallidula*). At the scale of the Aeolian Islands, intraspecific divergences varied between ant subfamilies, with Myrmicinae showing relatively high and Formicinae low levels of intraspecific maximum genetic distances. When comparing genetic distances of specimens to conspecific samples from Sicily and mainland Italy, the Island of Panarea appeared to have the genetically most distinct fauna from both potential source populations. Overall, DNA barcoding is a useful-albeit not perfect-method for classifying poorly studied groups of organisms, and ants in particular.

Data availability All data generated or analysed during this study are included in this published article and its supplementary information files and accessible via the BOLD Taxonomy Browser (project AEANT).

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