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RESEARCH ARTICLE

Phylogeographic structure of Italian *Formica pratensis* (Retzius 1783) populations in the framework of the species Eurasian range

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Abstract

The phylogeography and demographical history of Italian *Formica pratensis* populations were examined and compared with the Eurasian-wide dataset available for this species and the other red wood ant species *Formica lugubris*. Forty-eight workers belonging to eight populations from both Alps and Apennines were analysed sequencing a 1.5-kilobase mitochondrial DNA fragment, including the cytochrome b gene and part of the NADH dehydrogenase subunit 6 gene. A total of 127 sequences were screened, scoring 53 different haplotypes amongst all specimens, with five new haplotypes discovered in the Italian populations. All the Italian haplotypes clustered in a monophyletic clade, underlining a clear phylogeographical separation of this group from the other Eurasian groups and suggesting a glacial separate forest refugia and different post-glacial colonisation patterns. The haplotypes from the Alps and the Apennines showed a high genetic proximity, pointing out an ancient (Pleistocene) wide distribution of this species across all these areas and common ancestral lineages. No shared haplotypes were scored between Northern and Central Apennine populations, but the low inter-population genetic distance indicated similar post-glacial selective processes acting on these groups. The diversity we recorded may be influenced by the actual fragmentation of *F. pratensis* populations across its entire Eurasian range, and by the limited geographical origin and sample dimension of the dataset analysed. Future studies with a more extensive sampling in the Alps and Eastern Europe are needed to confirm our result.

Keywords Red wood ants · mtDNA · Phylogeography · Cytochrome b gene · Haplotypes network

Introduction

Red wood ants (RWAs, *Formica rufa* group) are one of the dominant ecosystem components in cool and temperate coniferous woodlands of the Palearctic Region (Risch et al. 2016). Fourteen morphologically similar species can be found in this region with at least six species recorded in Europe, where most of them can often be found in sympatry and even generate hybrids (Seifert 2021). The distribution of European RWA species is well-known, given the large number of studies focussing on these taxa (Stockan et al. 2016). However, the frequent occurrence of hybrid colonies makes it more difficult to understand the actual geographical range: for example, many RWA populations of Great

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Britain are supposed to consist of hybrids between *F. rufa* and *F. polyctena*, since 95% of all samples collected present intermediate phenotypic traits between the two species (Seifert 2021).

The *F. rufa* group origin dates back to around 15 million years ago when the group diverged from the other *Formica* species. According to the mitochondrial DNA analysis, this clade is monophyletic and is divided into a Nearctic and a Palearctic sub-clade (Goropashnaya et al. 2012). In particular, the Palearctic species have been characterised by a reticulate evolution largely driven by hybridisation and introgression events (Seifert and Goropashnaya 2004; Trager 2016; Romiguier et al. 2018). Many of these hybridisation events are currently ongoing between sympatric populations of RWA species (Satokangas et al. 2023), but they are difficult to detect without a molecular screening approach due to the extreme intraspecific polymorphism and occurrence of local morphological variants (Seifert, 2021).

RWAs occupy a keystone position in the habitats they colonise, influencing their nests and foraging activity important biotic and abiotic ecosystem components (Frizzi et al. 2018; Di Nuzzo et al. 2022; Guariento and Fiedler 2021). Some of their ecological functions, such as nutrient recycling, trophobiosis, seed dispersal, soil aeration, and predation on other arthropods are fundamental for forest ecosystem functioning (Frouz et al. 2016; Guariento et al. 2021; Balzani et al. 2022a), furthermore, their nests host a huge variety of arthropod species representing a real biodiversity hot spot (Frizzi et al. 2020; Castellucci et al. 2022). In the last 50 years, local decline or even extinction events have been recorded in many areas of Europe, with agricultural activities, industrialization, and habitat fragmentation representing the main threats, together with climate change (Sorvari 2016; Çamlıtepe and Aksoy 2019). Therefore, in 1996, the International Union for Conservation of Nature (IUCN), listed five RWA species [*F. rufa*, *F. lugubris*, *F. polyctena*, *F. aquilonia*, and *F. pratensis* (plus *F. uralensis*)] as Near Threatened at a global level. However, after more than 20 years, no further official assessment with species status updating was carried out, nor any other RWA species has been included in the Red List (IUCN Red List 2023). Although the standing recognition of their importance prompted these species for legal protection in several European countries (Cherix et al. 2012), there is not a unique reference framework; therefore, the development of wide-scale conservation actions is needed (Sorvari 2016; Balzani et al. 2022b).

In Italy, RWA species are widespread along the Alps and only the more thermophilic *Formica pratensis* (Retzius, 1783) naturally occurs at lower latitudes in the Apennine mountains. In those areas additional RWA species (*F. lugubris, F. rufa, F. paralugubris,* and *F. aquilonia*) may be found, originating from past intentional introductions, with *F. paralugubris* being the most common species (Masoni et al. 2019), as biocontrol agents against the outbreaks of forest insect pests (Ronchetti and Groppali 1995). Some of these populations are still extant, are expanding and show signs of genetic diversification from their source populations (Masoni et al. 2022). Other populations, instead, underwent rapid decline and went extinct in a few years (Ronchetti et al 1986; Frizzi et al. 2018).

Formica pratensis mainly lives in open grassland habitats, often at the edge of coniferous or mixed coniferous-deciduous forests and compared to the other RWAs, it occurs at lower elevations, forming smaller colonies in open sunny habitats (Véle et al.2009). Monogynous colonies of this species predominate, although polygyny (Seifert 1991; Pirk et al. 2001; Aksoy and Camlitepe 2018) and, rarely, supercoloniality (Kiss and Kóbori 2010) may also occur. Polydomy is also described in this species, which allows colonies to create new nests without going through the single-queen nest foundation (Seifert 1991; Ellis and Robinson 2014).

The distribution of *F. pratensis* populations along the Italian peninsula is unknown, as are their ecology and conservation status. The scant information about the presence and distribution of the species is limited to outdated publications (Pavan 1981, Fig. S1), where it was reported as separated into two species (*F. nigricans* and *F. pratensis,* see below), and some recent records on scientific blogs or social networks (iNaturalist, https://www.inaturalist.org/; Antwiki, https://antwiki.org/). At the beginning of the last century, Emery (1909) described the Apennine populations as belonging to *F. nigricans*, which differed from the Alpine and European populations (*F. pratensis*) by having darker head and thorax. However, morphological studies carried out by Seifert since 1991 rejected this separation, and recently Seifert (2021) confirmed that the former *F. nigricans* is to be considered as an ecomorph of the *F. pratensis* originally described by Retzius in 1783. Considering the rapid decline of this species in recent decades across its entire European range (e.g. Dekoninck et al. 2010; Çamlıtepe and Aksoy 2019), many Apennine populations may have experienced the same fate, disappearing, decreasing in number, or forming scattered isolated populations.

In this study, we analysed the geographical pattern of mitochondrial cytochrome b gene (Cyt-b) variation amongst eight populations of *F. pratensis*, seven located along the Apennine and one in the Italian Alps. This mitochondrial region was chosen given the high variability that was observed in the genus *Formica* L. in previous phylogeography and population structure studies (Goropashnaya et al. 2004a; Antonov and Bukin 2016) and a Eurasian-wide dataset based on this marker is available for both *F. lugubris* and *F. pratensis* (Goropashnaya et al. 2004b). We first analysed the relationships between haplotypes of the Italian populations and their geographical distribution, then we compared

the Italian populations with the Eurasian ones investigated by Goropashnaya et al. (2004a) and stored in Gene Bank, NCBI (http://www.ncbl.nlm.nih.gov/). Our results will improve the knowledge of the *F. rufa* group speciation and spread. They can also help to turn the spotlight on the importance of the Italian populations of *F. pratensis,* prompting urgent conservation plans.

Materials and methods

Sample collection

Fieldwork was carried out between March 2021 and September 2023. We collected samples from eight *F. pratensis* populations (See Table 1) distributed across the Alps and the Apennines. These populations strongly differ in elevation and habitat selection. For each population, 20 workers were sampled from six different nests. To avoid sampling ants from non-independent nests belonging to the same polydomous colony as much as possible, we used only nests located at least 30 m away and not connected by trails of workers. All ants from each nest were preserved in absolute ethanol and divided into two groups, one stored at − 80 °C until DNA extraction, and the other one used for species identification (Seifert 2021).

DNA Extraction, amplification and sequencing

Total genomic DNA was extracted from the leg tissue of a single ant per nest, using a Nucleospin® DNA insect kit (Macherey–Nagel) following the manufacturer's instructions. To compare the newly sampled Italian populations with the Eurasian dataset by Goropashnaya et al. (2004b), a highly variable 1571 bp mitochondrial DNA fragment including the complete cytochrome b gene (Cyt-b, 1125 bp long) and the first part of the NADH dehydrogenase subunit 6 (ND-6, 446 bp long) has been amplified via PCR and sequenced. A microsatellite characterised by the repetition of a TTA motif is found in the intragenic region between the Cyt-b gene and the ND-6. Two primer couples were chosen to amplify two overlapping fragments (Goro1 and Goro2, see Suppl. Tab. S1), subsequently assembled to reconstruct the whole 1571 bp sequence. Amplification via PCR was carried out with an initial denaturation step of 2 min at 94 °C, 45–50 PCR cycles (50 s at 94 °C, 45 s at Ta, 50 s at 72 °C) and a final extension step of 5 min at 72 °C. PCR products were checked via electrophoresis on a 1% agarose gel and purified using ExoSAP-IT Product Cleanup Reagent (Thermo Fisher Scientific). Sanger sequencing of the forward and reverse strands was performed by Macrogen Europe (Amsterdam, The Netherlands). Chromatograms were inspected and edited with the software SeqTrace v.0.9.0 (Stucky 2014). The search for potential contaminants was carried out using BLASTn (Zhang and Madden 1997) on the NCBI database. The obtained sequences were submitted to NCBI GenBank (accession numbers PP179335-PP179375).

Phylogenetic and haplotype network analyses

We obtained sequences from a total of 48 workers. These were, then, added to the 79 *F. pratensis* + *F. lugubris* sequences from Goropashnaya et al. (2004b) Palearctic dataset, stored in GenBank, NCBI (PopSet accession number: 46403874 and 45,934,296), to build the final dataset. Alignment was performed using the automatic detection of the best-fit algorithm on MAFFT v7.503 (Katoh and Standley 2013). The microsatellite close to the 5'-end of the Cyt-b gene was excluded from the analyses to facilitate comparison of the results. Sites with missing data or alignment gaps were removed and haplotypes were retrieved running the R software v 4.1.2 (R Core Team 2021), using the package "haplotypes" (Aktas 2020). A single sequence for each of the observed haplotypes was maintained in the final dataset.

Table 1 Populations of *F. pratensis* analysed in this work

Localities	Pop ID	Altitude	Habitat	N° nest	Latitude	Longitude
Alps						
Bozen province (Trentino-Alto Adige)	BOL	1200–1400	Open areas/Fir Forest	> 10	46°49′12.01″N	11°9′27.00″E
Northern Apennine						
La Consuma (Florence, Tuscany)	CO	1000–1100	Open areas/Fir Forest	< 10	43°45′57.65″N	11°35′18.35″E
Vallombrosa (Florence, Tuscany)	VAL	1000–1100	Fir Forest	< 10	43°73′38.34″N	11°55′70.38″E
La Verna (Arezzo, Tuscany)	LV	1000–1200	Open areas/Fir Forest	> 10	43°42′51.71″N	11°56′38.32″E
Orecchiella (Lucca, Tuscany)	ORE	1100–1250	Fir Forest	< 10	44°12′21.03″N	10°20′45.38″E
Ticchiano (Parma, Emilia-Romagna)	TIC	950–1100	Fir Forest	< 10	44°26′6.46″N	10° 6′7.42″E
Central Apennine						
Barrea (Aquila, Abruzzo)	BAR	1200–1300	Open areas	< 10	41°46′2.43″N	13°59′40.56″E
Scanno (Aquila, Abruzzo)	SCA	1100–1300	Open areas	< 10	41°53′29.81″N	13°55′7.10″E

Model selection and maximum likelihood phylogenetic reconstruction were carried out using IQ-TREE (Minh et al. 2013) and nodal support was estimated with 1000 replicates of UltraFast bootstrap.

The same dataset used for phylogenetic reconstruction was used to build a haplotype network with PopART (Leigh and Bryant 2015), implementing the Median Joining method.

Genetic distances within and between *F. pratensis* clades were computed with MEGA v.11.0.13 (Tamura et al. 2021) using the Tamura and Nei (1993) substitution model.

Results

The final dataset consisted of 127 sequences of 1431 bp, excluding sites with missing data, alignment gaps and the microsatellite found in the intergenic region of Cytb according to Goropashnaya et al. (2004b). The TTA motif of this microsatellite was repeated four to thirteen times in the Italian haplotypes and four to seven times in Eurasian ones, underlining the first clear separation of the Italian populations. Overall, 53 different haplotypes were scored amongst all specimens, with five new haplotypes discovered in the Italian populations of *F. pratensis* (Table 2).

The maximum likelihood tree obtained on the haplotypes of both species (Fig. 1) was comparable to the neighbour-joining tree by Goropashnaya et al. (2004b) for the *F. pratensis* clade. Two major, well-supported clades were identified (bootstrap > 90), separating haplotypes belonging to *F. pratensis* and *F. lugubris* with few exceptions. As in Goropashnaya et al. (2004b), few haplotypes (H2, H3, H4, and H6) found in populations from the Urals and the Pyrenees, morphologically identified as *F. pratensis,* clustered within the *F. lugubris* clade, and one haplotype (H5) was found to be shared between populations of both species. Concerning the Italian populations, all haplotypes clustered in a strongly supported clade (bootstrap = 100), which was found in sister relation to all other Eurasian *F. pratensis* haplotypes (Fig. 1). Within this clade, no clear distinctions could be found between populations from the Alps (haplotypes H49 and H50) and the Apennines (haplotypes H49, H51, H52 and H53), with haplotype H49 being shared between populations sampled on the two mountain ranges.

The median-joining network showed a clear distinction between the *F. pratensis* and the *F. lugubris* haplotype groups (Fig. 2), differentiated by at least seven nucleotide substitutions. The *F. lugubris* haplotypes were organised in a star-like sub-network, with haplotype H5 in a central position. This haplotype was scored in populations from Russia, Sweden, and the Pyrenees, but also in *F. pratensis* populations from the Urals. Moreover, haplotypes H2, H3 and H4, belonging to *F. pratensis* specimens from the Pyrenees, and haplotype H6, belonging to *F. pratensis* specimens from Urals, were nested into the *F. lugubris* group, as already evidenced in the phylogenetic tree (Fig. 1). The remaining *F. pratensis* haplotypes, on the other hand, were included in a sub-network showing a more branched pattern. Here, the Italian haplotypes (coloured circles in Fig. 2) appeared to form a distinct group from the other Eurasian ones, being differentiated by a high number of substitutions and several missing haplotypes inferred by the median-joining method.

Surprisingly, Central Apennine populations seemed closer to the Alpine populations than to the ones in the Northern Apennines (Fig. 3). Haplotype H49 was indeed shared by the Central Apennine populations from Barrea and Scanno and the population from the Alps (Fig. 3). Regarding the Northern Apennine populations, haplotype H51 was shared by all the localities, whilst haplotypes H52 and H53 were private for the populations of Ticchiano and Vallombrosa, respectively. Haplotype H50 was found only in the Alpine population.

We evaluated the level of divergence, considering the genetic distances amongst the Eurasian *F. pratensis* haplotypes and the Italian haplotypes group. The genetic distance was higher in the European cluster ($d = 0.00637$) compared to the Italian ones ($d = 0.00126$), as expected considering the different number of haplotypes in the two groups and the widespread geographical distribution. Interestingly, the genetic distance between the two groups ($d = 0.0104$) resulted higher than the within-group, underlining a divergence of the Italian populations from all the others.

Discussion

The phylogeography and demographical history of Italian *Formica pratensis* populations were examined and compared with the Eurasian haplotypes dataset (Goropashnaya et al. 2004b). We included in our analysis also the published *F. lugubris* haplotypes, given the close relationship between these two species, and the already reported genetic evidence for past hybridization events (Goropashnaya et al. 2004b). The analyses based on a 1.5-kilobase mitochondrial DNA fragment, including the Cyt-b gene and part of the ND-6 gene, suggested no evidence of past or recent mitochondrial DNA exchange between Italian *F. pratensis* and *F. lugubris*. The exchanges with other species are difficult in the Apennines, where *F. pratensis* is the only RWA species naturally occurring in these areas since the last glaciation. Interactions with other introduced RWAs (mainly *F. paralugubris*), although possible, have never been documented and, in our opinion, are unlikely. In the Foreste Casentinesi National Park (Northern Tuscany), where one of the largest introduction campaigns was carried out in the past and there are several extant

Table 2 List of the scored haplotypes. Concerning the Italian populations, the number of individuals of each population that own the given haplotype is reported in brackets

Haplotype	Species	Localities	Ref.seq database
H1	*F. pratensis*	Finland	AY584199.1
H2	*F. pratensis*	Pyrenees	AY584232.1
H3	*F. pratensis*	Pyrenees	AY584231.1
H4	*F. pratensis*	Pyrenees	AY584230.1
H5	*F. pratensis-F.lugubris*	Urals + Sweden + Russia + Pyrenees	AY573860.1
H6	*F. pratensis*	Urals	AY584227.1
H7	*F. pratensis*	Sweden	AY584226.1
H8	*F. pratensis*	Sweden	AY584225.1
H9	*F. pratensis*	Sweden	AY584224.1
H10	*F. pratensis*	Romania	AY584223.1
H11	*F. pratensis*	Romania	AY584222.1
H12	*F. pratensis*	Russia	AY584221.1
H13	*F. pratensis*	Russia	AY584220.1
H14	*F. pratensis*	Sweden	AY584219.1
H15	*F. pratensis*	Urals	AY584218.1
H16	*F. pratensis*	Urals	AY584217.1
H17	*F. pratensis*	Finland	AY584216.1
H18	*F. pratensis*	Sweden	AY584215.1
H19	*F. pratensis*	Finland	AY584214.1
H20	*F. pratensis*	Finland + Russia	AY584212.1
H21	*F. pratensis*	Urals	AY584211.1
H22	*F. pratensis*	Russia	AY584209.1
H23	*F. pratensis*	Urals	AY584208.1
H24	*F. pratensis*	Urals	AY584207.1
H25	*F. pratensis*	Urals	AY584206.1
H26	*F. pratensis*	Russia	AY584203.1
H27	*F. pratensis*	Sweden	AY584202.1
H28	*F. pratensis*	Denmark	AY584201.1
H29	*F. pratensis*	Russia	AY584198.1
H30	*F. pratensis*	Finland	AY584197.1
H31	*F. pratensis*	Finland	AY584196.1
H32	*F. lugubris*	Russia + Urals	AY573873.1
H33	*F. lugubris*	Sweden + Switzerland	AY573870.1
H34	*F. lugubris*	Russia	AY573866.1
H35	*F. lugubris*	Pyrenees	AY573874.1
H36	*F. lugubris*	Russia	AY573872.1
H37	*F. lugubris*	Urals	AY573871.1
H38	*F. lugubris*	UK	AY573869.1
H39	*F. lugubris*	UK	AY573868.1
H40	*F. lugubris*	UK	AY573867.1
H41	*F. lugubris*	Urals	AY573865.1
H42	*F. lugubris*	Urals	AY573864.1
H43	*F. lugubris*	Russia	AY573863.1
H44	*F. lugubris*	Russia	AY573862.1
H45	*F. lugubris*	Sweden	AY573861.1
H46	*F. lugubris*	Sweden	AY573859.1
H47	*F. lugubris*	Sweden	AY573858.1
H48	*F. lugubris*	Pyrenees	AY573856.1
H49	*F. pratensis*	Italy: BAR (6), BOL (1), SCA (6)	this study
H50	*F. pratensis*	Italy: BOL(5)	this study

Table 2 (continued)

Haplotype	Species	Localities	Ref.seq database
H51	*F. pratensis*	Italy: CO (6), LV (6), ORE (6), TIC (5), VAL (5)	this study
H52	*F. pratensis*	Italy: TIC (1)	this study
H53	*F. pratensis*	Italy: VAL (1)	this study

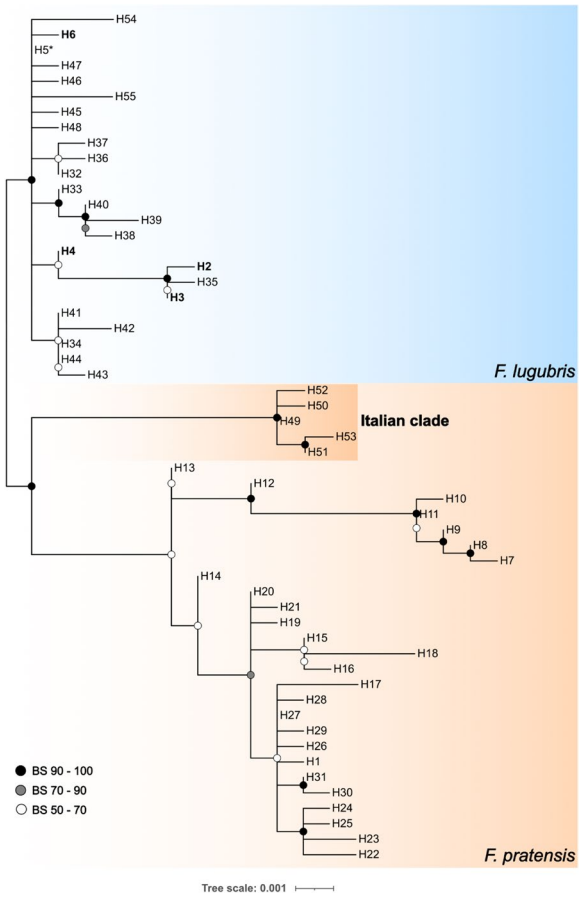

Fig. 1 Maximum likelihood tree of *Formica pratensis* and *F. lugubris* built using IQ-TREE with 1000 ultrafast bootstrap replicates. *BS* bootstrap values. Haplotypes in bold were detected in populations morphologically identified as *F. pratensis* but clustered with haplotypes of *F. lugubris*. The haplotype H5 marked with a * was shared by populations belonging to both species

F. paralugubris populations, repeated surveys of the area found no evidence of contact between the two species. The closest known *F. pratensis* population is the one in La Verna (Province of Arezzo), more than 20 km apart. Moreover, the two species have different environmental requirements, grasslands for *F. pratensis*, closed canopy stands for *F. paralugubris*, which further limits the possibility of contact. None of the other *F. pratensis* populations used in this study have other RWAs populations in their proximity. Considering the Alpine range, where several RWA species live in sympatry and such events are more likely to occur (Bernasconi et al. 2011), we sampled only one population; therefore, future studies with a more extensive sampling are needed to confirm this result.

An interesting outcome concerns the monophyletic clade that characterised the Italian samples, supported by both phylogenetic analyses, with maximum bootstrap support, and the haplotype network. Alpine and Apennine *F. pratensis* haplotypes clustered in the same clade, and the haplotype H49 was shared by the two groups. The phylogeographic separation of the Italian population from the other Eurasian ones implied a different vicariant history, with the divergence dating before the diversification of the other Eurasian populations into the suggested western and eastern clades, and in any case in the last glacial period (Desalle et al. 1987). The current western distribution of *F. pratensis* has been hypothesised to belong to populations that survived the glaciations in the Carpathian Mountains refugia (Sumegi & Krolopp 2002), but this theory is only supported by the analysis of a few haplotypes from Romania and Sweden (Goropashnaya et al. 2004a). Conversely, we did not find any genetic evidence (i.e. shared haplotype) that supports a potential proximity of the Italian cluster with the Western one. Instead, the genetic distance of the first group from the other Eurasian populations suggested a possible different scenario: both the Alps, especially the south-western part, and Apennine areas may represent other possible glacial refugia for this species as it was reported for many animals (Schebeck et al. 2019; Korábek et al. 2023) and plants (Záveská et al. 2021; Parisod 2022). The continuous S-shaped mountain-hill system comprising both mountain chains (37° to 48° of latitude) encompasses the glacial refugia of the Italian Peninsula (Dapporto et al., 2019) and mountain areas covered by ice caps during glacial maxima (Menchetti et al. 2021). This area is considered a single biogeographic unit for many endemisms (Petit et al. 2003; Drovetski et al. 2018; but see Menchetti et al. 2021) and our results agreed with this notion. Furthermore, the haplotypes from the Alps and the Apennines showed high genetic proximity, with one shared haplotype, suggesting an ancient (Pleistocene) wide distribution of this species across all these areas. Moreover, after the last glaciation, the physical barrier of the Alps may have limited the northward dispersal exchanges with Central European populations (Hewitt 1999; Drovetski

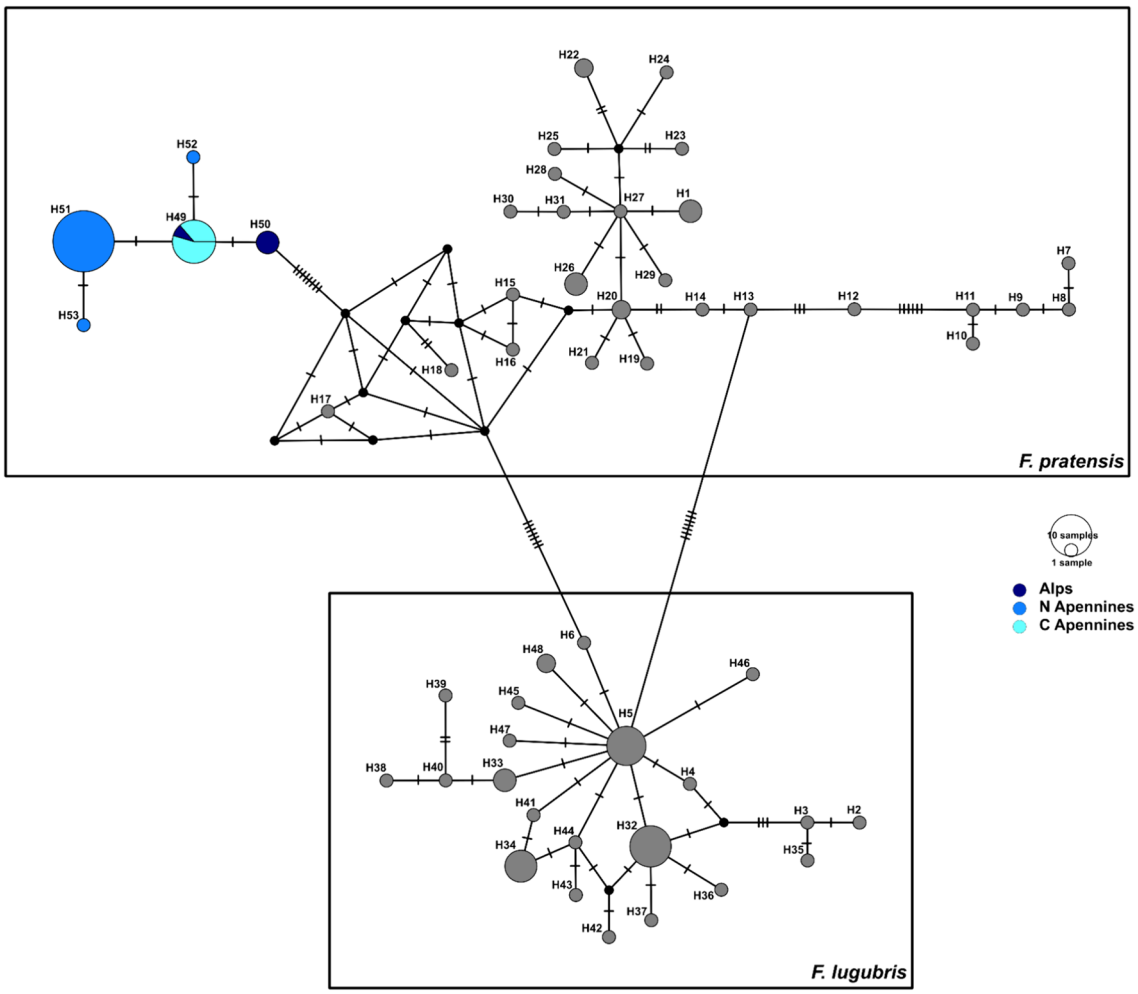

Fig. 2 Median-joining network of *Formica pratensis* and *F. lugubris* built with popART. Italian haplotypes are coloured in different shades of blue according to mountain range of origin. Black circles represent inferred missing haplotypes, whilst the number of small transversal dashes on the lines connecting two haplotypes represents the number of nucleotide substitutions differentiating them

et al. 2018), thus isolating the Italian *F. pratensis* populations from the other European ones, as suggested by the lack of haplotypes shared between these groups.

Considering the genetic structure of Apennine samples (Fig. 3), no haplotypes were shared between Northern and Central populations, but the haplotype H52 from Ticchiano was genetically closer to the Central group than to its native group, underlining the sharing of common ancestral lineages and the wide distribution along the inter-refugium areas of these species. The low interpopulation genetic distance amongst these groups (one missing haplotype) can suggest possible similar post-glacial evolutionary history and selective process acting on these two groups, which can explain the geographical patterns recorded. This partly contrasts with the evolutionary dynamics recorded in these areas for other cold-adapted insect species (Martín-Bravo et al 2010; Lecocq et al. 2013), which experienced more intense intraspecific differentiation processes leading sometimes to speciation events, as happened for *Bombus monticola mathildis* and *B. konradini* (Martinet et al 2018).

In addition, the diversity we recorded is greatly influenced by the actual fragmentation of *F. pratensis* populations because of the rapid decline that this species has been experiencing for many decades in Italy (e.g. at the time we are submitting this article, the population sampled in Vallombrosa has gone extinct, personal observation) and across the entire Eurasian range (Kiss and Kóbori 2010; Stockan et al. 2016; Çamlıtepe and Asksoy 2019). The same situation was recorded in the last 50 years also for other RWA species, with agricultural activities, industrialization and habitat fragmentation representing the main threats (Mäki-Petäys and Breen 2007; Dekoninck et al. 2010), together with climate change (Sorvari 2016). In 1996 the International Union for Conservation of Nature (IUCN), listed five RWA species (*F. rufa*, *F. lugubris, F. polyctena, F. aquilonia*, and *F. pratensis* (plus *F. uralensis*)) as Near Threatened at a global

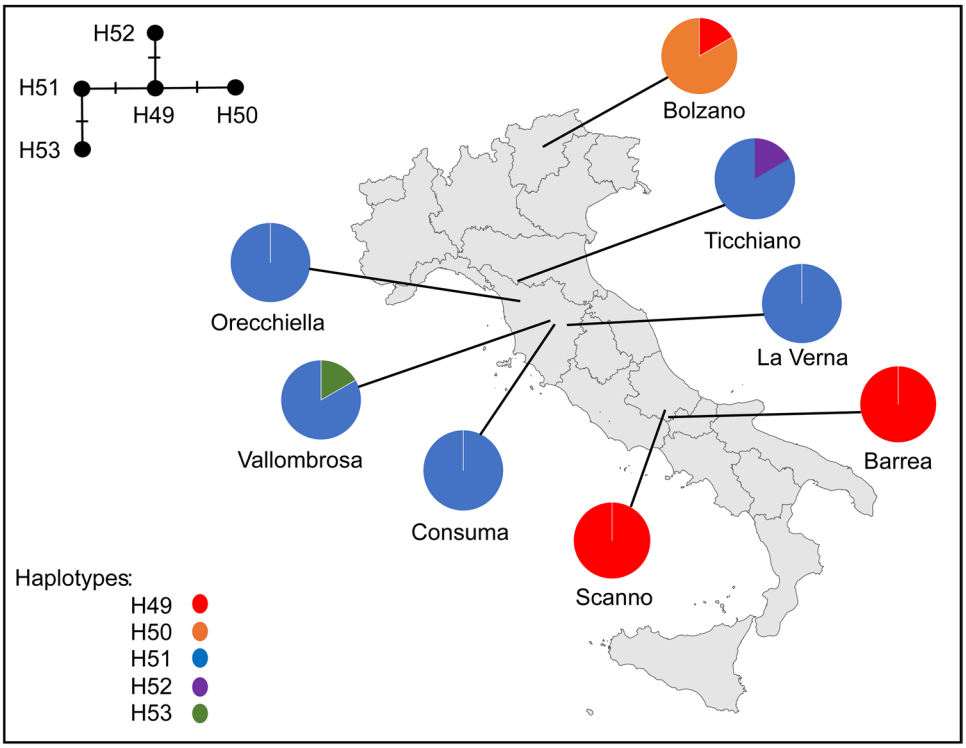

Fig. 3 Haplotypes distribution maps and network of Italian *Formica pratensis* populations. The different colours represent the haplotypes ID whilst the pie chart portions their frequencies ($n = 6$ individuals x populations)

level. However, after more than 20 years, no further official assessment with species status updating was carried out, or any other RWA species listed (IUCN Red List 2023). It is therefore necessary to take rigorous measures to protect and facilitate the survival and dispersal of this and the other RWA species (Balzani et al. 2022b).

In conclusion, the results of this study provided a description of the phylogeographic relationship amongst Italian *F. pratensis* populations and those from Central and North Europe. The results also generated several open-ended questions, answering which will require the extension of the sample size, with more populations from the Alps and intermediate areas between Italy and Europe like Slovacchia, Germany, Poland, but also a different approach. In particular it might be useful to employ different genetic markers to screen both mitochondrial and nuclear DNA to better detect the genetic diversity amongst and between these populations, which is crucial for understanding species genetic structure and defining evolutionary and management units for future conservation planning.

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Data availability The sequences generated and analysed in the present study are available from NCBI database.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interest to disclose.

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