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# Complex population genetic structure of the bark beetle predator *Thanasimus formicarius* (L.) (Coleoptera: Cleridae) across its European range

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## Abstract

1. *Thanasimus formicarius* (L.) (Coleoptera: Cleridae) is an important bark beetle predator and can reduce bark beetle population densities of some of the most severe forest pests in Europe.
2. We analysed the population genetics and phylogeography of *T. formicarius* across its European range, using mitochondrial COI data from 187 individuals sampled

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from 23 locations. Our extensive sampling from the Fennoscandian to the Mediterranean region and from Iberia to the Middle East provides insights into the genetic structure of *T. formicarius*.

3. We found a high genetic diversity, revealing 119 haplotypes across the sampling range. Two main clades, an Atlantic and a Continental, were identified, suggesting the presence of at least two glacial refugia during the last ice ages.
4. An isolated population was discovered on the island of Corsica, suggesting that a limited number of individuals, probably from mainland France, may have colonized Corsica and a founder effect occurred.
5. These findings increase our understanding of the recent evolutionary history of *T. formicarius*, which was shaped by Pleistocene events and certain species-specific life-history traits.

**KEYWORDS**
ant beetle, Cleridae, COI, glacial refugia, natural enemy, phylogeography, Pleistocene, population genetics

## INTRODUCTION

The ant beetle *Thanasimus formicarius* (L.) is one of the most relevant, abundant and well-studied bark beetle predators in Europe (Heidger, 1995; Hilszczański et al., 2007; Kenis et al., 2004; Schroeder, 1999b). It preys on over 20 species across 8 genera, including spruce bark beetles, such as *Dendroctonus micans* (Kug.) (Coleoptera: Curculionidae), *Ips typographus* (L.) (Coleoptera: Curculionidae) and *Pityogenes chalcographus* (L.) (Coleoptera: Curculionidae), as well as pine bark beetles, such as *Ips acuminatus* (Gyll.) (Coleoptera: Curculionidae), *Ips sexdentatus* (Börner) (Coleoptera: Curculionidae), *Orthotomicus erosus* (Woll.) (Coleoptera: Curculionidae), *Tomicus minor* (Hart.) (Coleoptera: Curculionidae) and *Tomicus piniperda* (L.) (Coleoptera: Curculionidae), and bark beetles of deciduous trees (Gauss, 1954; Kenis et al., 2004). Thus, *T. formicarius* is a natural enemy of some of the most severe forest pests in Europe. Although the prey of *T. formicarius* breeds in spruce, pine and broadleaf trees, *T. formicarius* is most abundant in pine stands (Thomaes et al., 2017; Warzée, 2005; Warzée & Grégoire, 2003), probably because it prefers *Pinus* species for pupation (Warzée, 2005).

The ant beetle is native to Eurasia and was intentionally introduced to North America in 1892 and in 1998 for the biological control of *Dendroctonus frontalis* (Zimm.) (Coleoptera: Curculionidae) and *T. piniperda* (Klimaszewski et al., 2017; Niehuis, 2013; Opitz, 2002; Simpson et al., 2022). Since 2006, *T. formicarius* has been reared in laboratories and released as third-instar larva for the biological control of bark beetles in Turkey (Akyol & Sarikaya, 2017), and since 2018 also in Ukraine (Meshkova et al., 2021). Otherwise, the role of human-mediated dispersal in *T. formicarius* is not clear, but there is a possibility that *T. formicarius* has been dispersed unintentionally by human activities, e.g., via timber transport (larvae in bark) or hitchhiking on vehicles, as it has been described for other insects (Gippet et al., 2019). Both larvae and adults of *T. formicarius* feed on larvae and adults of bark beetles and can reduce the brood of *T. piniperda* by over 80% (Schroeder, 1996) and of *I. typographus* between 18% (Mills, 1985) and 60% (Weslien, 1994), indicating a significant effect on bark beetle population densities. Females of *T. formicarius* can lay between 106 (Heidger, 1995) and 439 eggs (Schlup, 1987), while females of *I. typographus* deposit up to 80 eggs (Schebeck et al., 2023; Wermelinger, 2004), making *T. formicarius* more fertile than some of its prey species. *Thanasimus formicarius* predatory rates combined with its high fecundity, mobility (adults have been described as highly mobile and as good dispersers) and long lifespan (adults live up to 10 months) make it an effective predator of bark beetles (Gauss, 1954; Heidger, 1995; Kenis et al., 2004; Niehuis, 2013; Schlup, 1987; Schroeder, 1999b). There is no specific information on sex-biased dispersal in *T. formicarius*. However, equal sex ratios (47%–50% males) observed in traps baited with *I. typographus* pheromones or alpha-pinene + ethanol over two years (Schroeder, 2003) suggests that both males and females have a high dispersal capacity and that there is no skewed dispersal. Attracted by tree volatiles and bark beetle pheromones, *T. formicarius* adults approach trees and feed on adult bark beetles on the tree surface (Bakke & Kvamme, 1978, 1981; Kohnle & Vité, 1984; Tømmerås, 1985, 1988; Ye, 1998). After reproduction, females lay eggs in bark crevices of bark beetle-infested trees, and larvae feed on bark beetle offspring below the bark (Kenis et al., 2004; Schroeder, 1999b; Ye, 1998).

Adults of *T. formicarius* are active from early spring to late summer and can live for several months, allowing them to prey on numerous species with different phenologies (Schroeder, 2003; Schroeder & Dalin, 2017; Ye, 1998). Depending on climatic conditions, the development of *T. formicarius* in Europe takes one to two and a half years, with shorter generation times in lower latitudes and elevations (Dippel et al., 1997; Heidger, 1994; Schroeder & Dalin, 2017). *Thanasimus formicarius* overwinters as larva, pre-pupa or adult under the bark of bark beetle-infested pine or spruce trees, or in the forest litter (Schroeder & Dalin, 2017; Warzée & Grégoire, 2003). Univoltine

individuals pupate in late summer and overwinter as adults, followed by dispersal flights in early spring, which last several months (Schroeder, 1999a, 2003; Schroeder & Dalin, 2017). Semivoltine individuals overwinter as larvae or pre-pupae, and pupate in late summer or autumn of the following year; adults overwinter and mate in spring of the third year (Schroeder, 1999b; Schroeder & Dalin, 2017).

The biology and ecology of *T. formicarius*, e.g., kairomone response, flight period or interactions with prey, have been extensively studied (Bakke & Kvamme, 1978, 1981; Gauss, 1954; Heidger, 1995; Kenis et al., 2004; Mills, 1985; Schroeder, 1999b), whereas its phylogeography and population genetics have only been investigated once by Gerstmeier et al. in 2019. The authors focused on the species status of European *Thanasimus* spp., i.e., *Thanasimus pectoralis* (Fuss) (Coleoptera: Cleridae), *Thanasimus rufipes* (Brahm) (Coleoptera: Cleridae), *Thanasimus femoralis* (Zett.) (Coleoptera: Cleridae) and *T. formicarius*, therefore only a limited number of *T. formicarius* individuals were studied (Gerstmeier et al., 2019). In general, data on the phylogeography and population genetic structure can give us insights into a species' recent evolutionary history, population dynamics and dispersal activities (Bertheau et al., 2013; Mayer et al., 2015; Sallé et al., 2007). This is of particular interest for an important bark beetle antagonist such as *T. formicarius*, as it provides insights into its species identity, genetic diversity, population genetic structure and adaptive potential, which is useful for biodiversity monitoring and can optimize release strategies.

Pleistocene cycles of glacial and interglacial periods shaped the phylogeography and population genetic structure of many species. During the last glacial maximum (LGM), which occurred about 20,000 years ago, most of Europe was covered by ice and permafrost, making large parts of the continent uninhabitable for many animal and plant species (Clark et al., 2009; Ehlers et al., 2018; Ehlers & Gibbard, 2003; Habel et al., 2010; Hewitt, 1996; Hewitt, 2000; Mix et al., 2001; Schmitt, 2007; Taberlet et al., 1998). Organisms migrated to glacial refugia, life-sustaining and ice-free areas, such as the Apennine Peninsula, the Iberian Peninsula, the Carpathian Mountains or the Balkan Peninsula (Habel et al., 2010; Hewitt, 1999; Schmitt & Varga, 2012; Varga, 2009). The retraction to geographically distinct and isolated refugia was accompanied by limited gene flow and genetic differentiation among populations. Following the last ice age, which ended about 12,000 years ago, the entire continent became inhabitable again, and species spread across Europe, facilitating genetic exchange among formerly isolated populations (Habel et al., 2010; Hewitt, 1996, 2000; Taberlet et al., 1998; Willis et al., 2004). In addition to Pleistocene events, certain life-history traits, such as a strong host plant dependency, predator–prey relationships and dispersal capacity, can affect a species' genetic structure (Barbosa et al., 2012; Coll et al., 1994; Hewitt, 1996; Omondi et al., 2011; Sallé et al., 2007; Schmitt, 2007; Stauffer et al., 1999; Tavares et al., 2015).

The population genetic structure and phylogeography of the ant beetle *T. formicarius* was probably affected by Pleistocene events, its strong dependency on prey and host tree species, and its high mobility (Barbosa et al., 2012; Gerstmeier et al., 2019; Omondi et al., 2011; Schroeder, 1999b). By analysing mitochondrial COI data of *T. formicarius*, Gerstmeier et al. (2019) found a high genetic diversity and an Atlantic-Continental structuring across Europe. However, the study by Gerstmeier et al. (2019) was limited by its small sample size, as they analysed 27 *T. formicarius* individuals and the Atlantic clade consisted of only one individual from southern England. As *T. formicarius* is highly dependent on its major prey and host trees, it might have shared certain glacial refugia with these species. The evolutionary history of *T. formicarius*' prey, i.e., economically and ecologically relevant bark beetle species such as *I. typographus*, *P. chalcographus* or *T. piniperda*, has been intensively studied (Avtzis et al., 2008; Bertheau et al., 2013; de Becquevort et al., 2024; Horn et al., 2009; Kerdelhué et al., 2006; Krascsenitsová et al., 2013; Mayer et al., 2015; Papek et al., 2024; Ritzerow et al., 2004; Schebeck et al., 2018, 2023). For example, the spruce bark beetle *I. typographus* has a shallow population genetic structure and the exact number and location of major glacial refugia is unknown, but two small glacial refugia in the Carpathians have been described. It is proposed that it shared refugial areas with its main host tree species *P. abies*, e.g., the Apennines, the Carpathians, the Balkan Peninsula or the Russian plain (Bertheau et al., 2013; Krascsenitsová et al., 2013; Mayer et al., 2015; Papek et al., 2024; Schebeck et al., 2023). Compared to *I. typographus*, *P. chalcographus* has a clearer genetic structure; its main glacial refugia were the Russian plain, the Carpathians, the Apennines and the Dinaric Alps (Avtzis et al., 2008; Bertheau et al., 2013; Schebeck et al., 2018, 2019, 2023). Research on the population genetics of the pine bark beetle *T. piniperda* in continental Europe and the United Kingdom has shown high genetic heterogeneity and several glacial refugia (shared with its main hosts *Pinus* spp.) have been proposed, e.g., in the Iberian Peninsula, North-Central Europe, the Apennine Peninsula, in the Balkan area and in Scotland (de Becquevort et al., 2024; Horn et al., 2009; Kerdelhué et al., 2006; Ritzerow et al., 2004). Hence, these above-mentioned refugial areas can also be hypothesised for *T. formicarius*.

Here, we analysed the population genetic structure and phylogeography of the ant beetle *T. formicarius* across its European range, covering a large part of the continent. A total of 187 individuals from 23 locations were sampled and a fragment of the mitochondrial COI gene was analysed to investigate its glacial and post-glacial history. In addition, we focused on the presence of an Atlantic and a Continental clade in European *T. formicarius* as proposed by Gerstmeier et al. (2019). Our large-scale phylogeographic analysis of the important bark beetle antagonist *T. formicarius* provides information on its recent evolutionary history and can be used to infer certain life history traits that help to assess its potential to control bark beetle populations.

## METHODS

### Sampling of beetles

To analyse the phylogeography, genetic structure and recent evolutionary history of *T. formicarius* across the species' European range,

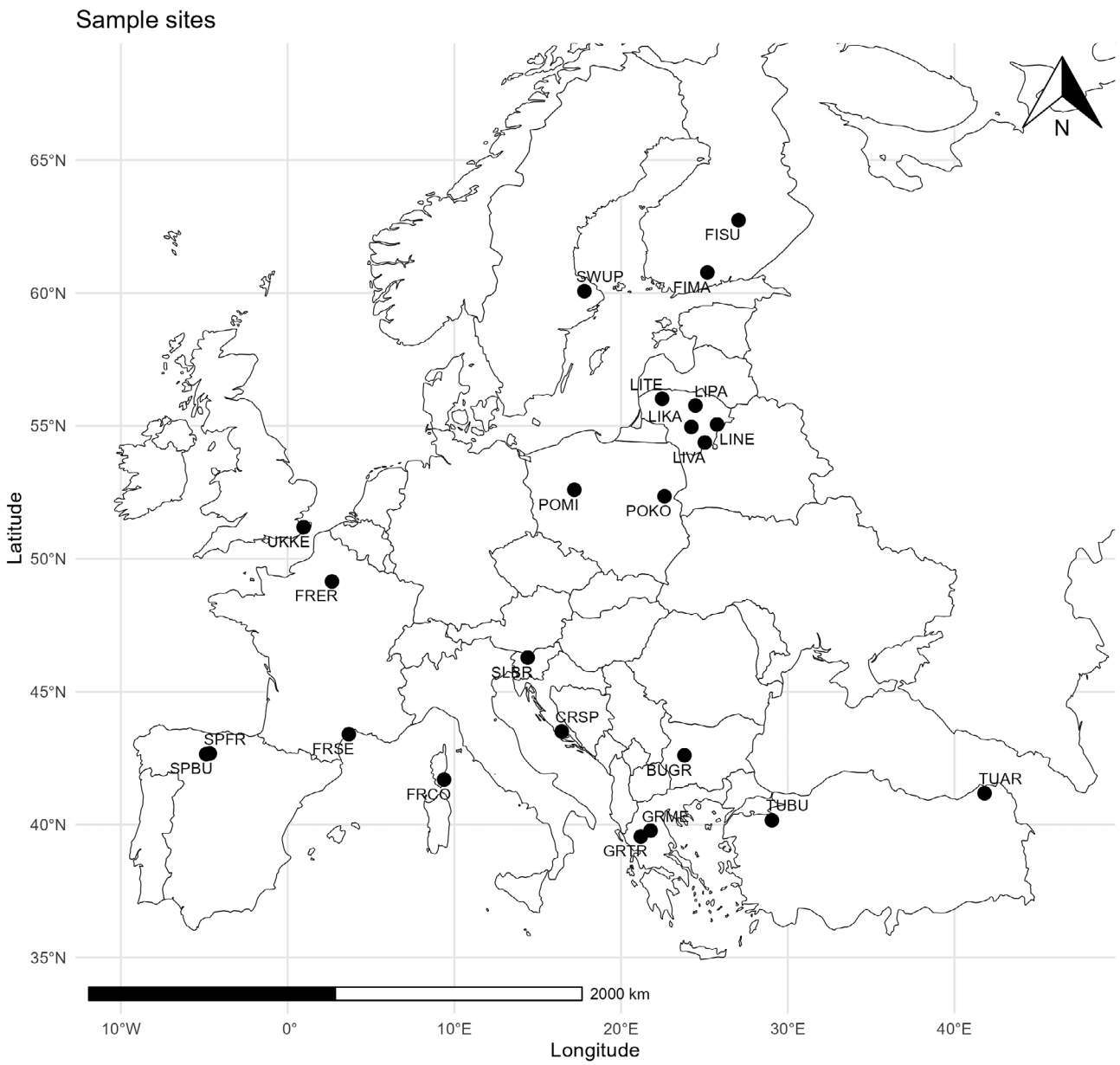


**FIGURE 1** Sampling locations of *Thanasimus formicarius*, covering a large part of the species' European range, see Table 1 for details.

beetles were collected from 23 different locations, originating from 12 countries, covering large parts of the continent (Figure 1, Table 1). Between 2015 and 2020, 2–15 individuals per location were sampled as by-catch in the course of monitoring programmes from traps baited with either bark beetle pheromones or cerambycid pheromone blends supplemented with ethanol and alpha-pinene (Table 1). Traps were filled with either water and detergent, 70% ethanol, or with no sampling liquid, and were emptied at intervals ranging from once per day to four weeks. After collection, beetles were transferred to 70%–100% ethanol, stored at −20°C and sent to BOKU University for subsequent analyses. Before DNA extraction, individuals were morphologically determined to species-level as described in Gerstmeier (1998), Thomaes et al. (2017) and Gerstmeier et al. (2019).

## DNA extraction, PCR amplification and sequencing

The collection and storage of samples highly impacted DNA quality (i.e., 70% ethanol or no sampling liquid, collection intervals of up to 4 weeks). Therefore, different approaches for DNA extraction and PCR amplification have been applied to obtain high-quality DNA. For each sample, DNA was extracted from different body parts, i.e., head, thorax muscle tissue and the whole abdomen. The GenElute Mammalian Genomic DNA miniprep kit (Sigma) was used for most DNA extractions (from 176 individuals), and the Gentra Puregene kit (Qiagen) was used for DNA extractions from 11 individuals, both according to the manufacturer's instructions. DNA was eluted in 100 μL hydration solution and stored at 4°C. For PCR amplification the DNA from the body parts with the highest DNA concentration

**TABLE 1** Overview of sampling locations, location abbreviations (either used in the main text, tables or figures, respectively) and coordinates of *Thanasimus formicarius* collections.

| Country | Location | Abbreviation | Coordinates |
|---|---|---|---|
| Bulgaria | Golema Rakovitsa | BulgariaGR/BUGR | 42.60866, 23.80232 |
| Croatia | Split | CroatiaSP/CRSP | 43.50799, 16.44020 |
| Finland | Mäntsälä | FinlandMA/FIMA | 60.77143, 25.17877 |
| Finland | Suonenjoki | FinlandSU/FISU | 62.73830, 27.04880 |
| France | Coti-Chiavari (Corsica) | FranceCO/FRCO | 41.69725, 9.38566 |
| France | Ermenonville | FranceER/FRER | 49.14983, 2.66049 |
| France | Sète | FranceSE/FRSE | 43.40469, 3.67164 |
| Greece | Metsovo | GreeceME/GRME | 39.77642, 21.768369 |
| Greece | Trikala | GreeceTR/GRTR | 39.55493, 21.182810 |
| Lithuania | Kaunas | LithuaniaKA/LIKA | 54.95950, 24.21729 |
| Lithuania | Nemencine | LithuaniaNE/LINE | 55.05451, 25.75847 |
| Lithuania | Panevezys | LithuaniaPA/LIPA | 55.76729, 24.46257 |
| Lithuania | Telsiai | LithuaniaTE/LITE | 56.02292, 22.46151 |
| Lithuania | Varena | LithuaniaVA/LIVA | 54.37582, 25.02359 |
| Poland | Korczew | PolandKO/POKO | 52.35360, 22.60469 |
| Poland | Miaczynek | PolandMI/POMI | 52.59880, 17.19760 |
| Slovenia | Brdo | SloveniaBR/SLBR | 46.28667, 14.40000 |
| Spain | Fresno del Río | SpainFR/SPFR | 42.67667, −46.66722 |
| Spain | Buenavista de Valdavia | SpainBU/SPBU | 42.65506, −48.85167 |
| Sweden | Uppland | SwedenUP/SWUP | 60.06764, 17.80256 |
| Turkey | Artvin | TurkeyAR/TUAR | 41.18270, 41.804133 |
| Turkey | Bursa | TurkeyBU/TUBU | 40.16569, 29.047052 |
| United Kingdom | Kent | UnitedKingdomKE/UKKE | 51.19247, 0.95883 |

was used, the most successful extractions were from the thorax muscle tissue or the whole abdomen using the GenElute Mammalian Genomic DNA miniprep kit (Sigma). A ~650 bp fragment of the mitochondrial COI gene was PCR-amplified using the forward primer LCO1490 and the reverse primer HCO2198 (Folmer et al., 1994). Due to differences in DNA quality, four different reaction mixes and four different PCR protocols were applied (details see in Tables S1 and S2). Each reaction mix either contained bovine serum albumin (BSA) (Farell & Alexandre, 2012; Rejili et al., 2016) or 10% trehalose (Spiess et al., 2004) to increase amplification success. All reactions were performed in a total volume of 20 μL. The most commonly used PCR reaction mix was *mix 2*, which consisted of 12.2 μL dd$H_2O$, 2 μL BSA, 2 μL 20 mM $MgCl_2$, 0.4 μL 10 mM dNTPs, 0.6 μL of each primer, 0.2 μL OneTaq (New England Biolabs) polymerase (5 U/μL) and 2 μL template DNA. The predominant PCR protocol was *protocol 2*, comprising an initial denaturation step at 95°C for 3 min, followed by four cycles of (95°C—30 s, 45°C—40 s, 72°C—60 s), then 34 cycles of (95°C—30 s, 51°C—30 s, 72°C—60 s), and followed by a final extension step at 72°C for 7 min. To confirm successful PCR amplification, PCR products were run on a 1.5% agarose gel stained with Gel Red Nucleic Acid Stain (Biotinum). The PCR products with the highest DNA concentrations (estimated using NanoDrop 2000C) were both cleaned and Sanger-sequenced (in forward direction) by an external provider (Eurofins Genomics, Vienna, Austria). Singleton sequences were confirmed by an additional PCR run and re-sequencing in forward and reverse direction, to eliminate the presence of artefacts, i.e., errors during PCR amplification or sequencing runs.

## Data analysis

The quality of all sequences (unambiguous peaks, no double peaks) was visually examined in Chromas 2.6.6 (Technelysium, 2018). These high-quality sequences were trimmed to equal fragment length using GeneRunner 6.5.52x64 Beta (Buquicchio & Spruyt, 2019), and aligned with ClustalW implemented in MEGA X (Kumar et al., 2018). Haplotypes and haplotype distribution were estimated in R Studio 2023.09.1 (R-Coreteam, 2023) using the package 'haplotypes' (Aktas, 2020). Maps (sample locations and haplotype distribution) were plotted in R Studio 2023.09.1 (R-Core team, 2023) using the packages 'rnaturalearth' and 'ggplot2' (South et al., 2024; Wickham, 2011). Nucleotide diversity and haplotype diversity were calculated in DnaSP 6.12 (Rozas et al., 2017). The number of parsimony-informative sites was calculated in POPART 3 (Leigh & Bryant, 2015). The evolutionary divergence between haplotypes (pairwise distances) and the overall mean distance between haplotypes

**TABLE 2** Sample sizes (*n*), distribution of haplotypes and intra-population diversity indices (nucleotide diversity and standard deviation [$\pi$ ± SD], and haplotype diversity and standard deviation [Hd ± SD]) of European *Thanasimus formicarius*.

| Location | Sample size (*n*) | Number of haplotypes | Haplotypes | $\pi$ ± SD | Hd ± SD |
|---|---|---|---|---|---|
| BUGR | 6 | 5 | H7, H28, H33, H34, H93 | 0.00843 ± 0.00253 | 0.933 ± 0.122 |
| CRSP | 8 | 4 | H27, H35, H36, H94 | 0.01209 ± 0.00357 | 0.821 ± 0.101 |
| FIMA | 11 | 7 | H13, H37, H38, H39, H40, H41, H42 | 0.00272 ± 0.00052 | 0.873 ± 0.089 |
| FISU | 9 | 6 | H7, H13, H38, H43, H44, H45 | 0.00335 ± 0.00062 | 0.917 ± 0.073 |
| FRCO | 10 | 4 | H49, H50, H51, H52 | 0.00156 ± 0.00067 | 0.533 ± 0.180 |
| FRER | 7 | 5 | H46, H48, H97, H98, H99 | 0.03947 ± 0.01037 | 0.905 ± 0.103 |
| FRSE | 9 | 5 | H46, H47, H48, H95, H96 | 0.04621 ± 0.00566 | 0.861 ± 0.087 |
| GRME | 7 | 7 | H1, H2, H3, H4, H5, H6, H7 | 0.00649 ± 0.00081 | 1.000 ± 0.076 |
| GRTR | 5 | 5 | H100, H101, H102, H103, H104 | 0.01829 ± 0.00351 | 1.000 ± 0.126 |
| LIKA | 10 | 10 | H7, H8, H9, H10, H11, H12, H13, H14, H75, H76 | 0.00938 ± 0.00209 | 1.000 ± 0.045 |
| LINE | 8 | 7 | H7, H13, H15, H16, H17, H77, H78 | 0.01202 ± 0.00294 | 0.964 ± 0.077 |
| LIPA | 11 | 9 | H7, H13, H18, H19, H20, H21, H22, H23, H78 | 0.00913 ± 0.00212 | 0.945 ± 0.066 |
| LITE | 4 | 4 | H24, H25, H26, H76 | 0.01362 ± 0.00387 | 1.000 ± 0.177 |
| LIVA | 5 | 4 | H7, H27, H28, H76 | 0.00739 ± 0.00333 | 0.900 ± 0.161 |
| POKO | 2 | 2 | H57, H58 | 0.00973 ± 0.00486 | 1.000 ± 0.500 |
| POMI | 7 | 7 | H7, H13, H42, H53, H54, H55, H56 | 0.01149 ± 0.00400 | 1.000 ± 0.076 |
| SLBR | 9 | 9 | H31, H59, H60, H61, H62, H63, H64, H65, H66 | 0.01211 ± 0.00338 | 1.000 ± 0.052 |
| SPBU | 11 | 9 | H105, H107, H108, H111, H112, H113, H114, H115, H116 | 0.01677 ± 0.00238 | 0.945 ± 0.066 |
| SPFR | 10 | 6 | H105, H106, H107, H108, H109, H110 | 0.01608 ± 0.00243 | 0.911 ± 0.062 |
| SWUP | 15 | 12 | H7, H29, H30, H31, H32, H67, H68, H69, H70, H71, H72, H73 | 0.00530 ± 0.00071 | 0.962 ± 0.040 |
| TUAR | 5 | 5 | H79, H80, H81, H82, H83 | 0.02840 ± 0.00518 | 1.000 ± 0.126 |
| TUBU | 13 | 10 | H79, H84, H85, H86, H87, H88, H89, H90, H91, H92 | 0.00718 ± 0,00096 | 0.962 ± 0.041 |
| UKKE | 5 | 5 | H48, H74, H117, H118, H119 | 0.00623 ± 0.00142 | 1.000 ± 0.126 |
| Total | 187 | 119 | | 0.03064 ± 0.00189 | 0.985 ± 0.004 |

were calculated in MEGA X using the Tajima-Nei model by iterating 1000 bootstrap replicates (Kumar et al., 2018; Tajima & Nei, 1984). To visualize the relationships between haplotypes, a minimum spanning haplotype network was constructed in POPART 3 (Leigh & Bryant, 2015).

The relationships among haplotypes were reconstructed in MEGA X, using the Neighbour Joining (NJ) method and the Maximum Likelihood (ML) method. The evolutionary distances in the NJ tree were calculated using the Tajima-Nei method, using 1000 bootstrap iterations (Felsenstein, 1985; Kumar et al., 2018; Saitou & Nei, 1987; Tajima & Nei, 1984). The evolutionary distances in the ML tree were determined using the ML method and the Tamura–Nei model; relationships among haplotypes were calculated using 1000 bootstrap replicates and the tree with the highest log likelihood was shown (Felsenstein, 1985; Kumar et al., 2018; Saitou & Nei, 1987; Tajima & Nei, 1984). A COI sequence of *T. femoralis* (GenBank accession number: MZ657595.1) was used as an outgroup for the NJ and the ML tree (Roslin et al., 2022).

To determine the origin of genetic variation (among groups, among or within localities), a nested analysis of molecular variance (nested AMOVA) was calculated in POPART 3 (Leigh & Bryant, 2015). The 23 locations were clustered into three groups: western Europe/Atlantic Europe (FranceER, FranceSE, SpainFR, SpainBU and UnitedKingdomKE), Corsica (FranceCO) and rest of Europe/Continental Europe (BulgariaGR, CroatiaSP, FinlandMA, FinlandSU, GreeceME, GreeceTR, LithuaniaKA, LithuaniaNE, LithuaniaPA, LithuaniaTE, LithuaniaVA, PolandKO, PolandMI, SloveniaBR, SwedenUP, TurkeyAR and TurkeyBU) (abbreviations see Table 1). The three groups were defined by the results of the NJ and ML trees, the haplotype network and their geographic location: the western locations (Atlantic), the isolated island location Corsica and the Continental locations.

## RESULTS

The final dataset comprised 187 sequences with a length of 514 bp from 23 locations. In total, there were 100 polymorphic sites,

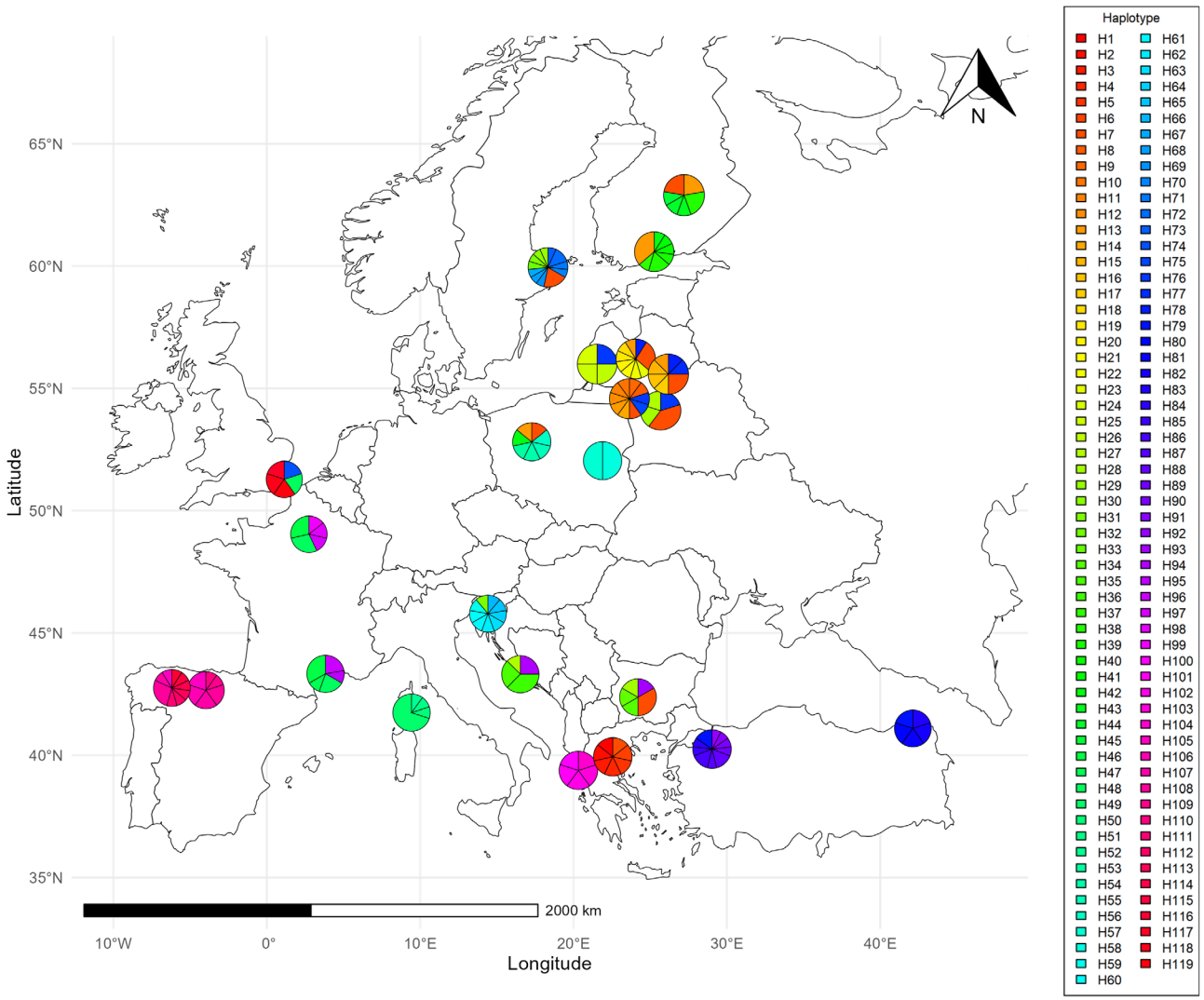


**FIGURE 2** Geographic distribution of *Thanasimus formicarius* COI haplotypes. The haplotypes are colour-coded and each pie chart shows the distribution and frequency of haplotypes per location.

22 singleton variable sites (all confirmed by and additional PCR run and re-sequencing in both directions) and 76 parsimony-informative sites, resulting in 119 different haplotypes. Among these mutations, 94.34% were transitions and 5.66% were transversions. The overall nucleotide frequencies were 36.2% Thymine, 19.0% Cytosine, 27.6% Adenine and 17.2% Guanine. No insertions or deletions were found. The overall mean distance among haplotypes, calculated from pairwise distances, was 0.03264 (±0.00422), and the pairwise distances ranged from 0.00195 to 0.08743. The overall nucleotide diversity was 0.0306 and the overall haplotype diversity was 0.985 (Table 2). The location with the lowest haplotype and nucleotide diversity was Coti-Chiavari in Corsica (FranceCO), an isolated island location, while the location with the highest nucleotide diversity was Sète (FranceSE) in mainland France. The locations with the highest haplotype diversities were GreeceME, GreeceTR, LithuaniaKA, LithuaniaTE, PolandKO, PolandMI, SloveniaBR, TurkeyAR and UnitedKingdomKE (Table 2).

The most common haplotype was H7 (9.09%), present in BulgariaGR, FinlandSU, GreeceME, LithuaniaKA, LithuaniaNE, LithuaniaPA, LithuaniaVA, PolandMI and SwedenUP, followed by H13 (5.35%), which was found in FinlandMA, FinlandSU, LithuaniaKA, LithuaniaNE, LituaniaPA and PolandMI. Both, H7 and H13 were exclusively present in the Continental European part of our study area. H49 (3.74%) was the third most abundant haplotype, although it was only present in the isolated island location Coti-Chiavari in Corsica (FranceCO) (Figure 2, Table 2). H46, H48 and H108 with 2.67% each were found in the mainland French locations (FranceER and FranceSE), in the mainland Spanish locations (SpainBU and SpainFR) and in the location in Kent (UnitedKingdomKE), i.e., in the Atlantic European part of the study area (Figure 2, Table 2). H38 (2.14%) was present in the two locations from Finland (FinlandMA and FinlandSU). The haplotypes H36, H76, H79, H105 and H107 with 1.6% each and H27, H28, H31, H35, H42, H72, H78, H86, H88, H94, H96 and H106 with 1.07%

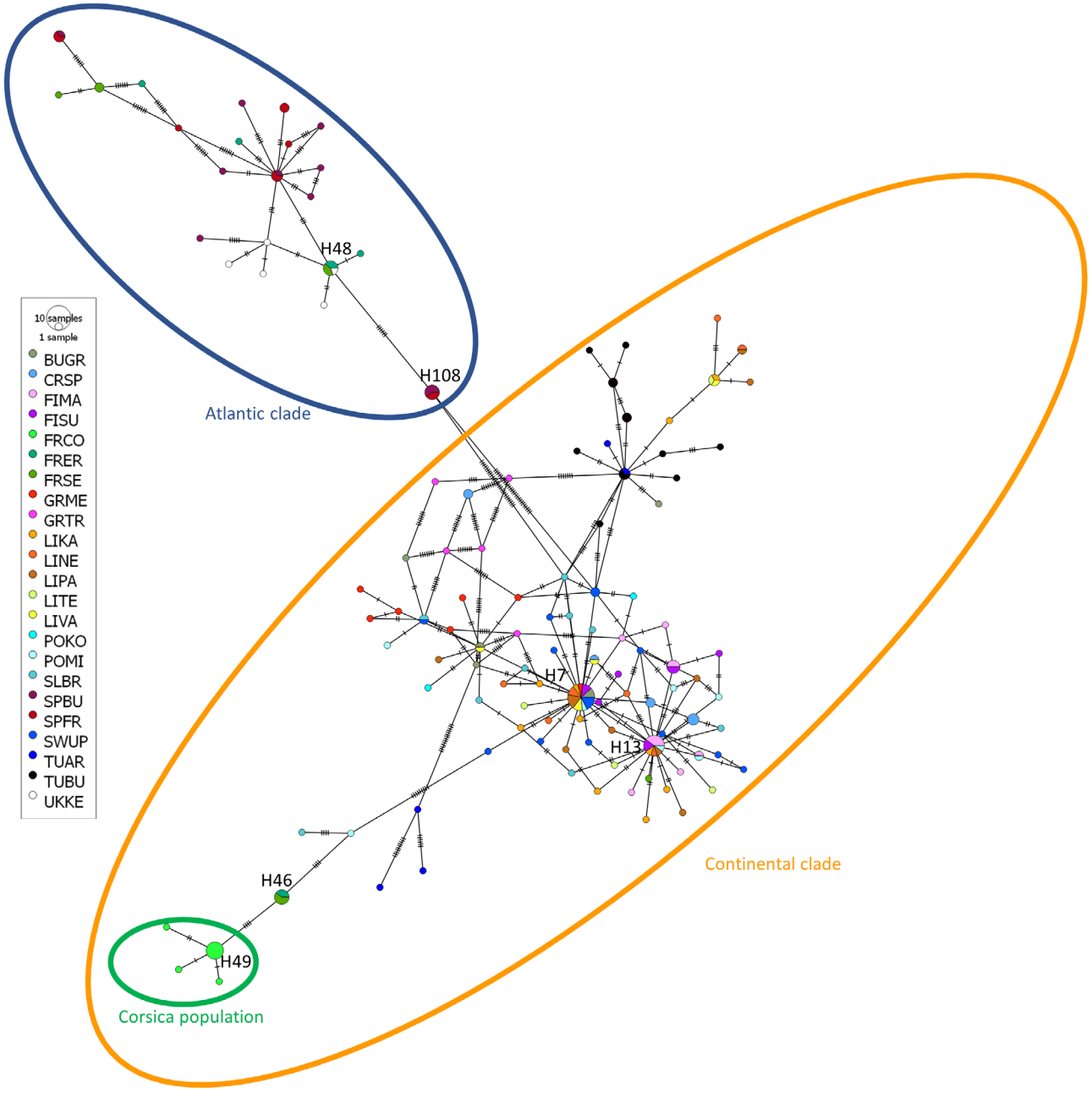


**FIGURE 3** Minimum spanning haplotype network of *Thanasimus formicarius* mitochondrial COI haplotypes. The discs are colour-coded (based on location) and the diameter of discs is proportional to haplotype frequency. The haplotypes which are frequently distributed (at least three individuals) are labelled. The number of dashes on the branches represents the number of single nucleotide polymorphisms (SNPs) between haplotypes. The coloured ellipses highlight the three major groups, i.e., the Continental European clade (orange), the Atlantic clade (blue) and the Corsican population (green).

each were present in one or two locations in either Continental or Atlantic Europe. The remaining haplotypes (H1–H6, H8–H12, H14–H26, H29–H30, H32–H34, H37, H39–H41, H43–H45, H47, H50–H71, H73–H75, H77, H80–H85, H87, H89–H93, H95, H97–H104 and H109–H119) were singletons, thus the most prevalent distribution is one haplotype per one individual, showing the high genetic diversity of *T. formicarius* (Table 2, Figure 2). We found no geographic distribution pattern among singletons, as they were randomly dispersed across sampling locations.

However, the distribution of haplotypes is indicating an Atlantic-Continental pattern (Figure 2, Table 2). The haplotype network (Figure 3) shows the complexity of the haplotype distribution and visualizes the isolated position of the island population Coti-Chiavari in Corsica (FranceCO) and its private haplotypes. It also indicates that the FranceCO haplotypes originated from H46, which was present in the French mainland locations (FranceER and FranceSE). Furthermore, the haplotype network illustrates the distinct positions of the Atlantic-Continental European haplotypes, emphasizing the presence

of two distinct major clades (Figure 3). In addition, haplotypes from our easternmost study location TurkeyAR form a group within the Continental clade, as do haplotypes from TurkeyBU, together with some haplotypes from all Lithuanian locations (LithuaniaKA, LithuaniaNE, LithuaniaPA, LithuaniaTE and LithuaniaVA) (Figure 3).

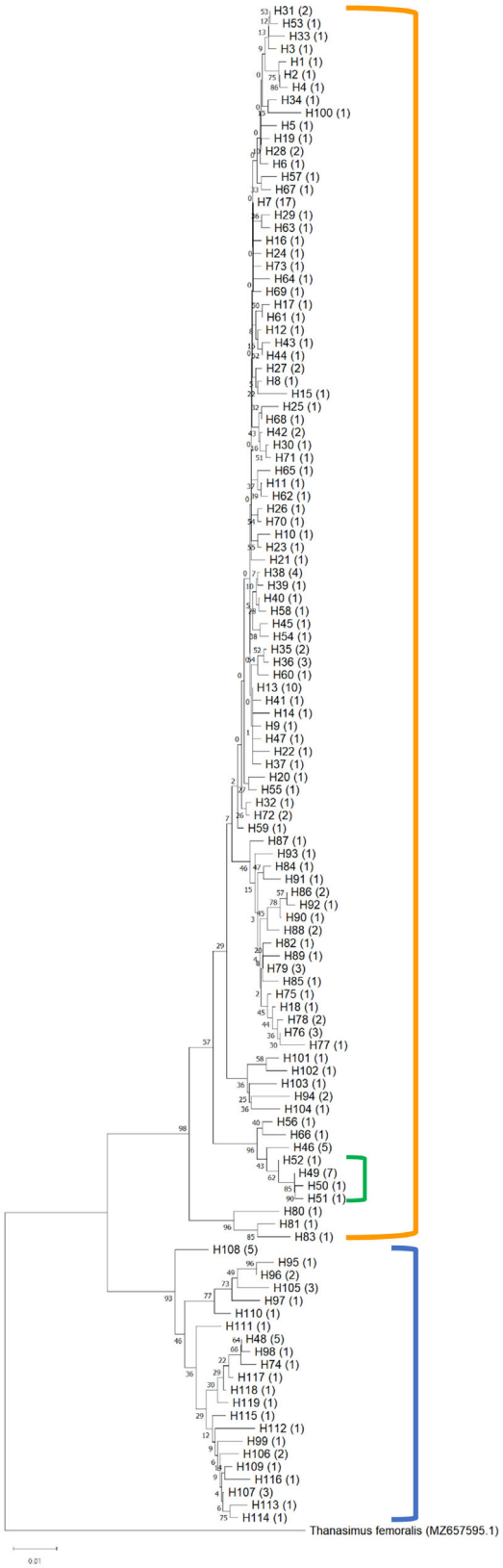


**FIGURE 4** Neighbour Joining tree of 119 *Thanasimus formicarius* COI haplotypes with *Thanasimus femoralis* as an outgroup. Bootstrap values (calculated from 1000 bootstrap replicates) are shown above or below the nodes. The frequency of each haplotype (number of individuals) is given in parentheses at the end of each tip, and the GenBank accession number is given in parentheses for the outgroup *T. femoralis*. The Atlantic clade is highlighted in blue, the Corsica population (FranceCO) within the Continental clade is marked in green, and the Continental clade is highlighted in orange.

The *T. formicarius* haplotype NJ tree with *T. femoralis* as outgroup revealed two major clades, i.e., an Atlantic and a Continental clade, containing several smaller clades (Figure 4; the topology of the ML tree resembles that of the NJ tree, Figure S2). The Atlantic/western clade is strongly supported by a bootstrap value of 93%; it contains the majority of haplotypes from Atlantic Europe, except H46 (FranceER and FranceSE) and H47 (FranceSE). The Continental haplotypes build another major clade, which consists of all Continental European haplotypes and two haplotypes from Atlantic Europe, i.e., H46 (FranceER and FranceSE) and H47 (FranceSE). Another well-supported clade (bootstrap value of 96%) within the Continental clade contains haplotypes H80, H81 and H83 from the easternmost location of our study range TurkeyAR. Additionally, a clade including haplotypes from multiple locations is also well-supported with a bootstrap value of 96%: FranceER (H46), FranceSE (H46), PolandMI (H56), SloveniaBR (H66) and FranceCO (H49, H50, H51 and H52). The haplotypes from FranceCO form a separate Corsica clade with a bootstrap value of 62%.

The Atlantic-Continental structure and the isolated position of Corsica are further supported by the results of the nested AMOVA, as it detected a significant differentiation ($p$ value <0.001) among the three defined groups, i.e., Atlantic Europe (FranceER, FranceSE, SpainBU, SpainFR and UnitedKingdomKE), Continental Europe (BulgariaGR, CroatiaSP, FinlandMA, FinlandSU, GreeceME, GreeceTR LithuaniaKA, LithuaniaNE, LithuaniaPA, LithuaniaTE, LithuaniaVA, PolandKO, PolandMI, SloveniaBR, SwedenUP, TurkeyAR and TurkeyBU) and Corsica (FranceCO), as well as among populations and within populations (Table 3). 82.58% of the differentiation is explained within groups, 13.30% within populations and 4.12% among populations (Table 3).

## DISCUSSION

We used a part of the mitochondrial COI gene to study the phylogeography and genetic structure of the bark beetle predator *T. formicarius* across the species' European range. Our large-scale analysis (187 individuals from 23 locations and 12 countries, sampled from Fennoscandia to the Mediterranean region and from Iberia to the Middle East) revealed a high genetic diversity and the presence of 119 haplotypes, which showed a clear distribution pattern across the sampling range.

**TABLE 3** Results of the nested analysis of molecular variance (nested AMOVA). *Thanasimus formicarius* populations are grouped in Corsica, Atlantic Europe and Continental Europe (see Methods section for details of groupings). Significant p values are in bold. Phi CT measures the genetic differentiation among defined groups, relative to the total genetic variation, Phi SC quantifies the genetic differentiation among populations within groups, and Phi ST estimates the total genetic differentiation among all populations.

| Source of variation | df (degrees of freedom) | Sums of squares | Variance component | % Total variation | Fixation index | *p* value |
|---|---|---|---|---|---|---|
| Among groups | 2 | 25,638.118 | 317.140 | 82.58 | Phi_CT 0.82579 | **<0.001** |
| Among populations | 20 | 3566.195 | 15.820 | 4.12 | Phi_ST 0.86698 | **<0.001** |
| Within populations | 164 | 8377.858 | 51.085 | 13.30 | Phi_SC 0.23646 | **<0.001** |
| Total | 123 | 37,582.171 | 384.045 | 100 | | |

Several lines of evidence support the proposed genetic structure of European *T. formicarius*. The distribution of haplotypes (Figures 2 and 3, Table 2) indicates an Atlantic and Continental European structuring, as haplotypes are either present in Atlantic or Continental Europe, i.e., there are no common haplotypes between the two groups. Furthermore, the haplotype network shows the presence of three groups: the Atlantic group, the Continental group and the isolated FranceCO population, with some haplotypes from Turkey and Lithuania clustering within the Continental group (Figure 3). The nested AMOVA showed that the origin of genetic variation was mostly within defined groups (Atlantic, Continental and Corsican), supporting the existence of an Atlantic and a Continental group, and a Corsican population (Table 3). The Corsican population is further supported by the presence of private/isolated haplotypes (GreeceTR and PolandKO also have private haplotypes, but the isolated genetic position of FranceCO is supported by multiple analyses performed here) and its low haplotype and nucleotide diversity (Figures 3 and 4, Table 2). Although two haplotypes from Atlantic Europe lie within the Continental clade, the phylogenetic analyses (NJ and ML tree) also support the structuring in Atlantic and Continental European clades (Figures 4 and S2).

The genetic structure of *T. formicarius* was probably affected by Pleistocene climatic cycles, the predator's strong host dependency (prey and trees) and certain additional life-history traits (Barbosa et al., 2012; Ehlers et al., 2018; Gerstmeier et al., 2019; Hewitt, 1996, 1999; Omondi et al., 2011; Schroeder, 1999b). The presence of an Atlantic and a Continental clade indicates that there were at least two major refugia for *T. formicarius* during the LGM, one in Atlantic Europe and one in Continental Europe. In Continental Europe several different glacial refugia for animal and plant species have been described, e.g., the Apennine Peninsula, the Carpathians and the Balkan Peninsula. In Atlantic Europe, the Iberian Peninsula and Ireland/Scotland have been identified as glacial refugia (de Becquevort et al., 2024; Habel et al., 2010; Hewitt, 1999; Prus-Głowacki et al., 2012; Schmitt, 2007; Schmitt & Varga, 2012; Smout, 2014; Tóth et al., 2017; Varga, 2009). *Thanasimus formicarius* probably shared its refugial areas with at least one of its major prey species, e.g., *I. typographus*, *P. chalcographus* or *T. piniperda*, and with one of its main host tree species, i.e., *Pinus* spp. or *P. abies*. For bark beetle species, it has been proposed that they perhaps shared at least one glacial refugium with their host tree species (Avtzis et al., 2008; Bertheau et al., 2013; de Becquevort et al., 2024; Horn et al., 2009; Kerdelhué et al., 2006; Krascsenitsová et al., 2013; Mayer et al., 2015; Papek et al., 2024; Ritzerow et al., 2004; Schebeck et al., 2018, 2023). Norway spruce endured the last ice ages in four major glacial refugia: the Apennine Peninsula, the Balkan Peninsula, the Carpathians and the Russian Plain (Giannini et al., 1991; Lagercrantz & Ryman, 1990; Schmidt-Vogt, 1977; Terhürne-Berson, 2005; Tollefsrud et al., 2009). The evolutionary histories of the spruce bark beetles *I. typographus* and *P. chalcographus* have been studied using mitochondrial and nuclear markers. For *I. typographus* two small refugia in the Carpathians have been described, but other main refugia remained elusive (Bertheau et al., 2013; Krascsenitsová et al., 2013; Mayer et al., 2015; Papek et al., 2024; Schebeck et al., 2023). Major glacial refugia of *P. chalcographus* were the Dinaric Alps in the Balkans, the Carpathians, the Apennine Peninsula and the Russian plain (Avtzis et al., 2008; Bertheau et al., 2013; Schebeck et al., 2018, 2019, 2023). Based on mitochondrial data and pollen records, pine species survived the LGM in Central Europe, the Alps, in various Mediterranean refugia and the Iberian Peninsula (Bucci et al., 2007; Burban & Petit, 2003; Sinclair et al., 1999; Soranzo et al., 2000). Moreover, a glacial refugium of *Pinus* spp. in Northern Europe (Scandinavia or Scotland/Ireland) was identified (de Becquevort et al., 2024; Prus-Głowacki et al., 2012; Smout, 2014; Tóth et al., 2017). The pine bark beetle *T. piniperda* survived the LGM in several refugia, i.e., the Iberian Peninsula, Northern Europe (Scandinavia or Ireland/Scotland) and Central Europe (de Becquevort et al., 2024; Horn et al., 2009; Kerdelhué et al., 2006; Ritzerow et al., 2004). Taken together, with our findings, possible glacial refugia of *T. formicarius* during the LGM could have been the Iberian Peninsula and/or Ireland/Scotland reflecting the Atlantic clade, and the Apennine Peninsula, Central Europe, the Balkan Peninsula or Scandinavia, representing the Continental clade. Due to the high genetic diversity in most of our study sites, it is not possible to draw final conclusions on the number and location of refugia. Perhaps the combination of nuclear and mitochondrial markers can facilitate the accurate determination of *T. formicarius* refugia. All locations, except Corsica, show a high genetic diversity. This, the phylogenetic tree and the haplotype network indicate that a limited number of individuals

have colonized Corsica from French mainland, i.e., that the limited genetic diversity in the Corsican population is the result of a founder effect, which could have been reinforced by the presence of only one host tree genus, i.e., *Pinus* (Caudullo et al., 2016; Garnas, 2018). Moreover, data from the INPN (Inventaire Nationale du Patrimoine Naturel) show that *T. formicarius* was present in mainland France before it arrived in Corsica in the Mesolithic period, about 15,000–5000 years ago (https://inpn.mnhn.fr/espece/cd_nom/11867/tab/archeo), supporting a French origin and subsequent colonization of Corsica.

Life-history traits, such as *T. formicarius'* high mobility, flight capacity, longevity, large prey spectrum and high fecundity, can affect its population genetic structure. These characteristics may have contributed to the high genetic diversity across its European range. High mobility enables *T. formicarius* to overcome long distances, facilitating gene flow among geographically isolated populations, as it was described for other species, e.g., *I. typographus* or *P. chalcographus* (Bertheau et al., 2013; Krascsenitsová et al., 2013; Mayer et al., 2015; Papek et al., 2024; Sallé et al., 2007; Schebeck et al., 2018, 2019). Moreover, the prey spectrum of *T. formicarius* might have shaped its genetic structure. It has been observed that generalist species have a higher genetic diversity than specialist species in the same genus (Li et al., 2014). The ability of *T. formicarius* to prey on more than 20 bark beetle species, living in different host trees, makes it a generalist, which may also explain the high genetic diversity within Europe (Gauss, 1954; Kohnle & Vité, 1984; Niehuis, 2013). Furthermore, *T. formicarius* has a high fecundity which was described to enhance genetic diversity (De Kort et al., 2021). Taken together, numerous environmental and species-specific factors may have affected the species' glacial and post-glacial history and contributed to the high genetic diversity in European *T. formicarius*. In the closely related clerid beetle, *Thanasimus dubius* (F.) (Coleoptera: Cleridae), the analysis of the mitochondrial COI gene revealed a similar genetic diversity to our study, with 60 unique haplotypes in 85 sampled individuals and a high haplotype diversity in 8 out of 10 sampled populations (ranging from 0.795 to 1.000) (Schrey et al., 2005). Our study detected 119 haplotypes in 187 analysed individuals and high haplotype diversities in 22 out of 23 locations (ranging from 0.821 to 1.000). *Thanasimus dubius* populations show significant genetic differentiation between northern and southern regions of North America, likely influenced by the geographic variation in *T. dubius'* response to prey pheromones (*D. frontalis*) and the discontinuous distribution of pine tree habitats, which may act as a barrier to gene flow (Schrey et al., 2005). A similar effect of responses to various prey species might also have shaped the genetic structure of *T. formicarius* in Europe, but this remains elusive. Although the prey spectrum of *T. formicarius* is broader than that of *T. dubius* and their geographical distribution is different, they have similar life histories, as predators of several bark beetle species. This suggests that they may be subject to similar selection pressures that affected their genetic structure. Whereas *T. formicarius* preys on species of eight bark beetle genera infesting pine, spruce and deciduous trees (Gauss, 1954; Kenis et al., 2004), *T. dubius* has been observed preying on bark beetle species of two genera infesting pine and spruce trees (Aukema & Raffa, 2002; Mignot & Anderson, 1969; Thatcher & Pickard, 1966). While *T. dubius* is native to North America (Schenk & Benjamin, 1969; Thatcher & Pickard, 1966), *T. formicarius* is native to Eurasia and was introduced to North America for the biological control of bark beetles (Klimaszewski et al., 2017; Niehuis, 2013; Opitz, 2002; Simpson et al., 2022). The rearing of *T. formicarius*, its introduction to North America (Klimaszewski et al., 2017; Opitz, 2002; Simpson et al., 2022), and its release in Turkey (Akyol & Sarikaya, 2017) and Ukraine (Meshkova et al., 2021) as a biological control agent of bark beetles, as well as the possible unintentional human-mediated spread of *T. formicarius*, e.g., through timber transport (Gippet et al., 2019), could have influenced the population genetic structure of *T. formicarius*.

Gerstmeier et al. (2019) confirmed the species status of *T. formicarius* in Central Europe and showed that *T. pectoralis* and *T. rufipes* are synonymous with *T. femoralis*. *Thanasimus femoralis* and *T. formicarius* are sympatric in parts of Europe; therefore, comparing the genetic structure of these congeneric species can offer new insights into their evolutionary histories and whether they were shaped by similar biotic and abiotic drivers.

Taken together, our study revealed a complex and diverse genetic structure of European *T. formicarius*. Two distinct main clades were identified: an Atlantic and a Continental one, suggesting the presence of at least two European glacial refugia; we also discovered an isolated island population in Corsica that was likely colonized from mainland France. This large-scale genetic analysis not only improves our understanding of the population genetic structure and recent evolutionary history of European *T. formicarius*, but can also suggest potential benefits for biodiversity management and forestry. Our data support the predator's high mobility and fecundity, which can help assess its potential to regulate bark beetle populations. The presence of two major clades and regional genetic differences between locations highlight the importance of considering the origin of individuals used for biological control. Introducing individuals from a different region could be less effective, as there might be regional variations in host tree usage, adaptations to local climate or volatile responses to different prey species. Further research will be needed to explore these potential local differences to improve the efficiency of *T. formicarius* as a biological control agent in bark beetle management.

## AUTHOR CONTRIBUTIONS

**Eva Papek:** Formal analysis; investigation; visualization; writing – original draft; writing – review and editing. **Amina Derlić:** Methodology; writing – review and editing. **Markus Melin:** Resources; writing – review and editing. **Alain Roques:** Resources; writing – review and editing. **Dragos Cocos:** Resources; writing – review and editing. **Martin L. Schroeder:** Resources; writing – review and editing. **Milan Pernek:** Resources; writing – review and editing. **Dimitrios N. Avtzis:** Resources; writing – review and editing. **Paulius Zolubas:** Resources; writing – review and editing. **David T. Williams:** Resources; writing – review and editing. **Juan A. Pajares:** Resources. **Oğuzhan Sarikaya:** Resources; writing – review and editing. **Roman Pavlin:** Resources; writing – review and editing. **Sevdalin Belilov:** Resources;

writing – review and editing. **Martin M. Gossner:** Resources; writing – review and editing. **M. Lukas Seehausen:** Resources; writing – review and editing. **Anna Wierzbicka:** Resources; writing – review and editing. **Lara Kundtner:** Methodology; writing – review and editing. **Christian Stauffer:** Conceptualization; writing – review and editing. **Martin Schebeck:** Conceptualization; supervision; writing – review and editing.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available on Dryad at https://doi.org/10.5061/dryad.hdr7sqvst and on Zenodo at https://doi.org/10.5281/zenodo.14203579.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Table S1.** Details of four different PCR reaction mixes used for DNA amplification.

**Table S2.** Details of four different PCR protocols used for DNA amplification in this study.

**Figure S1.** Haplotype distribution *Thanasimus formicarius* among populations (details on location abbreviations see Methods section in the main manuscript).

**Figure S2.** Maximum Likelihood tree of 119 *Thanasimus formicarius* COI haplotypes with *Thanasimus femoralis* as an outgroup. The bootstrap values are shown next to the branches. The frequency of each haplotype (number of individuals) is given in parentheses at the end of each tip, and the GenBank accession number is given in parentheses for the outgroup *T. femoralis*.

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