

Phylogeny of the *Formicoxenus* genus-group (Hymenoptera: Formicidae) reveals isolated lineages of *Leptothorax acervorum* in the Iberian Peninsula predating the Last Glacial Maximum

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ABSTRACT

The *Formicoxenus* genus-group comprises six genera within the tribe Crematogastrini. The group is well known for repeated evolution of social parasitism among closely related taxa and cold-adapted species with large distribution ranges in the Nearctic and Palearctic regions. Previous analyses based on nuclear markers (ultraconserved elements, UCEs) and mitochondrial genes suggest close relationship between *Formicoxenus* Mayr, 1855, *Leptothorax* Mayr, 1855 and *Harpagoxenus* Forel, 1893. However, scant sampling has limited phylogenetic assessment of these genera. Also, previous phylogeographic analyses of *L. acervorum* (Fabricius, 1793) have been limited to its West-Palearctic range of distribution, which has provided a narrow view on recolonization, population structure and existing refugia of the species. Here, we inferred the phylogenetic history of genera within the *Formicoxenus* genus-group and reconstructed the phylogeography of *L. acervorum* with more extensive sampling. We employed three datasets, one data set consisting of whole mitochondrial genomes, and two data sets of sequences of the COI-5P (658 bp) with different number of specimens. The topologies of previous nuclear and our inferences based on mitochondrial genomes were overall congruent. Further, *Formicoxenus* may not be monophyletic. We found several monophyletic lineages that do not correspond to the current species described within *Leptothorax*, especially in the Nearctic region. We identified a monophyletic *L. acervorum* lineage that comprises both Nearctic and Palearctic locations. The most recent expansion within *L. acervorum* probably occurred within the last 0.5 Ma with isolated populations predating the Last Glacial Maximum (LGM), which are localized in at least two refugial areas (Pyrenean and Northern plateau) in the Iberian Peninsula. The patterns recovered suggest a shared glacial refugium in the Iberian Peninsula with cold-adapted trees that currently share high-altitude environments in this region.

KEY WORDS

Phylogenomics; mitogenomes; ant; *Leptothorax*; *Formicoxenus*; Last Glacial Maximum refugia; biogeography; phylogeography.

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INTRODUCTION

Several invertebrate groups have species with Holarctic distributions, including beetles (Larson and Nilsson, 1985), Lepidoptera (Landry et al., 2013), spiders (Marusik & Koponen, 2005) and ants (Schär et al., 2018). Among ant species, only three species (*Camponotus herculeanus* Linnaeus, 1758, *Formica gagatoides* Ruzsky, 1904 and *Leptothorax acervorum* Fabricius, 1793) are known to have monophyletic lineages with a Holarctic distribution (Schär et al., 2018). The latter of these ant species belongs to the tribe Crematogastrini Emery, 1914 within the subfamily Myrmicinae, with Crematogastrini comprising some 6,630 species (Blaimer et al., 2018; Ward et al., 2015). There, recent phylogenomic analyses based on ultraconserved elements (UCEs) have consistently recovered a monophyletic lineage of six genera (*Vombisidris* Bolton 1991, *Gauromyrmex* Menozzi, 1993, *Harpagoxenus* Forel, 1893, *Formicoxenus* Mayr, 1855, *Temnothorax* Mayr, 1861 and *Leptothorax* Mayr, 1855) referred to informally as the *Formicoxenus* genus-group (Branstetter et al., 2017; Blaimer et al., 2018). These analyses have recovered a close relationship between *Formicoxenus* and *Leptothorax*. However, all these studies have been focused on higher taxonomic relationships and few studies have included a comprehensive sampling of species within each genus (Heinze and Gratiashvili, 2015; Prebus, 2017; Schär et al., 2018). Among the *Formicoxenus* genus-group, *Leptothorax* is the second largest genus with an estimated 20 species (AntWeb ver. 8.42, <https://www.antweb.org>, accessed 29 October 2020). The genus has a Holarctic distribution and it has been inferred to have originated in the Nearctic and dispersed in the Palearctic within the last 2 Ma (Schär et al., 2018). Relationships within *Leptothorax* have not been fully resolved and previous phylogenetic analyses indicate the presence of multiple undescribed and non-monophyletic taxa, particularly in the Nearctic (Heinze & Gratiashvili, 2015; Schär et al., 2018). At least seven species have been described in the Palearctic region, most of them with limited distribution and only *L. acervorum* with a distribution both in the Nearctic and the Palearctic regions (Schär et al., 2018).

Taxa that span large geographic regions in both the Nearctic and Palearctic are expected to have experienced variable connectivity because of the fluctuating presence of the land bridge of Beringia between Eurasia and North America (DeChaine, 2008). Also, climate oscillations during the Quaternary (last 2 Ma), characterized by pronounced cycles between cold glacial (ca. 100,000 years) and warm interglacial periods (ca. 20,000 years) during the last 700,000 years, altered the geographic distribution of species in the northern hemisphere (Nearctic and Palearctic) (Hewitt, 2000). During these glacial episodes, species ranges contracted to refugia in suitable areas in the southern part of their distribution. As the climate warmed and glaciers retreated, species with a temperate range of distribution expanded and reconnected. In contrast, the range of distribution for boreal cold-adapted species likely reduced and fragmented (Hewitt, 1996; Petit et al., 2003). Evidence from several ant species have suggested the presence of several refugia during the Pleistocene (2.58–0.012 Ma) in the southern Mediterranean peninsulas, the Caspian-Caucasus region and further east in southern East Asia (Goropashnaya et al., 2004; Pusch et al., 2006; Schlick-Steiner et al., 2007; Beibl et al., 2007; Leppänen et al., 2011, 2013). In addition, populations of cold-adapted ant species could have also survived in more northerly refugia near the permafrost (Leppänen et al., 2011, 2013). Indeed, *L. acervorum* is among the very few cold-adapted species that extend their distribution above the polar circle both in the Nearctic and the Palearctic (Heinze et al., 1996, 1998; Berman et al., 2010). In the Palearctic, this species occurs in the boreal zone from the Atlantic Ocean to Japan, and in the mountains of southern Europe, the Caucasus, and the Tien-Shan and Pamir (Czechowski et al., 2012; Seifert, 2018). Thus, the climatic fluctuations of the Quaternary have likely played a significant role in shaping its current distribution, connectivity, and genetic diversity. Populations located near the permafrost and those located on the southern range of its distribution were likely affected differently.

The most recent phylogenetic analysis of *Leptothorax* indicate that *L. acervorum* originated about 2 Ma, with the most recent populations located in the Nearctic region. Within the Palearctic, populations situated in the Iberian Peninsula were

inferred to be the most basal among the specimens included (Schär et al., 2018), which might have been located in refugia during the glacial cycles. In addition, more detailed analyses based on mitochondrial DNA (COI-3P region) and microsatellites (SSRs) have been used to infer the phylogeographic history and population structure of this species in the western part of its distribution (West Palearctic). These analyses have found generally less population structure in *L. acervorum* compared to other closely related species within *Leptothorax*, e.g. *L. muscorum* (Nylander, 1846) and *Harpagoxenus sublaevis* (Nylander, 1849) (Brandt et al., 2007; Foitzik et al., 2009; Trettin et al., 2016), but also evidence of divergent haplotypes has been found in the Pyrenees and Southern France (Trettin et al., 2016). Given the large distribution range across the Holarctic and the extensive variation in the latitudinal range in western Europe (from the Iberian Peninsula to North Cape in Norway) (Heinze & Holldobler, 1994), approaches that combine analyses at different taxonomic levels with extensive sampling are necessary to understand the phylogenetic relationships and evolutionary history of *Leptothorax* species. Here we present the most comprehensive sampling of members of the *Formicoxenus* genus-group with an emphasis on the phylogenetic relationships within *Leptothorax* and the biogeography of *L. acervorum* across its range of distribution in the Holarctic region with a special focus on the Iberian populations. Our specific objectives are: 1) to infer relationships among the six genera of the *Formicoxenus* genus-group using whole mitochondrial genomes and assess their correspondence with previous topologies obtained with nuclear genes, 2) to clarify the relationships of *Leptothorax* species and the timing of divergence of the Palearctic species, 3) to determine the phylogenetic position of the Iberian Peninsula specimens of *L. acervorum* and if they diverged before the Quaternary.

MATERIAL AND METHODS

Taxon sampling and datasets

The sampling strategy used in this study was developed to represent the *Formicoxenus* genus-

group at three different hierarchical levels. The first dataset consisted of 49 specimens representing all six genera: *Temnothorax* (2 spp.), *Leptothorax* (4 spp.), *Formicoxenus* (1 spp.), *Harpagoxenus* (1 spp.), *Gauromyrmex* (2 spp.) and *Vombisidris* (1 spp.) currently recognized within this group (Blaimer et al., 2015; Prebus, 2017). This sampling represents 17% of genera within the Crematogastrini. We also included outgroups from Myrmicinae (all tribes), Dolichoderinae and Ponerinae. In this data set we used whole mitochondrial genomes to explore the major relationships within *Formicoxenus* genus-group at the genus level. We used only one representative specimen per species within each genus, except for *L. acervorum*, where we included six specimens from the Iberian Peninsula and the UK (Table S1). In the second dataset, we gathered specimens representing eight out of the 20 *Leptothorax* species currently recognized (AntWeb ver. 8.42, <https://www.antweb.org>, accessed 29 October 2020), two *Formicoxenus* species and *H. sublaevis*. In this dataset we sequenced the section of the mitochondrial cytochrome c oxidase (COI-5P region, 658 bp) in 96 specimens (Table S2). The third dataset consisted of 113 specimens of *L. acervorum* across its distribution range in the Holarctic region, where we sequenced the same gene region (COI-5P region, 658 bp) as the previous dataset. This dataset was complemented with available sequences from public repositories (Table S3). All specimens sequenced in this study were identified by one of us and using the current species delimitation of *Leptothorax* (Czechowski et al., 2012; Seifert, 2018).

Whole mitochondrial sequencing and assembly

Mitochondrial genomes were newly generated for six specimens of *L. acervorum* from six different populations in Spain and the UK (Spain: Valdelinares (V), Orihuela del Tremedal (OT), Larra (L), Neila Refuge (NR), Pla de la Font (PF); UK: Santon Downham (SD) (Table S3). A *de novo* mitochondrial genome was identified as part of a whole genome sequencing project from a single adult male (PF population, sample: PF18_15_M1) using 10x linked reads assembled with Supernova 2.1.1 (Weisenfeld et al., 2018). The scaffold

containing the mtDNA genome was identified by a BLASTn query of the assembled genome with two published *L. acervorum* mtDNA sequences (query 1: COXI – tRNA - Leu - COXII: GenBank: KU245569 (Trettin et al., 2016); query 2: COB: GenBank: HQ259995 (Gill et al., 2009). These two sequences, located ~6Kb apart in the canonical hymenopteran mtDNA genome, were used to minimize erroneous matches to nuclear genomic scaffolds containing translocated mtDNA (NUMTs). Only two scaffolds (102,807 and 104,071) showed convincing matches to both query sequences (E value = 0, bit scores > 1000). However, mapping re-sequenced samples (see below) showed scaffold 102,807 had 40 times higher coverage (200x-400x) than 104,071 (~5x-10x coverage) with the latter having similar coverage to the rest of the presumed nuclear genome. Furthermore, scaffold 102,807 was 17Kb in length (the expected size of the mtDNA genome) whereas scaffold 104,071 was longer than expected at 24Kb. These lines of evidence clearly show scaffold 102,807 contains the *L. acervorum* mtDNA genome whereas scaffold 104,071 is a transposition of mtDNA sequences to the nuclear genome (a NUMT).

To genotype single individuals in the six populations (V, OT, L, NR, PF, and SD), short-read sequence data (Illumina HiSeq 2x150bp paired-end reads) were, after quality control steps, aligned to the draft genome with Bowtie2 2.3.5 (Langmead and Salzberg, 2014) and processed with SAMtools (Li et al., 2009) to produce bam files. Bam files were then subset to only include the identified mtDNA scaffold (scaffold: 102,807) with SAMtools. These mtDNA alignments were converted to mpileup with BCFtools (—max-depth 1000) and BCFtools call used to produce vcf files. Vcf files were indexed and normalized and variants within 5bp of any indels removed with BCFtools. Finally, a fasta file for each alignment was produced with BCFtools consensus.

In addition, mitochondrial genomes of the taxa within the *Formicoxenus* genus-group were extracted and assembled from ultra-conserved elements (UCE) libraries from previous studies (Branstetter et al., 2017; Prebus, 2017) using MitoFinder (Allio et al., 2020). Outgroup species within subfamilies Myrmicinae, Dolichoderinae and Ponerinae were downloaded from Genbank, previously published in several studies (Ciconardi

et al., 2020; Du et al., 2019; Duan et al., 2016; Gotzek et al., 2010; Hasegawa et al., 2011; Liu et al., 2016; Park et al., 2021, 2020b, 2020a, 2019; Rodovalho et al., 2014).

Phylogenomic analyses using whole mitochondrial genomes

All 49 whole mitochondrial genomes were aligned using MAFFT ver. 7.310 (Katoh and Kuma, 2002) with default parameters. Visual inspection and further adjustment were performed with AliView (Larsson, 2014) and summary statistics of the alignment were obtained with AMAS (Borowiec, 2016). We only included regions that were aligned across all the mitochondrial of all species and excluding ambiguous regions. Phylogenetic analysis was performed with maximum likelihood (ML) as implemented in IQ-TREE 1.6.1 (Nguyen et al., 2015) with ultrafast likelihood bootstrap with 1000 replicates. The final tree was visualized and edited with FigTree (Rambaut, 2016).

DNA extraction, PCR amplification and sequencing of cytochrome c oxidase (COI)

We collected either pupae or adults of workers, males, or queens from different colonies of *H. sublaevis*, *L. acervorum*, *L. kutteri* (Buschinger, 1965) and *L. muscorum* (Table S2). DNA was extracted from legs or whole specimens using the salt extraction method (Aljanabi and Martinez, 1997) and we sequenced the portion of the mitochondrial COI using primers and PCR conditions previously reported (Folmer et al., 1994). The sequences obtained were edited, visually inspected using Sequencher (Gene Codes), and aligned with AliView (Larsson, 2014).

Phylogenetic analyses and dating estimation within Formicoxenus-Leptothorax based on the COI gene region

To infer the phylogenetic relationships within the *Formicoxenus-Leptothorax*, we used the 5' region of COI (658 bp, ranging from 5442-6601 in the *L. acervorum* mitochondrial genome assembly). First, we performed an explorative analysis based on a comprehensive sampling from

this gene region using the sequences generated in this study and available sequences from GenBank and BOLD: The Barcode of Life Data System (www.barcodinglife.org). This previous analysis was performed to select the most appropriate specimens from public repositories representing the species within the *Formicoxenus-Leptothorax*. The matrix was aligned and manually edited with AliView (Larsson, 2014), with summary statistics obtained with AMAS (Borowiec, 2016). This preliminary analysis was based in a total of 747 specimens of *Formicoxenus* (2 spp.), *Leptothorax* (8 spp.) and specimens of *H. sublaevis* (outgroup). We used maximum likelihood (ML) as implemented in IQ-TREE 1.6.1 (Nguyen et al., 2015) with ultrafast likelihood bootstrap with 1000 replicates (Minh et al., 2013). Based on the results from this analysis (data not shown), we selected representative specimens of the major Nearctic lineages identified (>80 bootstrap support) and all the specimens in the Palearctic lineage of *L. acervorum*. This dataset consisted of 96 specimens representing eight *Leptothorax* spp., specimens labelled as *Leptothorax* sp., *L. muscorum* complex, *Leptothorax* sp. AF CAN, two *Formicoxenus* spp. and *H. sublaevis* as an outgroup. This dataset consisted of the newly generated sequences in this study and available sequences from previous publications (Smith et al., 2009; Stahlhut et al., 2013; Hebert et al., 2016; Prebus, 2017; Schär et al., 2018) (Table S2). The best nucleotide substitution model, the partition of the data, and the ML analysis were inferred with IQ-TREE 1.6.1. Clade support was assessed with ultrafast likelihood bootstrap with 1000 replicates. In addition, we also performed a Bayesian inference (BI) as implemented in MrBayes 3.2.6 (Huelsenbeck & Ronquist, 2001; Ronquist et al., 2012) with four chains, two runs of 20 million generations with the GTR+I+Gamma model of nucleotide substitution and a sample frequency of 1000. We used TRACER 1.7 (Rambaut et al., 2018) to verify whether effective sample sizes (ESS values) were higher than 200 for all parameters.

To estimate divergence times among the lineages in *Formicoxenus-Leptothorax*, we used a simplified dataset (fewer specimens representing each species/lineage) with the same number of species as above, but fewer specimens (63) of these

two genera to simplify the analyses and the visualization of the results. We used BEAST 1.10.4 (Bouckaert et al., 2014; Suchard et al., 2018) with a strict clock model, GTR substitution model, and a constant population size under a coalescence model, similar to a previous analysis in this group (Schär et al., 2018). We employed the divergence time estimated in the Formicinae (Blaimer et al., 2015) by placing a prior in the divergence estimate of *Harpagoxenus* and *Formicoxenus-Leptothorax* of 8.89 (13.89-3.89) Ma. We ran two independent runs of 50 million generations each, sampling values every 1,000 steps. Output files were analyzed with TRACER 1.7 to assess chain convergence and LogCombiner 1.10.4 was used to combine independent runs. Finally, we used Treeannotator 1.10.4 to generate the maximum-clade-credibility tree. ML and BI consensus trees were visualized and edited with FigTree (Rambaut, 2016).

Phylogeography of *Leptothorax acervorum* in the Iberian Peninsula

To gain further insights into the geographic distribution of *L. acervorum* across its Holarctic distribution, we first determined the number of haplotypes, haplotype diversity (*H_d*) and defined haplotypes with DnaSP ver. 6.12 (Rozas et al., 2017). Then, we reconstructed the haplotype network of all 113 specimens (Table S3) using the COI-5P gene region (647 bp, dataset 3) with the statistical parsimony network using TCS (Clement et al., 2002) as implemented in popart ver. 1.7 (Leigh & Bryant, 2015). Given that our interest was focused on the populations distributed in the Iberian Peninsula, we explored in more detail five populations from this region and one population from the UK (Table S3).

Data Accessibility Statement

SRA sequences and the assembled mitochondrial genomes of *L. acervorum* are deposited in the NCBI Bioproject PRJNA634471. COI sequences are deposited in the NCBI Bioproject PRJNA634471, under accessions OL376482-OL376570. Details of the draft genomes recovered with MitoFinder are available in Table S4.

RESULTS

Phylogenomic analyses of the Formicoxenus genus-group

The final alignment of the mitochondrial genomes consisted of 14,351 bp with 13.32% missing data, 69% of sites variable and 59% of sites parsimony informative (Supplementary Information, Data S1). We recovered monophyletic lineages for all the tribes, except Attini within Myrmicinae, with most branches having moderate (>75%) to high (>85%) bootstrap support. Our phylogenetic analysis recovered all six genera of the *Formicoxenus* genus-group as a monophyletic lineage within Crematogastrini, with *Formicoxenus* as the most closely related genus to *Leptothorax*. The most closely related tribe was Solenopsidini (Fig. 1).

Relationships within Leptothorax and divergence times of the Palearctic species

The alignment matrix of the COI-5P region (dataset 2) consisted of 658 bp with 200 variable sites (30.4%), 148 parsimony informative sites (22.5%) and 1.42% of missing data. We recovered the species of *Formicoxenus* in different lineages within *Leptothorax*, suggesting that the former genus might not represent a monophyletic lineage. All the three Palearctic *Leptothorax* species we included (*L. muscorum*, *L. gredleri* Mayr, 1855 and *L. kutteri*) represent monophyletic lineages, whereas specimens assigned to *L. muscorum* from the Nearctic region represent several undescribed taxa (Fig. 2, Fig. S1). Similarly, we found non-monophyletic lineages for the other Nearctic species *L. canadensis* Provancher, 1887 and *L. calderoni* Creighton, 1950, but not for *L. retractus* Francoeur, 1986. Our divergence estimate suggests that the stem age lineages of Palearctic taxa (*L. gredleri*, *L. muscorum*, *L. kutteri* and *L. acervorum*) ranges between 1-1.6 Ma (Fig. 3). The crown age of the Palearctic lineage of *L. acervorum* was estimated at 0.56 Ma, with specimens from the Iberian Peninsula ranging in age between 0.1 and 0.5 Ma. The most recently diverged lineage (0.30 Ma) within *L. acervorum* comprises both specimens from the Nearctic and Palearctic distribution, including specimens at

high latitudes mainly from the West Nearctic (Figs. 4, 5).

Phylogeography of L. acervorum in the Holarctic region and the populations of the Iberian Peninsula

Our analyses based on the COI-5P region (dataset 3, excluding gaps and missing data), recovered 21 variable sites with 19 haplotypes ($Hd = 0.826$) among the 113 specimens examined (Table S3). Only two populations from the Iberian Peninsula (Larra and Pla de la Font) shared haplotypes with the rest of the populations in the West Palearctic, East Palearctic and the Nearctic. We also found a unique haplotype (H4) shared between the population in the UK and Switzerland, and the presence of unique haplotypes in Bulgaria and Kamchatka. The most widely distributed haplotype (H11) was shared across the entire geographic distributional range (Table 1). The haplotype network indicates that most populations in the Iberian Peninsula have been isolated from the remaining range of distribution in the West Palearctic (Fig. 6).

DISCUSSION

Congruence between phylogenomic inferences based on mitochondrial genomes and UCEs

Recent analyses using large sequence datasets from UCEs have been employed to resolve relationships among ant subfamilies (Blaimer et al., 2015; Branstetter et al., 2017; Li et al., 2018; Longino & Branstetter, 2021). Phylogenomic inferences using mitochondrial genomes have also been used in several ant subfamilies with limited congruence in topologies between nuclear (UCEs) and mitochondrial genomes (Allio et al., 2020). Incongruences between nuclear and mtDNA analyses in ants have been reported previously in phylogenetic and phylogeographic analyses (Lucky, 2011; Ueda et al., 2015; Darras & Aron, 2015; Eyer et al., 2016; Eyer & Hefetz, 2018), suggesting that caution should be placed on studies based only on mtDNA markers. Several characteristics of the mtDNA, such as its maternal inheritance, low recombination and overall higher mutation rates (Ballard & Whitlock, 2004; Bonnet

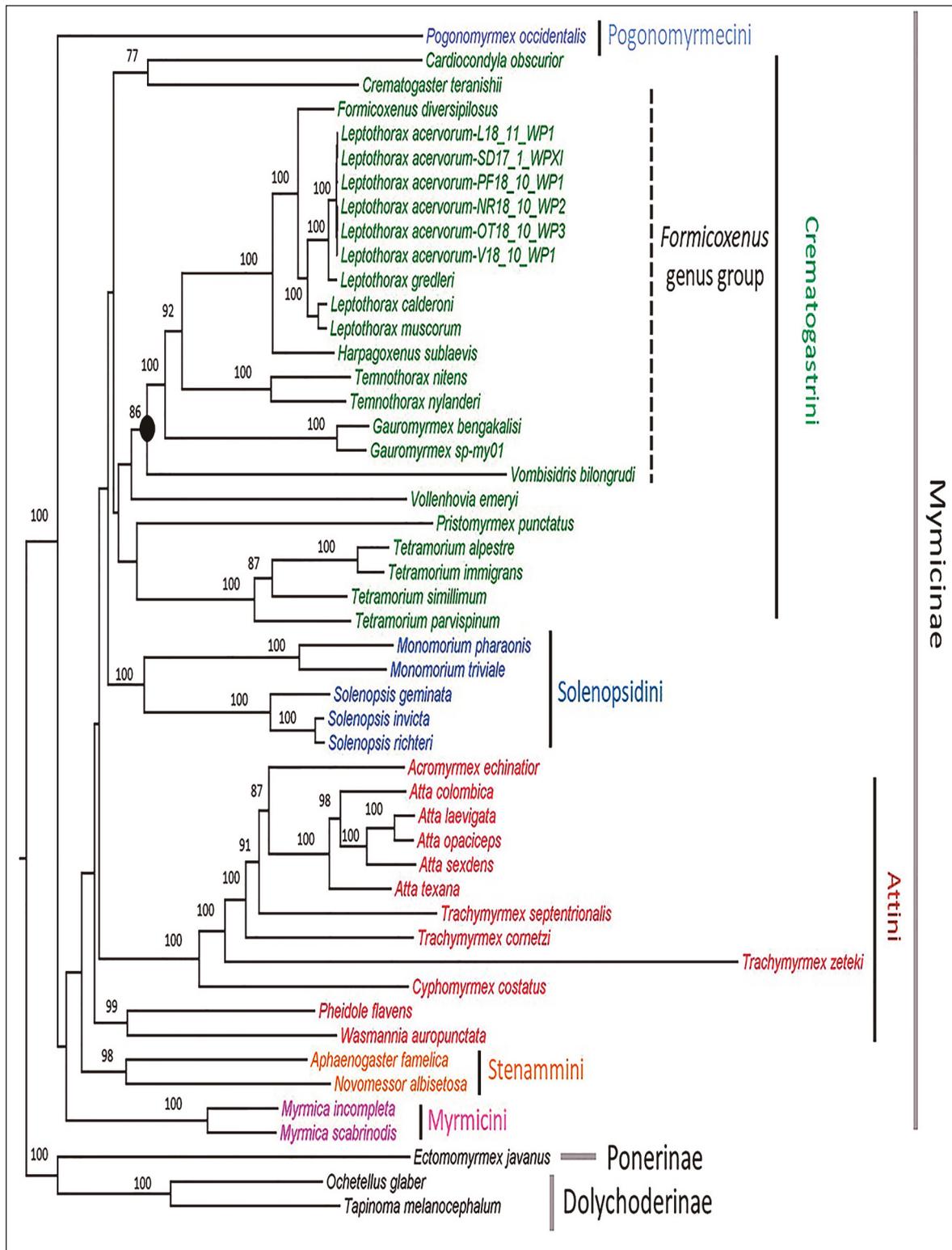
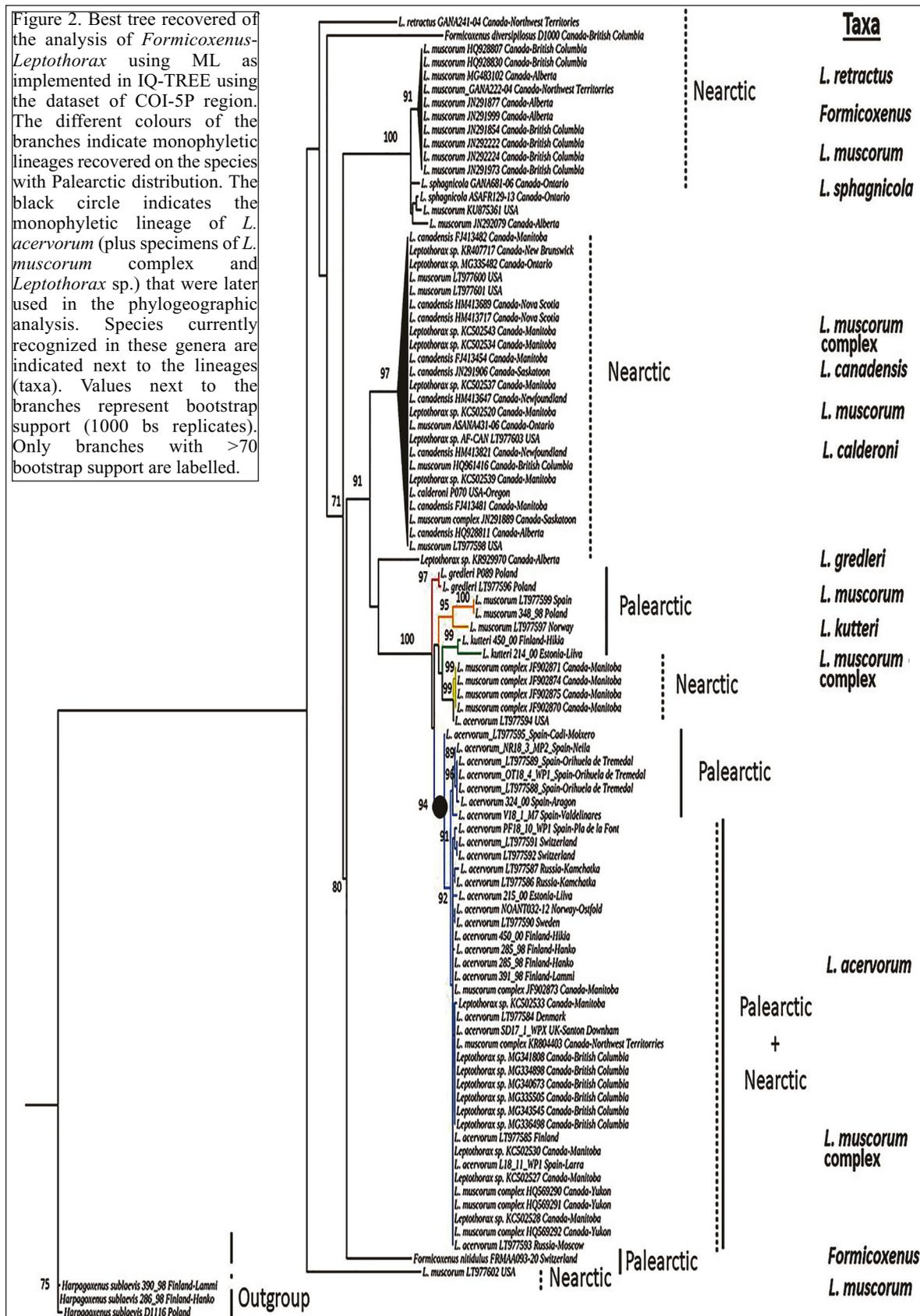


Figure 1. Best tree obtained in the phylogenetic analysis inferred with whole mitochondrial genomes of the *Formicoxenus* genus-group using ML as implemented in IQ-TREE. Values next to the branches represent bootstrap support (1000 bs replicates). Only branches with support over 70 are labelled. The black circle indicates the node with the genera of the *Formicoxenus* genus-group. Different colors indicate tribes within the Myrmecinae.

Figure 2. Best tree recovered of the analysis of *Formicoxenus-Leptothorax* using ML as implemented in IQ-TREE using the dataset of COI-5P region. The different colours of the branches indicate monophyletic lineages recovered on the species with Palearctic distribution. The black circle indicates the monophyletic lineage of *L. acervorum* (plus specimens of *L. muscorum* complex and *Leptothorax* sp.) that were later used in the phylogeographic analysis. Species currently recognized in these genera are indicated next to the lineages (taxa). Values next to the branches represent bootstrap support (1000 bs replicates). Only branches with >70 bootstrap support are labelled.



et al., 2017), particularly at low taxonomic levels where introgression among closely related species is higher. Although we did not have a comprehensive mitochondrial representation within each of the six tribes currently recognized within Myrmicinae (Borowiec et al., 2020), the topology we recovered from this analysis (Fig. 1) is congruent with the known relationships among these tribes inferred from nuclear genes (Ward et al., 2015; https://antwiki.org/wiki/Phylogeny_of_Myrmicinae). Crematogastrini harbors 40% of all Myrmicinae species and 45% of the genera belong to this tribe (Blaimer et al., 2018). Recent phylogenomic analyses based on UCEs (Prebus, 2017; Blaimer et al., 2018), as well as mitochondrial genomes (Park et al., 2021) have been used to increase resolution

among genera of Crematogastrini. Analyses using UCEs have recovered eight clades with high support within Crematogastrini, which have been treated as informal genus-groups.

The *Formicoxenus* genus group consists of six genera (*Formicoxenus*, *Leptothorax*, *Vombisidris*, *Gauromyrmex*, *Harpagoxenus* and *Temnothorax*) and the relationships among the genera are relatively well established (Prebus, 2017; Blaimer et al., 2018), but less attention has been paid to the *Leptothorax* genus group, which consists of *Formicoxenus*, *Leptothorax* and *Harpagoxenus*. Species within these genera are prone to develop social parasitism, a set of interrelated lifestyles where the parasitic species depend upon a free-living host to complete their life cycle (Beibl et al.,

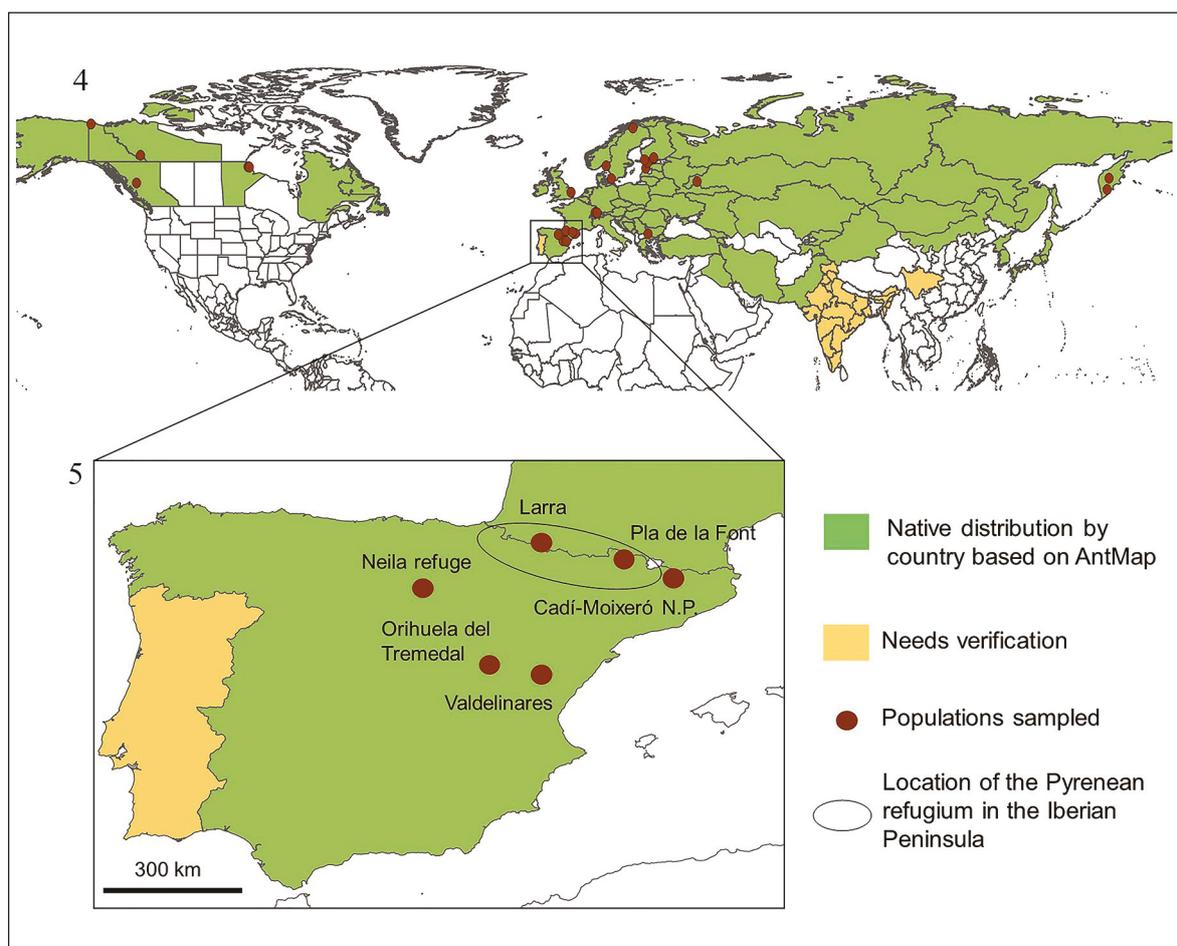


Figure 4. Distribution map of *L. acervorum* across the Holarctic region based on the Global Ant Biodiversity Informatics (GABI) database (Guénard et al., 2017). Locations of the different populations included in the phylogeographic analysis are indicated with red dots. Figure 5. Close-up of the populations located in the Iberian Peninsula. The black oval indicates the location of the Pyrenean refugium (Tinaut & Ruano, 2021). N.P. = National Park.

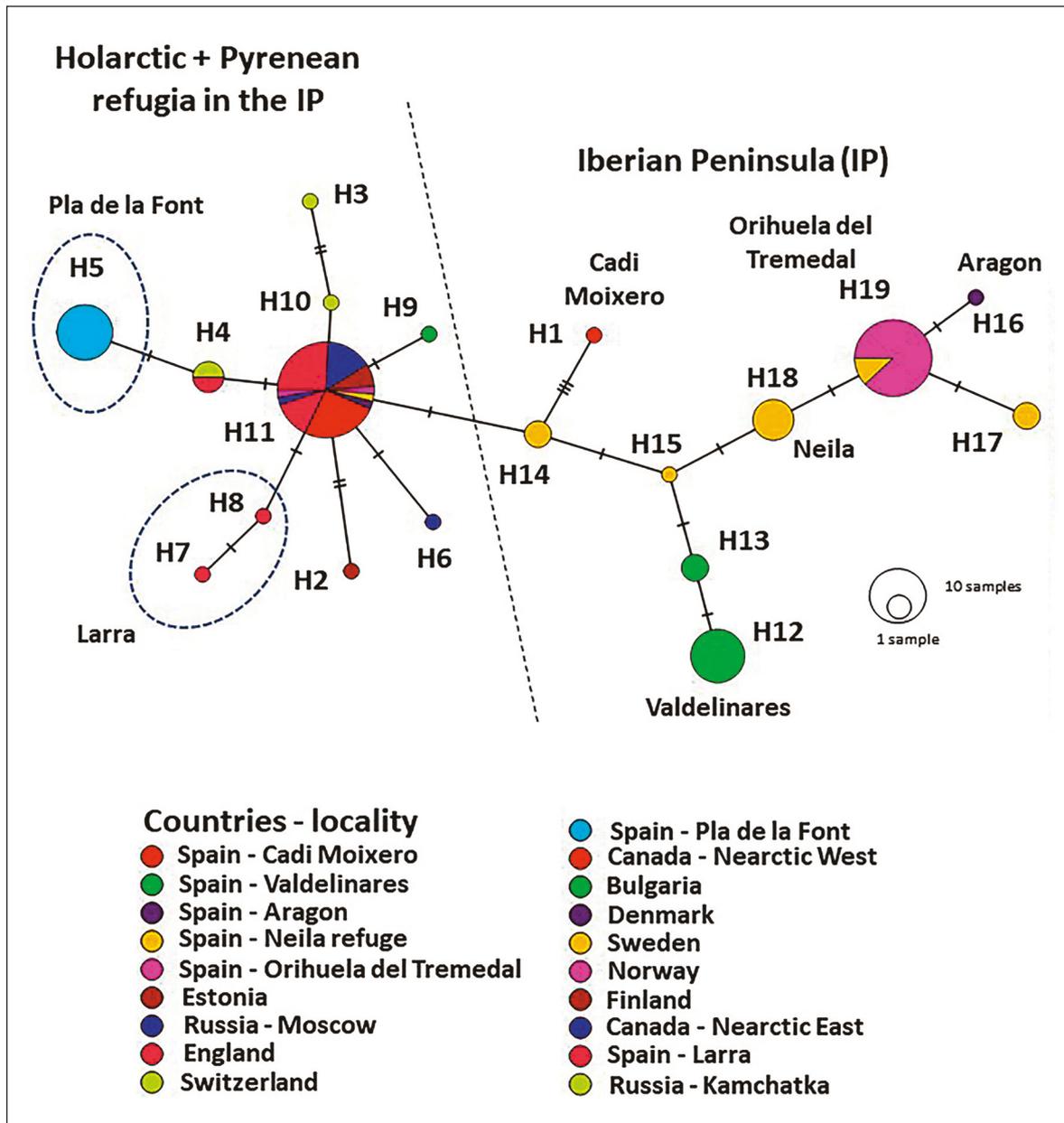


Figure 6. Haplotype network of *L. acervorum* across its Holarctic distribution range. Codes next to the circle indicate the haplotype classification and their distribution. Hatch marks represent mutation differences among the haplogroups. The dash line separates most of the Iberian Peninsula populations from the rest of distribution. Pla de la Font and Larra (in dash circles) are both located in the Pyrenean refugia.

2005; Heinze, 1995). These three genera, together with *Temnothorax*, are considered a hot spot for the evolution of social parasitism, where it has evolved at least five times among closely related taxa (Beibl et al., 2005; Prebus, 2017; Jongepier et al., 2021). The inclusion of more species within this group combined with additional mitochondrial genomes

and UCEs will provide a more complete understanding of the evolution of social parasitism in this group.

Evidence on non-monophyletic taxa within Leptothorax and monophyletic lineages within L. acervorum

Mitochondrial genes (cytochrome b and cytochrome oxidase subunit 1) alone or in combination with other nuclear markers have been previously used in phylogenetic inferences in *Leptothorax* genus (Baur et al., 1996, 1995; Beibl et al., 2005; Heinze & Gratiashvili, 2015; Schär et al., 2018), but with limited representation of its species or without using other genera within the *Formicoxenus* genus-group. Our more comprehensive sampling (42% of *Leptothorax* species, AntWeb ver. 8.42, <https://www.antweb.org>, accessed 29 October 2020 and multiple accessions of *L. acervorum*) supports a close relationship between *Formicoxenus* and *Leptothorax* (Fig. 2), similar to previous analyses based on limited taxa of this group (Ward et al., 2015; Prebus, 2017; Blaimer et al., 2018). As it has been previously suggested (Heinze & Ortius, 1991; Heinze & Gratiashvili, 2015; Schär et al., 2018), some taxa within the genus *Leptothorax*, particularly the Nearctic ones, represent species groups that deserve taxonomic adjustments. Our analyses suggest the presence of at least four Nearctic lineages (monophyletic groups with moderate to high support) comprising taxa currently assigned to *L. muscorum* (Nearctic), *L. canadensis*, *L. calderoni*, *L. sphagnicola* and specimens assigned to the *L. muscorum* complex. This latter complex seems to consist of a species group of three to four different taxa from the Nearctic that display a set of similar morphological characters and chromosome numbers (Brown, 1955; Heinze, 1989, 1991; Loiselle et al., 1990). One of these lineages also includes a specimen of *L. acervorum* (LT977594), which is more closely related to the Palearctic taxa *L. muscorum*, *L. gredleri* and *L. kutteri* (Fig. 2). This latter lineage deserves further exploration, as it involves determining whether *L. acervorum* in the Nearctic represents a separate lineage from the remaining samples we included.

In contrast, the Palearctic species *L. gredleri*, *L. muscorum* and the inquiline *L. kutteri* most likely represent monophyletic lineages (Fig. 2). The lineage of *L. acervorum* comprises both specimens from the Palearctic and Nearctic regions and our divergence age estimates suggest that this clade likely represents the most recent diversification event, within the last 0.5 Ma (Fig. 3). Despite the high support values observed for

most of the lineages in the Palearctic region, the support values for the mutual relationships of the lineages were low, and therefore more informative regions will be necessary to determine their relationships.

Evidence of isolated populations in the Iberian Peninsula with limited contribution to the most recent expansion of L. acervorum

Leptothorax acervorum is one of the only three ant species with Holarctic distribution (Schär et al., 2018); however, all current phylogeographic analyses of this species based on alloenzymes, SSRs, mtDNA and nuclear markers have included only Palearctic specimens (Stille & Stille, 1993; Heinze et al., 1994; Brandt et al., 2007; Foitzik et al., 2009; Gill et al., 2009; Trettin et al., 2016), thus providing an incomplete picture of the patterns of recolonization and populations structure. Previous phylogeographic analyses within *L. acervorum* species have been based on the COI 3'P region and indicate substantial genetic diversity within the species (Brandt et al., 2007; Foitzik et al., 2009; Trettin et al., 2016). The most recent analyses based on SSRs and mtDNA (COI 3'P region) have found the existence of multiple refugia in SW-Europe, and evidence of spatial genetic structure across the sampled area (Trettin et al., 2016). Our most extensive data set, including specimens we identified in the previous phylogenetic analyses (Fig. 2) from the Nearctic region and several populations from the Iberian Peninsula (IP), suggests that the IP populations represent the less divergent lineages and that they might have experienced fragmentation and isolation from the remaining Holarctic distribution (Fig. 6). *Leptothorax acervorum* is a cold-adapted species that in the IP inhabits mostly mountainous pinewoods and pine-dominated forest (*Pinus sylvestris*) above 1500 m.a.s.l (Felke & Buschinger, 1999; Gill et al., 2009). Our results indicate that all populations we included within the IP, except Larra and Pla de la Font, seem to have been more isolated from the remaining range of distribution of this species (Figs. 4, 5), supporting previous evidence based on SSRs, which have found evidence of bottlenecks and varying levels of connectivity in this area (Trettin et al., 2016). However, there are

Country	Locality	N	Haplotypes
Spain	Cadí-Moixeró N.P.	1	H1
	Neila refuge	16	H14, H15, H18, H17, H19
	Valdelinares	14	H13, H12
	Orihuela de Tremedal	19	H19
	Aragon	1	H16
	Pla de la Font	12	H5
	Larra	12	H11, H8, H7
UK	Santom Downham	7	H4, H11
Switzerland	Lausen, Wettingen	2	H4
Denmark	Nærum	1	H11
Norway	Østfold	1	H11
Sweden	Abisco	1	H11
Bulgaria	Panichishte	1	H9
Finland	Turku	1	H11
	Hikia	1	H11
	Lammi	1	H11
	Tvarminne	1	H11
Estonia	Liiva	1	H2
Russia	Kamchatka	2	H3
	Moscow	1	H10
Canada	Nearctic-West (Yukon, BC, NWT)	10	H11
	Nearctic-East (Manitoba)	5	H11

Table 1. Details of the sampling localities included in the phylogeographic analysis of *L. acervorum* across its Holarctic distribution and the haplotypes observed in each population. N = number of individuals in each locality, BC = British Columbia, NWT = Northwest Territories.

only a few mutations separating even the most divergent haplotypes among these populations, but these divergent haplotypes in the IP tend to be found in altitudinally restricted populations. In contrast, the most common haplotype (H11) is found in locations where *L. acervorum* is not altitudinally restricted (Fig. 6) and there is greater connectivity of suitable habitat. The lack of spatial genetic structure previously reported within *L. acervorum* using mtDNA (Brandt et al., 2007; Foitzik et al., 2009; Trettin et al., 2016) might be explained by the limited sampling outside the West-Palaearctic regions in previous studies, as well as that this lineage represents the group with the most recent expansion (Fig. 3). However, our sampling is still biased towards the West Palaearctic and East

Nearctic and our findings could be different when more samples from Alaska and Russia are included. Additional sampling across the Holarctic distribution with denser sampling among populations, together with the inclusion of additional markers, would be required to further expand the phylogeographic signal we recovered in our analyses.

Several refugia areas have been identified in the IP based on the ant species in this region (Tinaut & Ruano, 2021), and our results suggest that only populations from the Pyrenean refugia might have more recent connection with the rest of the West Palaearctic range of distribution (Figs. 4, 5). In contrast, the populations located in the Cantabric and the Northern Plateau (Tinaut & Ruano, 2021)

were likely more isolated from the rest of the populations. Cold-adapted species (boreal) with wide distribution in the Palearctic could survive in periglacial areas during the periods of maximum glacial expansion (e.g., during the LGM, 23-18 ka BP), expanding their range into southern areas. During periods of postglacial warming, southern populations of these species became isolated in

mountainous regions (Schmitt, 2009; Schmitt & Varga, 2012), surviving in southern refugia (Stewart et al., 2010). There is extensive evidence of the glacial-interglacial cycles during the Quaternary having influenced the individual genetic diversity and population structure of plants and animals in the West Palearctic (Bennett et al., 1991; Stewart et al., 2010; Schmitt & Varga, 2012; Morales-Barbero et

Subfamily	Species	Specimen code	Genbank	BioProject	Reference
MYRMICINAE Crematogastrini	<i>Vombisiridis bilongrudi</i>	D397		PRJNA393044	Prebus, 2017
	<i>Gauromyrmex bengkalisi</i>	CASENT0634314		PRJNA360290	Branstetter et al., 2017
	<i>Gauromyrmex</i> sp My01	P081		PRJNA393044	Prebus, 2017
	<i>Temnothorax nitens</i>	P017		PRJNA393044	Prebus, 2017
	<i>Temnothorax nylanderi</i>	P025		PRJNA393044	Prebus, 2017
	<i>Harpagoxenus sublaevis</i>	D1116		PRJNA393044	Prebus, 2017
	<i>Formicoxenus diversipilosus</i>	D1000		PRJNA393044	Prebus, 2017
	<i>Leptothorax acervorum</i>	L18-11-WP1		PRJNA634471	This study
	<i>Leptothorax acervorum</i>	SD17-1-WPXI		PRJNA634471	This study
	<i>Leptothorax acervorum</i>	PF18-10-WP1		PRJNA634471	This study
	<i>Leptothorax acervorum</i>	NR18-10-WP2		PRJNA634471	This study
	<i>Leptothorax acervorum</i>	OT18-10-WP3		PRJNA634471	This study
	<i>Leptothorax acervorum</i>	V18-10-WP1		PRJNA634471	This study
	<i>Leptothorax gredleri</i>	P089		PRJNA393044	Prebus, 2017
	<i>Leptothorax calderoni</i>	P070		PRJNA393044	Prebus, 2017
	<i>Leptothorax muscorum</i>	CASENT0639557		PRJNA360290	Branstetter et al., 2017
	<i>Vollenhovia emeryi</i>	VMER20160117	NC 030176	PRJNA321882	Liu et al., 2016
	<i>Tetramorium alpestre</i>	-	MK861065	-	Cicconardi et al., 2020
	<i>Tetramorium immigrans</i>	-	MK861067	-	Cicconardi et al., 2020
	<i>Tetramorium simillimum</i>	-	MK861069	-	Cicconardi et al., 2020
	<i>Tetramorium parvispinum</i>	-	MK861068	-	Cicconardi et al., 2020
	<i>Cardiocondyla obscurior</i>	COBS20161003	KX951753	-	Liu et al., 2019
	<i>Crematogaster teranishii</i>	-	NC 053534	PRJNA707849	Park et al., 2020a
<i>Pristomyrmex punctatus</i>	-	NC 015075	PRJNA62561	Hasegawa et al., 2011	
<i>Monomorium pharaonis</i>	-	MT038041	-	Park et al., unpublished	
<i>Monomorium triviale</i>	-	LC605004	-	Idogawa et al., 2021	
<i>Solenopsis geminata</i>	-	NC 014669	PRJNA60287	Gotzek et al., 2010	
<i>Solenopsis invicta</i>	-	NC 014672	PRJNA60287	Gotzek et al., 2010	
<i>Solenopsis ricteri</i>	-	NC 014677	PRJNA60287	Gotzek et al., 2010	
<i>Aphaenogaster famelica</i>	-	NC 049859	PRJNA648238	Park et al., 2019a	
<i>Novomesor albisetosa</i>	-	MK861057	-	Cicconardi et al., 2020	
<i>Pheidole flavens</i>	-	MG253276	-	Pires and Togawa, unpublished	
<i>Wasmannia auropunctata</i>	WA20160314	NC 030541	PRJNA328712	Duan et al., 2016	
<i>Cyphomyrmex costatus</i>	-	MK861059	-	Cicconardi et al., 2020	
<i>Tachymyrmex zeteki</i>	-	MK861060	-	Cicconardi et al., 2020	
<i>Tachymyrmex cornetzi</i>	-	MK861061	-	Cicconardi et al., 2020	
<i>Tachymyrmex septentrionalis</i>	-	MK861062	-	Cicconardi et al., 2020	
<i>Acromyrmex echinator</i>	-	MK861063	-	Cicconardi et al., 2020	
<i>Atta colombica</i>	-	KY950644	-	Almeida, unpublished	
<i>Atta laevigata</i>	-	KC346251	-	Rodovhalo et al., 2014	
<i>Atta opaciceps</i>	-	KY950643	-	Almeida, unpublished	
<i>Atta sexdens</i>	-	MG253258	-	Pires and Togawa, unpublished	
<i>Atta texana</i>	-	MF417380	-	Almeida, unpublished	
<i>Myrmica scabrinoides</i>	-	LN607806	-	Babbucci et al., 2014	
<i>Myrmica incompleta</i>	Ex808		-		
<i>Pogonomyrmex occidentalis</i>	SRR13003365	BK012408		Alli et al., 2020	
PONERINAE	<i>Ectomyrmex javanus</i>	-	NC 042678	PRJNA550746	Park et al., 2019b
DOLICHODELINAE	<i>Tapinoma melanocephalum</i>	-	MN397938	-	Du et al., 2019
	<i>Ochetelus glaber</i>	-	MN044390	-	Park et al., 2020b

Table S1. List of specimens used in the phylogenomic analysis of the *Formicoxenus* genus-group using whole mitochondrial genomes. The specimens represent the six genera currently recognized in the group and the outgroup species.

Species	Voucher ID	Year collected	Locality	Country	Co-lony	caste sex	Lat	Long	Genbank	Reference	Bioproject
<i>Harpagoxenus sublaevis</i>	286 98 FI TV	27.6.1998	Tvärminne Zoological Station, Hanko	Finland			59.844464	23.244397	OL376482	This study	PRJNA634471
<i>Harpagoxenus sublaevis</i>	390 98 FI L	24.6.1998	Lammi, Hämeenlinna	Finland			61.031732	24.978149	OL376483	This study	PRJNA634471
<i>Harpagoxenus sublaevis</i>	D1116			Poland						Prebus 2017	PRJNA393044
<i>Formicoxenus diversipilosus</i>	D1000		British Columbia	Canada						Prebus 2017	PRJNA393044
<i>Formicoxenus nitidulus</i>	FRMAA093-20	8.17.2013	Ticino	Switzerland			46.252	8.671			BOLD SYSTEM
<i>Leptothorax retractus</i>	GANAA241-04	7.27.2004	North Western Territories	Canada							BOLD SYSTEM
<i>Leptothorax sphagnicola</i>	GANAA681-06		Ontario	Canada							BOLD SYSTEM
<i>Leptothorax sphagnicola</i>	ASAFR-13		Ontario	Canada							BOLD SYSTEM
<i>Leptothorax canadensis</i>			Manitoba	Canada					FJ413482	Smith et al., 2009	PRJNA472144
<i>Leptothorax canadensis</i>			Nova Scotia	Canada					HM413717	iBOL, Unpub.	
<i>Leptothorax canadensis</i>			New Brunswick	Canada					KR407717	Hebert et al., Unpub.	PRJNA472144
<i>Leptothorax canadensis</i>			Manitoba	Canada					FJ413481	Smith et al., 2009	PRJNA472144
<i>Leptothorax canadensis</i>			Alberta	Canada					HQ928811	iBOL, Unpub.	
<i>Leptothorax canadensis</i>			Manitoba	Canada					FJ413454	Smith et al., 2009	PRJNA472144
<i>Leptothorax canadensis</i>			Saskatoon	Canada					JN291906	iBOL, Unpub.	
<i>Leptothorax canadensis</i>			Newfoundland	Canada					HM413821	iBOL, Unpub.	
<i>Leptothorax canadensis</i>			Newfoundland	Canada					HM413647	iBOL, Unpub.	
<i>Leptothorax calderoni</i>	P070		Oregon	USA						Prebus 2017	PRJNA393044
<i>Leptothorax kutteri</i>	214 00 EE	11.07.2000	Liiva Putla, Saare County (Saaremaa)	Estonia			58.3892	22.655535	OL376569	This study	PRJNA634471
<i>Leptothorax kutteri</i>	450 00 FI	23.7.2000	Hikiä, Hausjärvi	Finland			60.762284	24.904418	OL376570	This study	PRJNA634471
<i>Leptothorax muscorum</i>	348 98 PL	21.8.1998	Pieniny Mountains	Poland			49.413611	20.352778	OL376484	This study	PRJNA634471
<i>Leptothorax muscorum</i>				USA			65.22	-148.06	LT977601	Schar et al., 2017	PRJEB24435
<i>Leptothorax muscorum</i>				USA			40.56	-110.29	LT977600	Schar et al., 2017	PRJEB24435
<i>Leptothorax muscorum</i>				USA			46.91	-123.08	LT977598	Schar et al., 2017	PRJEB24435
<i>Leptothorax muscorum</i>				USA					LT977602	Schar et al., 2017	PRJEB24435
<i>Leptothorax muscorum</i>	GANAA222-04		North Western Territories	Canada							BOLD SYSTEM
<i>Leptothorax muscorum</i>			British Columbia	Canada					HQ928807	iBOL, Unpub.	
<i>Leptothorax muscorum</i>			British Columbia	Canada					HQ928830	iBOL, Unpub.	
<i>Leptothorax muscorum</i>			Alberta	Canada					MG483102	Dewaard Unpub.	PRJNA472144
<i>Leptothorax muscorum</i>			Alberta	Canada					JN291877	iBOL, Unpub.	
<i>Leptothorax muscorum</i>			British Columbia	Canada					JN291854	iBOL, Unpub.	
<i>Leptothorax muscorum</i>			British Columbia	Canada					JN291973	iBOL, Unpub.	
<i>Leptothorax muscorum</i>			British Columbia	Canada					JN292222	iBOL, Unpub.	
<i>Leptothorax muscorum</i>			British Columbia	Canada					JN292224	iBOL, Unpub.	
<i>Leptothorax muscorum</i>			Alberta	Canada					JN292079	iBOL, Unpub.	
<i>Leptothorax muscorum</i>				USA					KU875361	Sikes et al., 2017	
<i>Leptothorax muscorum</i>			British Columbia	Canada					HQ961416	Stahlhut et al., 2013	PRJNA37833
<i>Leptothorax muscorum</i>	ASANA431-06		Ontario	Canada							BOLD SYSTEM
<i>Leptothorax muscorum</i>				Norway			59.13	9.67	LT977597	Schar et al., 2017	
<i>Leptothorax muscorum</i>				Spain			42.27	1.7	LT977599	Schar et al., 2017	
<i>Leptothorax muscorum</i> complex			Saskatoon	Canada					JN291889	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex			Manitoba	Canada					JF902870	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex			Manitoba	Canada					JF902871	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex			Manitoba	Canada					JF902874	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex			Manitoba	Canada					JF902875	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	YUKIN-0425		Yukon	Canada			69.2988	-140.294	HQ569290	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	CHU06-ANT-506		Manitoba	Canada			58.75	-93.9	JF902873	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	YUKIN-0426		Yukon	Canada			69.2988	-140.294	HQ569291	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	YUKIN-0427		Yukon	Canada			69.2988	-140.294	HQ569292	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	BIOUG17365-B10		North Western Territories	Canada			61.607	-125.758	KR804403	Dewaard Unpub.	PRJNA472144
<i>Leptothorax sp.</i> AF CAN				USA			44.3	-71.28	LT977603	Schar et al., 2017	PRJEB24435

<i>Leptothorax gredleri</i>				Poland			51.15	17.08	LT977596	Schar et al., 2017	PRJEB24435
<i>Leptothorax gredleri</i>	P089			Poland						Prebus 2017	PRJNA393044
<i>Leptothorax acervorum</i>	215 00 EE	11.07.2000	Liiva Putla, Saare Countu (Saaremaa)	Estonia			58.3892	22.655535	OL376548	This study	PRJNA634471
<i>Leptothorax acervorum</i>	450 00 FI H	23.7.2000	Hikiä, Hausjärvi	Finland			60.762284	24.904418	OL376563	This study	PRJNA634471
<i>Leptothorax acervorum</i>	285 98 FI	27.6.1998	Tväminne Zoological Station, Hanko	Finland			59.844464	23.244397	OL376564	This study	PRJNA634471
<i>Leptothorax acervorum</i>	324 00 SP	31.7.2000	Broncahles, Teruel, Aragón,	Spain			40.503548	-1.633219	OL376503	This study	PRJNA634471
<i>Leptothorax acervorum</i>	391 98 FI	24.6.1998	Lammi, Hämeenlinna	Finland			61.031732	24.978149	OL376562	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NOANT032-12	20.06.2008	Ostfold	Norway			59	11		BOLD SYSTEM	PRJNA634471
<i>Leptothorax acervorum</i>			Alaska	USA			65.57	-144.84	LT977594	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>				Bulgaria			42.22	23.3	LT977583	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>				Denmark			55.83	12.56	LT977584	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>				Finland			60.51	22.27	LT977585	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Kamchatka	Russia			53.16	158.14	LT977587	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Kamchatka	Russia			55.94	158.69	LT977586	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Moscow	Russia			55.12	37.7	LT977593	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Cadi-Moixeró N.P.	Spain			42.3	1.92	LT977595	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Orihuela de Tremedal	Spain			40.52	-1.65	LT977588	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Orihuela de Tremedal	Spain			40.52	-1.65	LT977589	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>				Sweden			68.32	18.86	LT977590	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>				Switzerland			47.47	7.78	LT977592	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>				Switzerland			47.4	8.36	LT977591	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>	L18 11 WP1	2018	Larra	Spain	11	worker pupae	42.95238	-0.79397	OL376565	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 3 MP2	2018	Neila refuge	Spain	3	male pupae	42.0435	-3.04209	OL376505	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 4 WP1	2018	Orihuela de Tremedal	Spain	4	worker pupae	40.52867	-1.64288	OL376514	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 10 WP1	2018	Pla de la Font	Spain	10	worker pupae	42.602	1.07638	OL376533	This study	PRJNA634471
<i>Leptothorax acervorum</i>	SD17 1 WPX	2017	Santon Downham	UK	1	worker pupae	52.47132	0.67344	OL376549	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 1 M7	2018	Valdelinares	Spain	1	male	40.38473	-0.64038	OL376493	This study	PRJNA634471
<i>Leptothorax sp.</i>			Alberta	Canada			49.065	-113.778	KR929970	Hebert et al., 2016	PRJNA472144
<i>Leptothorax sp.</i>			New Brunswick	Canada			45.5894	-64.9556	KR407717	Hebert et al., 2013	PRJNA472144
<i>Leptothorax sp.</i>			Ontario	Canada			44.28	-78.529	MG335482	Dewaard Unpub.	PRJNA472144
<i>Leptothorax sp.</i>			Manitoba	Canada			58.678	-94.142	KC502543	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.731	-93.781	KC502534	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.678	-94.142	KC502537	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.786	-94.202	KC502520	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.678	-94.142	KC502539	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.758	-94.148	KC502527	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.758	-94.148	KC502530	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.758	-94.148	KC502528	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			Manitoba	Canada			58.661	-93.833	KC502533	Smith and Adamowicz, Unpub.	
<i>Leptothorax sp.</i>			British Columbia	Canada			54.7614	-126.931	MG340673	Dewaard Unpub.	PRJNA472144
<i>Leptothorax sp.</i>			British Columbia	Canada			54.7614	-126.931	MG343545	Dewaard Unpub.	PRJNA472144
<i>Leptothorax sp.</i>			British Columbia	Canada			54.7614	-126.931	MG341808	Dewaard Unpub.	PRJNA472144
<i>Leptothorax sp.</i>			British Columbia	Canada			54.7614	-126.931	MG336498	Dewaard Unpub.	PRJNA472144
<i>Leptothorax sp.</i>			British Columbia	Canada			54.7614	-126.931	MG334898	Dewaard Unpub.	PRJNA472144
<i>Leptothorax sp.</i>			British Columbia	Canada			54.7614	-126.931	MG335505	Dewaard Unpub.	PRJNA472144

Table S2. List of specimens employed in the phylogenetic analyses of *Formicoxenus* - *Leptothorax* using the cytochrome COI-5P region (658 bp).

Species	Voucher ID	Year collected	Population	Country	Colony	caste_sex	Lat	Long	Genbank	Reference	Bioproject
<i>Leptothorax acervorum</i>	215 00 EE	11.07.2000	Liiva Putla, Saare Countu (Saaremaa)	Estonia			58.3892	22.655535	OL376548	This study	PRJNA634471
<i>Leptothorax acervorum</i>	450 00 FI H	23.7.2000	Hikiä, Hausjärvi	Finland			60.762284	24.904418	OL376563	This study	PRJNA634471
<i>Leptothorax acervorum</i>	285 98 FI	27.6.1998	Tvärminne Zoological Station, Hanko	Finland			59.844464	23.244397	OL376564	This study	PRJNA634471
<i>Leptothorax acervorum</i>				Finland			60.51	22.27	LT977585	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>	391 98 FI	24.6.1998	Lammi, Hämeenlinna	Finland			61.031732	24.978149	OL376562	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NOANT032-12	20.06.2008	Ostfold	Norway			59	11		BOLD SYSTEM	
<i>Leptothorax acervorum</i>			Panichishte	Bulgaria			42.22	23.3	LT977583	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Nerum	Denmark			55.83	12.56	LT977584	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Kamchatka	Russia			53.16	158.14	LT977587	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Kamchatka	Russia			55.94	158.69	LT977586	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Moscow	Russia			55.12	37.7	LT977593	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Abisco	Sweden			68.32	18.86	LT977590	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Lausen	Switzerland			47.47	7.78	LT977592	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Wettingen	Switzerland			47.4	8.36	LT977591	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>	324 00 SP	31.7.2000	Bronchales, Teruel, Aragón,	Spain			40.503548	-1.633219	OL376503	This study	PRJNA634471
<i>Leptothorax acervorum</i>			Cadi Moixero	Spain			42.3	1.92	LT977595	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>	L18 11 WP1	2018	Larra	Spain	11	worker pupae	42.95238	-0.79397	OL376565	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 11 WP2	2018	Larra	Spain	11	worker pupae	42.95238	-0.79397	OL376566	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 2 WP1	2018	Larra	Spain	2	worker pupae	42.95238	-0.79397	OL376560	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 3 WP2	2018	Larra	Spain	3	worker pupae	42.95238	-0.79397	OL376567	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 5 WP1	2018	Larra	Spain	5	worker pupae	42.95238	-0.79397	OL376553	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 6 WP2	2018	Larra	Spain	6	worker pupae	42.95238	-0.79397	OL376554	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 7 AQ1	2018	Larra	Spain	7	alate queen	42.95238	-0.79397	OL376559	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 7 M2	2018	Larra	Spain	7	male	42.95238	-0.79397	OL376557	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 7 M3	2018	Larra	Spain	7	male	42.95238	-0.79397	OL376556	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 7 MP1	2018	Larra	Spain	7	male pupae	42.95238	-0.79397	OL376558	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 7 MP2	2018	Larra	Spain	7	male pupae	42.95238	-0.79397	OL376555	This study	PRJNA634471
<i>Leptothorax acervorum</i>	L18 9 WP1	2018	Larra	Spain	9	worker pupae	42.95238	-0.79397	OL376568	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 10 WP2	2018	Niela refuge	Spain	10	worker pupae	42.0435	-3.04209	OL376500	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 11 MP2	2018	Niela refuge	Spain	11	male pupae	42.0435	-3.04209	OL376501	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 12 WP2	2018	Niela refuge	Spain	12	worker pupae	42.0435	-3.04209	OL376508	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 14 WP1	2018	Niela refuge	Spain	14	worker pupae	42.0435	-3.04209	OL376510	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 16 MP1	2018	Niela refuge	Spain	16	male pupae	42.0435	-3.04209	OL376511	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 16 MP6	2018	Niela refuge	Spain	16	male pupae	42.0435	-3.04209	OL376512	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 17 WP1	2018	Niela refuge	Spain	17	worker pupae	42.0435	-3.04209	OL376545	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 1 WP2	2018	Niela refuge	Spain	1	worker pupae	42.0435	-3.04209	OL376504	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 2 WP2	2018	Niela refuge	Spain	2	worker pupae	42.0435	-3.04209	OL376502	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 3 MP2	2018	Niela refuge	Spain	3	male pupae	42.0435	-3.04209	OL376505	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 4 WP1	2018	Niela refuge	Spain	4	worker pupae	42.0435	-3.04209	OL376509	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 5 MP4	2018	Niela refuge	Spain	5	male pupae	42.0435	-3.04209	OL376506	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 5 WP2	2018	Niela refuge	Spain	5	worker pupae	42.0435	-3.04209	OL376507	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 7 M3	2018	Niela refuge	Spain	7	male	42.0435	-3.04209	OL376546	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 7 MP1	2018	Niela refuge	Spain	7	male pupae	42.0435	-3.04209	OL376547	This study	PRJNA634471
<i>Leptothorax acervorum</i>	NR18 9 WP2	2018	Niela refuge	Spain	9	worker pupae	42.0435	-3.04209	OL376499	This study	PRJNA634471
<i>Leptothorax acervorum</i>			Orihuela de Tremedal	Spain			40.52	-1.65	LT977588	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>			Orihuela de Tremedal	Spain			40.52	-1.65	LT977589	Schar et al., 2017	PRJEB24435
<i>Leptothorax acervorum</i>	OT18 10 WP3	2018	Orihuela de Tremedal	Spain	10	worker pupae	40.52867	-1.64288	OL376523	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 12 WP1	2018	Orihuela de Tremedal	Spain	12	worker pupae	40.52867	-1.64288	OL376522	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 14 WP1	2018	Orihuela de Tremedal	Spain	14	worker pupae	40.52867	-1.64288	OL376524	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 2 WP1	2018	Orihuela de Tremedal	Spain	2	worker pupae	40.52867	-1.64288	OL376561	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 3 M1	2018	Orihuela de Tremedal	Spain	3	male	40.52867	-1.64288	OL376516	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 3 W1	2018	Orihuela de Tremedal	Spain	3	worker	40.52867	-1.64288	OL376515	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 3 WP2	2018	Orihuela de Tremedal	Spain	3	worker pupae	40.52867	-1.64288	OL376528	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 4 MP1	2018	Orihuela de Tremedal	Spain	4	male pupae	40.52867	-1.64288	OL376514	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 4 WP1	2018	Orihuela de Tremedal	Spain	4	worker pupae	40.52867	-1.64288	OL376529	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 5 MP2	2018	Orihuela de Tremedal	Spain	5	male pupae	40.52867	-1.64288	OL376526	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 5 WP1	2018	Orihuela de Tremedal	Spain	5	worker pupae	40.52867	-1.64288	OL376530	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 6	2018	Orihuela de Tremedal	Spain	6	?	40.52867	-1.64288	OL376525	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 7 MP2	2018	Orihuela de Tremedal	Spain	7	male pupae	40.52867	-1.64288	OL376527	This study	PRJNA634471

<i>Leptothorax acervorum</i>	OT18 7 MP4	2018	Orihuela de Tremedal	Spain	7	male pupae	40.52867	-1.64288	OL376518	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 7 WP2	2018	Orihuela de Tremedal	Spain	7	worker pupae	40.52867	-1.64288	OL376519	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 8 M1	2018	Orihuela de Tremedal	Spain	8	male	40.52867	-1.64288	OL376513	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 8 M2	2018	Orihuela de Tremedal	Spain	8	male	40.52867	-1.64288	OL376517	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 8	2018	Orihuela de Tremedal	Spain	8	?	40.52867	-1.64288	OL376521	This study	PRJNA634471
<i>Leptothorax acervorum</i>	OT18 9 WP1	2018	Orihuela de Tremedal	Spain	9	worker pupae	40.52867	-1.64288	OL376520	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 10 WP1	2018	Pla de la Font	Spain	10	worker pupae	42.602	1.07638	OL376533	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 11 WP2	2018	Pla de la Font	Spain	11	worker pupae	42.602	1.07638	OL376534	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 14 M1	2018	Pla de la Font	Spain	14	male	42.602	1.07638	OL376535	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 15 W1	2018	Pla de la Font	Spain	15	worker	42.602	1.07638	OL376543	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 16 M1	2018	Pla de la Font	Spain	16	male	42.602	1.07638	OL376540	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 16 W1	2018	Pla de la Font	Spain	16	worker	42.602	1.07638	OL376541	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 2 WP2	2018	Pla de la Font	Spain	2	worker pupae	42.602	1.07638	OL376542	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 3 AQ1	2018	Pla de la Font	Spain	3	alate queen	42.602	1.07638	OL376544	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 4 WP5	2018	Pla de la Font	Spain	4	worker pupae	42.602	1.07638	OL376536	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 5 AQ3	2018	Pla de la Font	Spain	5	alate queen	42.602	1.07638	OL376537	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 6 WP1	2018	Pla de la Font	Spain	6	worker pupae	42.602	1.07638	OL376538	This study	PRJNA634471
<i>Leptothorax acervorum</i>	PF18 8 WP1	2018	Pla de la Font	Spain	8	worker pupae	42.602	1.07638	OL376539	This study	PRJNA634471
<i>Leptothorax acervorum</i>	SD17 1 WPXI	2017	Santon Downham	UK	1	worker pupae	52.47132	0.67344	OL376551	This study	PRJNA634471
<i>Leptothorax acervorum</i>	SD17 1 WPX	2017	Santon Downham	UK	1	worker pupae	52.47132	0.67344	OL376549	This study	PRJNA634471
<i>Leptothorax acervorum</i>	SD17 3 MPXIII	2017	Santon Downham	UK	3	male pupae	52.47132	0.67344	OL376531	This study	PRJNA634471
<i>Leptothorax acervorum</i>	SD17 3 MPXII	2017	Santon Downham	UK	3	male pupae	52.47132	0.67344	OL376532	This study	PRJNA634471
<i>Leptothorax acervorum</i>	SD17 5 QP2	2017	Santon Downham	UK	5	queen pupae	52.47132	0.67344	OL376550	This study	PRJNA634471
<i>Leptothorax acervorum</i>	SD17 5 QP6	2017	Santon Downham	UK	5	queen pupae	52.47132	0.67344	OL376552	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 10 WP1	2018	Valdelinares	Spain	10	worker pupae	40.38473	-0.64038	OL376497	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 11 WP2	2018	Valdelinares	Spain	11	worker pupae	40.38473	-0.64038	OL376496	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 13 WP1	2018	Valdelinares	Spain	13	worker pupae	40.38473	-0.64038	OL376488	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 14 WP2	2018	Valdelinares	Spain	14	worker pupae	40.38473	-0.64038	OL376498	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 15 WP2	2018	Valdelinares	Spain	15	worker pupae	40.38473	-0.64038	OL376490	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 16 WP2	2018	Valdelinares	Spain	16	worker pupae	40.38473	-0.64038	OL376491	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 1 M6	2018	Valdelinares	Spain	1	male	40.38473	-0.64038	OL376492	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 1 M7	2018	Valdelinares	Spain	1	male	40.38473	-0.64038	OL376493	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 2 MP3	2018	Valdelinares	Spain	2	male pupae	40.38473	-0.64038	OL376486	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 2 WP2	2018	Valdelinares	Spain	2	worker pupae	40.38473	-0.64038	OL376494	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 5 WP2	2018	Valdelinares	Spain	5	worker pupae	40.38473	-0.64038	OL376489	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 7 M7	2018	Valdelinares	Spain	7	male	40.38473	-0.64038	OL376493	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 8 MP2	2018	Valdelinares	Spain	8	male pupae	40.38473	-0.64038	OL376487	This study	PRJNA634471
<i>Leptothorax acervorum</i>	V18 9 AQP1	2018	Valdelinares	Spain	9	queen pupae	40.38473	-0.64038	OL376495	This study	PRJNA634471
<i>Leptothorax muscorum</i> complex	CHU06-ANT-506		Manitoba	Canada			58.75	-93.9	JF902873	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	YUKIN-0425		Yukon	Canada			69.2988	-140.294	HQ569290	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	YUKIN-0426		Yukon	Canada			69.2988	-140.294	HQ569291	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	YUKIN-0427		Yukon	Canada			69.2988	-140.294	HQ569292	iBOL, Unpub.	
<i>Leptothorax muscorum</i> complex	BIOUG17365-B10		North Western Territories	Canada			61.607	-125.758	KR804403	Dewaard Unpub.	PRJNA472144
<i>Leptothorax</i> sp.			Manitoba	Canada			58.758	-94.148	KC502527	Smith & Adamowicz, Unpub.	
<i>Leptothorax</i> sp.			Manitoba	Canada			58.758	-94.148	KC502530	Smith & Adamowicz, Unpub.	
<i>Leptothorax</i> sp.			Manitoba	Canada			58.758	-94.148	KC502528	Smith & Adamowicz, Unpub.	
<i>Leptothorax</i> sp.			Manitoba	Canada			58.661	-93.833	KC502533	Smith & Adamowicz, Unpub.	
<i>Leptothorax</i> sp.			British Columbia	Canada			54.7614	-126.931	MG340673	Dewaard Unpub.	PRJNA472144
<i>Leptothorax</i> sp.			British Columbia	Canada			54.7614	-126.931	MG343545	Dewaard Unpub.	PRJNA472144
<i>Leptothorax</i> sp.			British Columbia	Canada			54.7614	-126.931	MG341808	Dewaard Unpub.	PRJNA472144
<i>Leptothorax</i> sp.			British Columbia	Canada			54.7614	-126.931	MG336498	Dewaard Unpub.	PRJNA472144
<i>Leptothorax</i> sp.			British Columbia	Canada			54.7614	-126.931	MG334898	Dewaard Unpub.	PRJNA472144
<i>Leptothorax</i> sp.			British Columbia	Canada			54.7614	-126.931	MG335505	Dewaard Unpub.	PRJNA472144

Table S3. Specimens of *L. acervorum* used in the phylogeographic and genetic diversity analyses across its distribution range in the Holarctic region.

Species	Specimen code	Length (bp)	GC content (%)	BioProject	Reference
<i>Vombisiridis bilongrudi</i>	D397	15,518	23.84	PRJNA393044	Prebus, 2017
<i>Gauromyrmex bengalalis</i>	CASENT0634314	17,519	25.50	PRJNA360290	Branstetter et al., 2017
<i>Gauromyrmex</i> sp My01	P081	17,673	24.55	PRJNA393044	Prebus, 2017
<i>Temnothorax nitens</i>	P017	16,060	21.90	PRJNA393044	Prebus, 2017
<i>Temnothorax nylanderii</i>	P025	16,165	20.97	PRJNA393044	Prebus, 2017
<i>Harpagoxenus sublaevis</i>	D1116	15,699	21.75	PRJNA393044	Prebus, 2017
<i>Formicoxenus diversipilosus</i>	D1000	15,674	22.74	PRJNA393044	Prebus, 2017
<i>Leptothorax acervorum</i>	L18-11-WP1	17,077	21.84	PRJNA634471	This study
<i>Leptothorax gredleri</i>	P089	16,296	21.74	PRJNA393044	Prebus, 2017
<i>Leptothorax calderoni</i>	P070	16,227	23.17	PRJNA393044	Prebus, 2017
<i>Leptothorax muscorum</i>	CASENT0639557	15,999	23.56	PRJNA360290	Branstetter et al., 2017
<i>Myrmica incompleta</i>	Ex808	15,604	19.86	-	

Table S4. Sizes and GC content of the mitochondrial genomes assembled with MitoFinder.

al., 2018), including the presence of several periglacial and southern refugia of cold-tolerant of *Myrmica* (Leppänen et al., 2011, 2013) and *Formica* ant species (Goropashnaya et al., 2007, 2004). Emerging evidence seems to indicate that these glacial-interglacial cycles could also have shared refugia; for example, the congruent phylogeographic signal between *Myrmica* ants and *Betula* species (Maliouchenko et al., 2007; Leppänen et al., 2011), the leaf beetle *Gonioctena intermedia* and its boreal-temperate host trees *Prunus padus* and *Sorbus aucuparia* (Quinzin et al., 2017), and the similar patterns of isolated populations in the Iberian Peninsula observed between *L. acervorum* (Trettin et al., 2016) and *Pinus sylvestris* (Dering et al., 2017; Tyrmi et al., 2020).

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