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SPECIAL ISSUE ARTICLE

Phylogeography and cryptic speciation in the *Myrmica scabrinodis* Nylander, 1846 species complex (Hymenoptera: Formicidae), and their conservation implications

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Abstract. 1. *Myrmica scabrinodis* is one of the commonest European ant species, but field observations of variable ecology and behaviour have suggested the existence of several ecotypes or even cryptic species within this ant.

2. To address this hypothesis, we reconstructed the molecular phylogeny of *M. scabrinodis* and 15 related species based on 1089 base pairs of mitochondrial genes cytochrome B (Cyt-B) and cytochrome oxidase subunit I (COI).

3. We show that two major lineages occur throughout Europe. The observed sequence divergence between the two *M. scabrinodis* lineages is similar to or greater than that observed between the other investigated species.

4. On a local scale, the lineages are both observed at the wet and dry extremes of the overall *M. scabrinodis* niche distribution, but analysis of the *Myrmica* communities in two sympatric populations shows that lineage B tends to avoid the drier habitat patches.

5. Our inferred phylogenetic relationship of intra- and inter-specific mitochondrial lineages within the *M. scabrinodis* species group in general shows several inconsistencies with the presently accepted taxonomy, suggesting the potential existence of more unrecognised cryptic species.

6. The separate status of other species is not supported, particularly the differentiation between *Myrmica sabuleti* and *Myrmic*a *lonae*, and specimens identified as *Myrmica tulinae* have highly inconsistent mitochondrial haplotypes, suggesting that the morphology associated with this taxon does not reflect phylogeny.

7. The existence of multiple lineages within *M. scabrinodis*, and the apparent synonymy between *M. lonae* and *M. sabuleti* has implications for the conservation of *Maculinea* butterflies, for which these are major hosts.

Key words. cytochrome B, cytochrome oxidase I, post-glacial recolonisation, ants, *Maculinea*, *Phengaris*.

Introduction

Myrmica Latreille, 1804[1] is one of the most common and species-rich ant genera in the Holarctic region. In the old world, it has been divided into 17 species groups based on male and female morphology, of which seven are represented in Europe: the *rubra-, schencki-, lobicornis-* and *scabrinodis*-groups and the workerless socially parasitic *laurae-*, *karavajevi-*, *myrmicoxena*-groups (Radchenko & Elmes, 2010). Recent molecular phylogenies of *Myrmica* have supported these species groups (Jansen *et al*., 2009, 2010), although the morphologically specialised workerless social parasite groups are generally found within the species group of their free-living hosts (Vepsalainen *et al*., 2009; Jansen *et al*., 2010). The genus *Myrmica* is of interest for both sociobiological and ecological reasons. It has, for example, been studied because of (1) the highly variable number of queens per nest, and the resultant variation in relatedness (Elmes & Keller, 1993; Seppä, 1996; Evans, 1998; Pedersen &

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[1] Taxonomic authorities are given in "small caps" style throughout the text, to distinguish them from in-text citations. Those for the ant species used for phylogenetic reconstruction are given in Table 1 rather than in the text.

Boomsma, 1999a, 1999b). (2) The high frequency of inquiline social parasites with different stages of specialisation (Savolainen & Vepsalainen, 2003; Vepsalainen *et al*., 2009). (3) The recent emergence of at least one supercolonial invasive species – *Myrmica rubra* in North America (Groden *et al*., 2005; Gammans *et al*., 2018), a syndrome that may also occur in native populations of *M. rubra* and several other species (van der Hammen *et al*., 2002). (4) The extensive but highly variable suitability of species as hosts for *Maculinea* van Eecke, 1915 (= *Phengaris* Docherty, 1891) butterflies (Tartally *et al*., 2019; see also the other papers in this special edition), which are icons of insect conservation (Thomas *et al*., 2009; Stearns & Stearns, 2010).

New species of *Myrmica* have been described in most species groups in recent years, but the taxonomy of the *scabrinodis* group has been particularly debated, because morphological variation within species has often been confusingly large (Radchenko & Elmes, 2004, 2010; Seifert, 2011). Several intra-specific forms have been elevated to species level (Seifert, 2000, 2005; Radchenko *et al*., 2002; Radchenko & Elmes, 2004, 2010; Seifert *et al*., 2009) and other *scabrinodis*-group species have been described as new to science (Espadaler, 1996; Elmes *et al*., 2002; Radchenko & Elmes, 2004; Seifert *et al*., 2014). However, two decades of extensive morphological investigations of type material and samples from entire species ranges have also resulted in a number of synonymisations following substantial revisions of the *scabrinodis* group (Seifert, 1988, 2011; Dlussky *et al*., 1990; Radchenko, 1994a,b; Radchenko & Elmes, 2010). The result has been that the *scabrinodis* group presently comprises some 22 accepted species (Radchenko & Elmes, 2010; Seifert, 2011; Seifert *et al*., 2014).

The *scabrinodis* group is further subdivided into the *bergi-, rugulosa-, specioides-*, *sabuleti-* and *scabrinodis*-species complexes (Radchenko & Elmes, 2010). The *scabrinodis*-group species are distributed throughout the western Palæarctic, with an overall Eastern limit at lake Baikal, as only *Myrmica divergens* occurs further east (Radchenko & Elmes, 2010). The Southern limit of the species complex is the Mediterranean Sea, with the exception of *Myrmica cagnianti*, which is endemic to Northern Africa (Espadaler, 1996).

As a species, *Myrmica. scabrinodis* is one of the most common European *Myrmica* ants. Field studies have shown that its ecology, habitat preference, and behaviour are highly variable, so that the possible occurrence of several ecotypes or even cryptic species has been repeatedly suggested (Elmes *et al*., 1994, 1998; Radchenko & Elmes, 2010). *Myrmica. scabrinodis* is often associated with the wetter and cooler parts of the total range of possible *Myrmica* niches, with nests being situated just above water level in moist meadows and marshes, with *Myrmica. ruginodis* and *M. rubra* in the slightly drier patches. However, in dry habitats *M. scabrinodis* often switches position in this gradient with *M. rubra* and *M. ruginodis* to be found in almost as dry patches as the most xerothermophilic species *M. sabuleti* and *M. specioides* (Elmes *et al*., 1998; Radchenko & Elmes, 2010). Investigations of British hillsides have shown that *M. scabrinodis* can even occasionally be found in drier and warmer patches than *M. sabuleti* (Graham Elmes, pers. comm.).

The highly variable habitat preferences of *M. scabrinodis* have earlier led to the description of the separate species *Myrmica rugulosoides*, specialised on sphagnum bog habitat (Forel, 1915), but Seifert (1984) synonymised *M. scabrinodis* and *M. rugulosoides* because of insufficient morphological differentiation. Elmes *et al*. (1994) observed some degree of size and activity differentiation related to habitat, but detailed morphological investigations throughout Central Europe have failed to produce diagnostic traits that correlated with habitat or ecology (Seifert, 1988; Elmes *et al*., 1994; Radchenko, 1994a; Radchenko & Elmes, 2004, 2010); although a geometric analysis has recently identified a cryptic species (*M. martini* Seifert, Bagherian Yazdi et Schultz, 2014) within *M. scabrinodis,* which seems to be geographically limited to the Pyrenees and western Alps (Seifert *et al*., 2014). A pattern of variable habitat preferences correlated with worker morphology within *M. sabuleti*, however, did lead to the resurrection of *M. lonae* as an independent species (Seifert, 2000).

Myrmica species are host to a substantial number of myrmecophiles, social parasites and inquiline species (Donisthorpe, 1927; Savolainen & Vepsalainen, 2003; Vepsalainen *et al*., 2009; Witek *et al*., 2014). Considerable recent research effort has focused on the socially parasitic larvae of large blue butterflies of the genus *Maculinea*, which tend to specialise on one or a few species of *Myrmica* host ants whose distributions need to overlap with the specific food plants on which the adult butterflies oviposit (Tartally *et al*., 2019). Ambiguity about possible cryptic species within the *sabuleti* and *scabrinodis* complexes has therefore been considered a potential problem for *Maculinea* conservation (Elmes *et al*., 1998). As phylogenetic reconstructions based on mitochondrial sequence data have earlier proven to be highly valuable for assessing the evolutionary relationships of other *Myrmica* species groups (Savolainen & Vepsalainen, 2003; Steiner *et al*., 2006; Jansen et al., 2009, 2010; Vepsalainen *et al*., 2009), we therefore decided to use these genetic markers for a detailed analysis of the *scabrinodis* group throughout Europe.

Material and methods

Sampling and DNA extraction

Extensive *M. scabrinodis* worker samples collected in 95% ethanol were available, stemming from the EU-funded MacMan (*MAC*ulinea butterflies of the habitats directive and European red list as indicators and tools for habitat conservation and MANagement) project (Settele *et al*., 2002). A large number of additional samples, both alcohol-stored and dry specimens, were also provided by myrmecologists throughout Europe, including alcohol samples of the recently elevated species *Myrmica spinosior* Santschi, 1931 (Seifert, 2005) and dry paratype material of *Myrmica tulinae*, provided by Xavier Espadaler and Graham Elmes (Elmes *et al*., 2002). Additional field sampling of *scabrinodis*-group species for the present investigation was conducted in Germany, Austria, The Netherlands, Denmark and Sweden by JRE. Species identifications were carried out or confirmed by Graham Elmes or Sándor Csősz.

Initially, DNA was extracted from 101 *scabrinodis*-group specimens, consisting of 58 samples identified as *M. scabrinodis* from across the European range and 43 specimens of 12 additional *scabrinodis*-group species. We also sampled two *Myrmica* species outside the *scabrinodis*-group (*M. rubra* and *M. ruginodis*), as well as using 24 published *Myrmica* sequences and that of *Manica rubida* (as an outgroup) obtained from *GenBank* (Savolainen & Vepsalainen, 2003; Jansen *et al*., 2010, 2011). Samples, their origins and GenBank accession numbers are listed in the Supporting Information Table S1. The investigated data material thus consisted of a total of 17 species from the *scabrinodis*-group (Table 1), two European *Myrmica* species representing other species groups, and one *Manica* species. Additionally, DNA of 62 individuals of *M. scabrinodis* from two populations in The Netherlands and Denmark were extracted to evaluate local distribution and habitat use of intra-specific haplotype lineages based on restriction-enzyme assays (see below).

DNA was extracted from a single leg, which allowed the remaining body of the specimens to be stored as vouchers at the State Natural History Museum, Copenhagen. Individual legs were ground and incubated in 100 µl of 5% chelex solution with 5 µl of (0.75 units) proteinase K for 2 h at 56 °C, followed by 15 min of boiling. The higher DNA degradation of dry specimens necessitated higher numbers of PCR cycles for this material, increasing the risk of amplification of contaminating DNA. Negative controls for contamination of the PCR reactions of all dry material were therefore supplemented by a second independent extraction and sequencing of the ant tissue to confirm the origin of the amplified DNA.

Sequencing

For the reconstruction of the overall phylogenetic relationships of the *scabrinodis*-group species and their relatives, mitochondrial DNA fragments of cytochrome oxidase subunit I (COI) and cytochrome B (Cyt-B) were amplified and sequenced. 796 bp of COI were amplified from 30 specimens with the primers C1-J-2183 and TL2-N-3014 T and 409 bp of Cyt-B were amplified from 109 *Myrmica* specimens with the primers CB-J-10933 and CB-J-11367 (Simon *et al*., 1994; Table 2). A larger number of samples from across Europe were sequenced for Cyt-B to investigate the European distribution of haplotypes of *M. scabrinodis*, to increase the likelihood of finding a larger number of divergent lineages within the *scabrinodis* group, to evaluate the general level of intra- and inter-specific variation, and to evaluate the potential hybridisation and introgression among *M. scabrinodis* group species.

PCR reactions were performed in a Hybaid PCR thermocycler in 26 µl volumes containing 0.5 µM of each primer, 100 µM of

Table 1. *Myrmica scabrinodis*-group taxa and outgroups examined in this study, with authorities and notes on their synonymy for comparison with earlier molecular phylogenies.

Species complex	Species	Synonyms used in previous studies
bergi complex	*M. bergi* Ruzsky, 1902	
	M. divergens Karavaiev, 1930	
	M. gallienii Bondroit, 1920	
rugulosa complex	*M. constricta* Karavaiev, 1934	*M. hellenica**
	M. hellenica Finzi, 1926	*M. rugulososcabrinodis* Karavaiev, 1929†
	M. rugulosa Nylander, 1849	
sabuleti complex	*M. hirsuta* Elmes, 1978	
	M. lonae Finzi, 1926	
	M. sabuleti Meinert, 1861	
	M. spinosior Santschi, 1931	
	M. vandeli Bondroit, 1920	
scabrinodis complex	*M. aloba* Forel, 1909	
	M. scabrinodis Nylander, 1846	
	M. tulinae Elmes, Radchenko et Aktaç, 2002	
specioides complex	*M. salina* Ruzsky, 1905	*M. georgica* Seifert, 1987‡
		M. tobiasi Radchenko et Elmes, 2004‡
	M. curvithorax Bondroit, 1920	*M. slovaca* Sadil, 1952‡
		M. salina§
	M. specioides Bondroit, 1918	*M. sancta* Karavaiev, 1926‡
		M. turcica Santschi, 1931‡
Outgroups		
Myrmica	*M. rubra* (Linnaeus, 1758)	
	M. ruginodis Nylander, 1846	
Non-*Myrmica*	*Manica rubida* (Latreille, 1802)	

*Some specimens originally identified as *M. hellenica* were reclassified as *M. constricta* by Graham Elmes, following Seifert *et al*. (2009).
†Synonymised in the study by Radchenko & Elmes (2010).
‡Synonymised in the study by Seifert (2011).
§Western European specimens originally identified as *M. salina*, were re-assigned to *M. slovaca* after further examination by Graham Elmes - see Radchenko & Elmes (2010).

Table 2. Primer sequences used for amplification and sequencing of the 412 bp segment of Cyt-B and the 796 bp of COI.

Gene	Primer pairs	Direction	Sequence 5′ – 3′	Published	Edited sequence. Length
Cyt-B	CB-J-10933 (CB1)	F	TAT GTA CTA CCA TGA GGA CAA ATA TC	Simon *et al.* (1994)	409 bp
	CB-N-11367 (CB2)	R	ATT ACA CCT CCT AAT TTA TTA GGA AT	Simon *et al.* (1994)	
COI	C1-J-2183 (jerry)	F	CAA CAT TTA TTT TGA TTT TTT GG	Simon *et al.* (1994)	364 bp
	(Ben)	R	GCN ACN CAN TAA TAN GTA TCA TG	Villesen *et al.* (2004)	
COI	C1-J-2183 (jerry)	F	CAA CAT TTA TTT TGA TTT TTT GG	Simon *et al.*, 1994	796
	TL2-N-3014 T	R	CCA ATG CAC TAA TCT GCC ATA TTA	Simon *et al.* (1994)	

each dNTP, 2.5 mM MgCl, 1x GeneAmp PCR Gold buffer (15 mM Tris–HCl pH 8.0, 50 mM KCl; Applied Biosystems) and 0.75 units of Ampli*Taq* Gold Polymerase (Applied Biosystems). PCR programs included 5 min at 95 °C followed by 35 cycles of 40 s at 95 °C, 1 min annealing at 45° and 1 min extension at 72 °C, followed by 15 min of final extension at 72 °C. PCR products were separated on a 2% agarose gel to evaluate the final DNA concentration. For a few dry material samples, the number of PCR cycles was increased to 38 cycles to achieve sufficient product on the agarose gel. PCR products were cleaned with the QIAquick DNeasy PCR purification kit (QIAGEN) and sent to MWG Biotech (Germany) for sequencing. Sequence chromatograms were aligned, trimmed and the automatic base calls were evaluated for potentially ambiguous peaks with the program Geneious Prime 2019.0.4 (www.geneious.com). Final alignments were of 726 bp for COI and 393 bp for Cyt-B. The amplification of the COI fragment was unsuccessful in taxa for which only dried specimens were available, so that only Cyt-B sequence data are reported for these.

As a cheaper and quicker method to identify the interspecific *M. scabrinodis* lineages, we investigated the *M. scabrinodis* Cyt-B sequences to identify restriction enzyme sites specific for the two divergent lineages identified by sequencing. Two enzymes were identified that specifically cut haplotypes of one of the two lineages but not the other. Msp-I cuts the Cyt-B fragments of the lineage A haplotypes, resulting in a ≈320 bp and a ≈90 bp sub-fragments, and Dde-I cuts central European lineage B haplotypes resulting in two sub-fragments of ≈175 bp and ≈235 bp. By digesting the Cyt-B fragment obtained from sympatric populations in The Netherlands ($n = 20$) and Denmark ($n = 42$) with the two enzymes independently, a total of 62 individuals were assigned to haplotype lineages based on their segregation patterns on 2% agarose gel. After the investigation of the sympatric populations, we subsequently identified haplotype 17 (from Hungary), where changes have occurred within the Dde-I cutting site. We are confident that this observation did not, however, influence our investigation, as we worked with a double control, so the assignment of lineages was always based on digestion with one digestion enzyme and no digestion by the other and misidentification would need substitutions within both restriction enzyme sites.

Phylogenetic analysis

Phylogenies were inferred for Cyt-B and COI independently, and for the combined data for samples sequenced for both genes using MrBayes 3.2.6 (Ronquist & Huelsenbeck, 2003). Several samples had identical sequences for one or both genes, so only one representative sample was included in the alignment used for phylogenetic reconstruction. The most appropriate substitution model for each phylogeny was obtained by using MrModeltest (Posada & Crandall, 1998) in combination with PAUP* 4.10b (Swofford, 2002) and is given in the Supporting Information Table S2. For the inference of phylogenies we ran MrBayes for 1 000 000 MCMC (Markov Chain Monte Carlo) generations and sampled every 200th generation after a burn in of 500 000, which gave 10 000 sampled trees per run. Convergence of the MCMC chains after burn-in was evaluated from the average standard deviation of the split frequencies between two simultaneous MCMC runs. Fifty percent consensus trees with branch length estimates were constructed with the SUMT option in MrBayes, based on 10 000 collected trees and drawn using Geneious Prime. All three analyses produced trees with very similar topologies, so only the phylogeny based on the total data set is presented here. The gene-specific phylogenies are given in the Supporting Information Fig. S1 (COI) and Fig. S2 (Cyt-B), respectively. Phylogenetic relationships were also inferred with UPGMA neighbour-joining and maximum parsimony methods in Geneious Prime to evaluate potential inconsistencies between alternative methods of phylogenetic reconstruction.

Pairwise distances between samples were constructed with Bioedit 7.0.9.0 (Hall, 1999) from the larger Cyt-B data set, after which we used HapStar (Teacher & Griffiths, 2011) to construct a minimum-spanning haplotype network for the *sabuleti*- and *scabrinodis* complexes, with pie-charts of haplotype frequencies added in Adobe Illustrator.

Potential biases in the phylogenetic reconstruction originating from nuclear paralogues of the investigated sequences were investigated by comparing frequencies of synonymous and non-synonymous substitutions within the main branches of the terminal clades. Under the assumption of unconstrained nucleotide substitution within non-functional nuclear copies, a less biased ratio of synonymous to non-synonymous substitution would be expected within any putative pseudo gene branches. Ancestral sequences of the internal nodes were reconstructed with the software CRANN (Creevey & McInerney, 2003), based on the topology obtained from the MCMC phylogenies for the total data set, and synonymous (d_s) and non-synonymous substitution (d_n) were calculated to evaluate potentially changed constraints of nucleotide substitution within the main branches.

Habitat segregation between lineages of M. scabrinodis

Although habitat associations of sampled *scabrinodis*-group nests were not recorded directly, at two sites (in Denmark and the Netherlands) the GPS coordinates of nests were recorded together with those of all other *Myrmica* species encountered. Hence, it was possible to compare the distribution of different *M. scabrinodis* lineages with that of other *Myrmica* species of known habitat preference using spatial analysis by distance indices (SADIE) analysis (Perry *et al*., 1996) to infer habitat associations (Perry & Dixon, 2002).

Nests of *M. rubra*, *M. ruginodis*, *M. sabuleti*, *M. schencki* Viereck 1903 and *M. specioides* were found on the Danish site, and *M. rubra*, *M. ruginodis, M. lonae, M. sabuleti* and *M. schencki* on the Dutch site. To maximise the power of the association test, these were divided into two groups representing species associated with cold and damp habitats (*M. ruginodis*, *M. rubra* and *M. lonae*) and those occurring in warm and dry habitats (*M. sabuleti*, *M. schencki* and *M. specioides*), based on the classification made by Elmes & Thomas (1992), and their spatial association with the lineages of *M. scabrinodis* tested using the *n_a.exe* module of SADIE.

Results

Phylogenetic relationships of the scabrinodis group and its relatives

Our Bayesian analyses produced a phylogenetic tree (Fig. 1) that is generally consistent with the known morphologically defined species complexes (Radchenko & Elmes, 2010), and with previous molecular trees (Jansen *et al*., 2010). However, there are two types of mismatches. The first is that the European *M. scabrinodis* consists of two well supported independent clades, lineages A and B, with very limited (ca 0.5%) within-clade sequence divergence among western European haplotypes, but considerable (4–6%) divergence between them, and a higher level of within-clade divergence in the lineage B samples deriving from regions often associated with distinct glacial refugia. The two lineages form a clade with the *M. sabuleti* and the *M. vandeli* clades. The Spanish *M. sabuleti* haplotypes and the Balkan and Anatolian haplotypes of *M. scabrinodis* have diverged by ca. 2.5% from their Central European relatives (Fig. 1). The *specioides* complex appears as the sister group of the *sabuleti* and *scabrinodis* complexes, and the *rugulosa* and *bergi* complexes form the basal branches of the entire clade, with more distantly related *Myrmica* specimens as basal sister groups and *Manica rubida* as out-group.

The second mismatch between established morphological taxonomy and the mtDNA tree of Fig. 1 is that occasional specimens end up in what seems to be the wrong species complex. The *M. constricta* and *M. hellenica* GenBank sequences are found in the *specioides* complex instead of the expected *rugulosa* complex, in the former case with only 0.5% sequence divergence to *M. specioides*, suggesting recent exchange of haplotypes. Even more interesting is the positioning of *M. curvithorax* in the *bergi* complex rather than in the *specioides* complex. The positioning of *M. tulinae* specimens is very inconsistent, with specimens appearing throughout the tree; in the *specioides* complex (Fig. 1), sharing one of the *M. scabrinodis* B-lineage haplotypes (Cyt-B only, Fig. 2) and with the two paratype specimens clustering with the *sabuleti* complex (Cyt-B only, Fig. 2), despite all originating from Turkey. Finally, the branches within *M. gallienii* are very long and are rendered paraphyletic by *M. bergi*, and so are likely to represent cryptic species, whereas *M. lonae* does not appear to be differentiated sufficiently from *M. sabuleti* to justify species status when compared to the Spanish haplotype, the inquiline social parasite *M. hirsuta* and *M. spinosior.*

Constructing a haplotype network from the Cyt-B sequences of specimens from the polytomous *scabrinodis* and *sabuleti* complexes (Fig. 2) indicates that the level of sequence divergence across the polytomy of *M. vandeli*, *M. sabuleti* and the A and B lineages of *M. scabrinodis* is very similar, suggesting that these lineages arose at roughly the same time. The A lineage of *M. scabrinodis* appears to be rather homogeneous, whereas the B and *M. vandeli* lineages appear to have undergone more differentiation, although not as much as the *M. sabuleti* clade. The *M. tulinae* paratypes are close relatives of *M. sabuleti*, while additional Turkish samples of *M. tulinae* are highly divergent (Fig. 2). The *M. lonae* specimens all share the Cyt-B haplotype of the most common *M. sabuleti* specimens, supporting the idea that these do not represent a separate species.

Geographic distribution and habitat segregation between the A and B lineages of M. scabrinodis

The A and B lineages of *M. scabrinodis* appear to overlap largely in their extant European distributions (Fig. 3a). The two lineages were found to be sympatric at three of the investigated sample sites (The Netherlands, Poland and Denmark). Sympatry at other sites is also likely, given the low level of sampling in

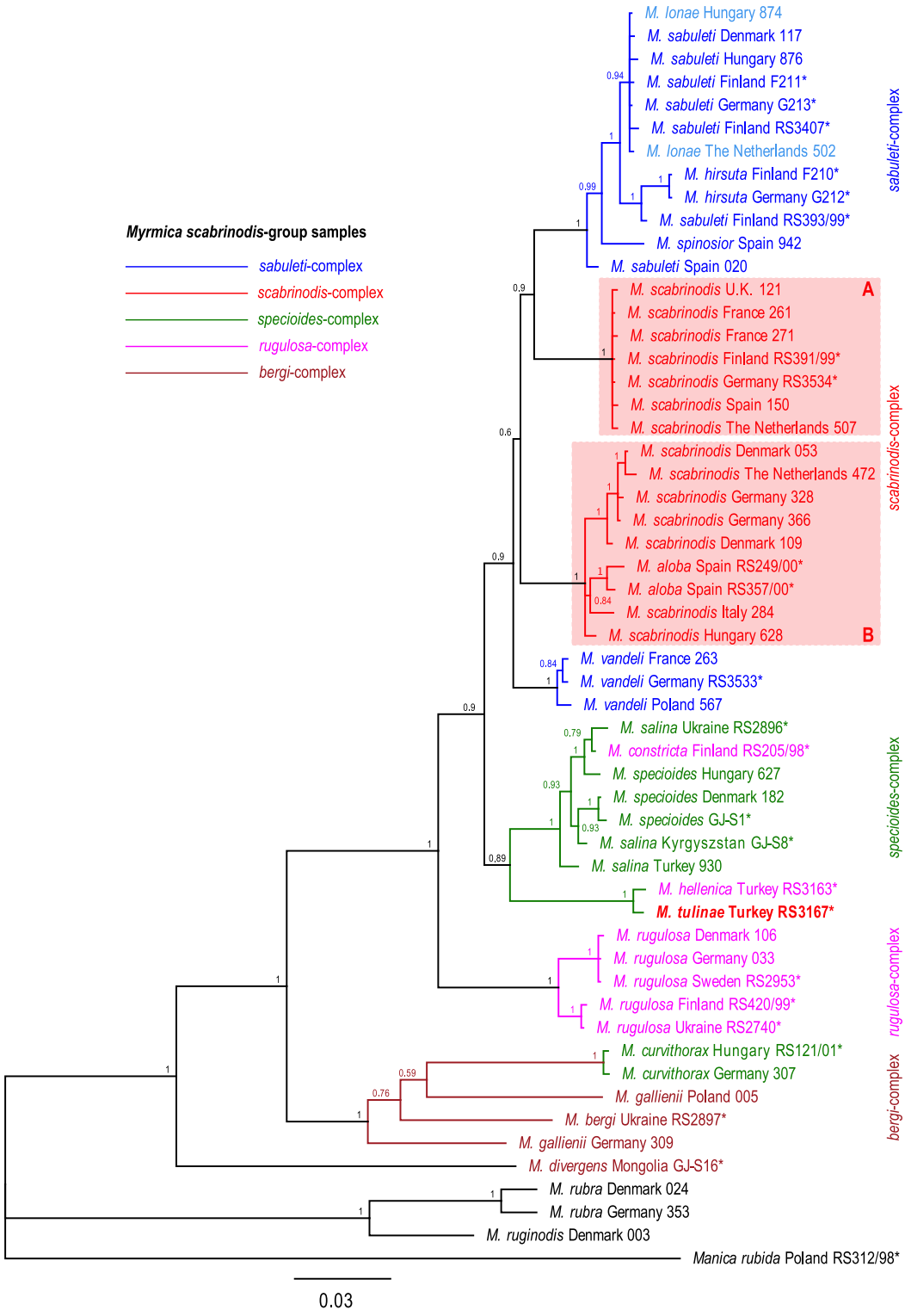

Fig. 1. Consensus tree based on the combined Cyt-B and COI sequence data (1098 bp) through Bayesian inference. Branch lengths reflect the genetic distances and have posterior probabilities assigned to them. Coloured branches indicate the commonly used subdivision of the *M. scabrinodis* species group based on morphological characteristics (Radchenko & Elmes, 2010). The inferred positions of *M. curvithorax*, *M. hellenica* and *M. constricta* are inconsistent with the morphological species complexes to which they are normally assigned, which are reflected by their colour coding. Asterisks next to sample names denote sequences obtained from GenBank. The two divergent lineages of *M. scabrinodis* are marked with shaded boxes. [Color figure can be viewed at wileyonlinelibrary.com]

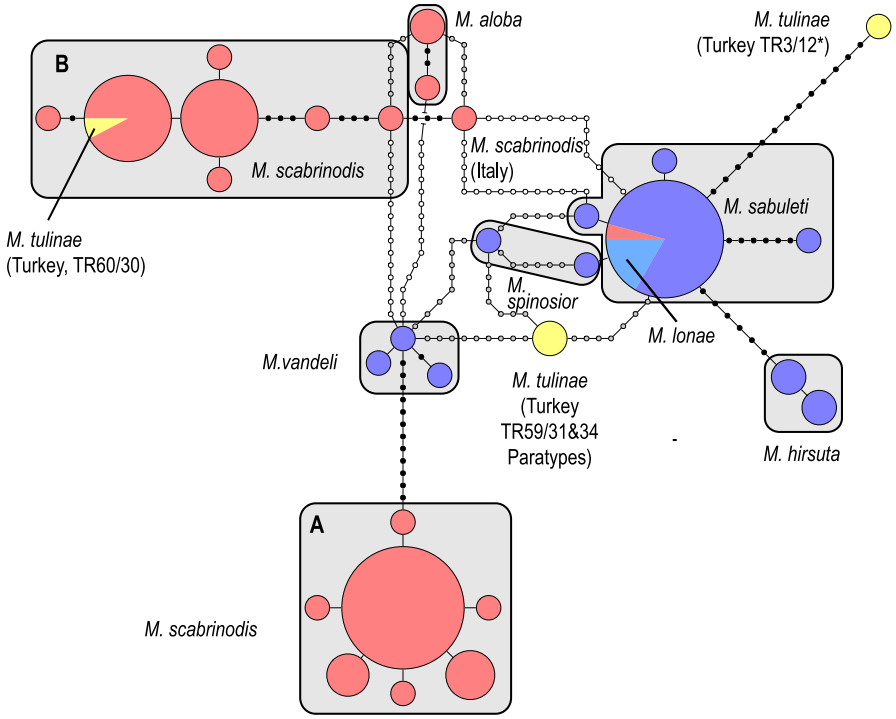

Fig. 2. Minimum spanning haplotype network based on 393 bp of Cyt-B sequence data from a total of 108 individuals representing 66 *M. scabrinodis* samples from across Europe (c.f. Fig. 3), along with samples of six closely related species from the *M. scabrinodis* and *M. sabuleti* species complexes. The area of each symbol is proportional to the frequency of that haplotype in samples. Symbols are coloured according to species complex (*scabrinodis* complex red and *sabuleti* complex blue), with specimens of *M. tulinae* coloured yellow. Inferred differences between haplotypes are shown as circles (representing unsampled potential haplotypes with single base-pair differences) along lines linking the observed haplotypes. Where there are equally parsimonious links between haplotypes they are shown as shaded circles (two equally probable paths) or open circles (four equally probable paths). The two divergent haplotype groups of *M. scabrinodis* are labelled "A" and "B". [Color figure can be viewed at wileyonlinelibrary.com]

most areas. For the sites in Denmark and the Netherlands, restriction enzyme digestion effectively allowed the identification of lineages of additional sampled nests without the necessity for full sequencing (Table 3). At these sites there seemed to be a tendency for lineage A to be associated with drier and warmer habitats similar to those in which *M. sabuleti* is normally found, but colonies with lineage A mitochondrial haplotypes have also been collected in wet *Sphagnum* bogs and along the shores of lakes, i.e. at the wet habitat extreme of the overall *M. scabrinodis* niche (Elmes *et al*., 1998). At the sympatric site in Denmark (Fig. 3b) there was a clear division of habitat types, with lineage B *M. scabrinodis* only being found in a wet depression to the south west of the site, and lineage A primarily in dry areas of the site. The picture was not so clear at the site in the Netherlands, however (Fig 3c), where lineage B was found near two small lakes, but also in drier heathland.

Our SADIE analysis showed that there was neither a significant positive nor significant negative association between lineage A and either *Myrmica* species associated with cold and damp areas ($X = +0.105$, $p = 0.208$) or those associated with warm and dry areas ($X = -0.093$, $p = 0.251$). There was also no significant association between lineage B and species occupying damp and wet habitats ($X = +0.189$, $p = 0.377$), but a highly significant negative association between lineage B nests and those of xerothermophilous *Myrmica* species ($X = -0.529$, $p < 0.0001$), suggesting that this lineage is not generally found in the hotter and drier habitats favoured by *M. schencki*, *M. sabuleti* and *M. specioides.*

Pseudogene sequences are highly unlikely to have affected the data

The 20 *M. scabrinodis* specimens sequenced for both Cyt-B and COI showed consistent phylogenetic clustering based on the two sequences independently (Supporting Information Figs. S1 and S2). Translation of the sequences resulted in uninterrupted sequences without frame-shifts or stop-codons. All chromatograms were further checked for indications of double peaks at the sites where the A and B lineages differed, but these were never observed. The rates of synonymous and non-synonymous substitutions within the major branches of the phylogeny (Supporting Information Fig. S3) did not show any difference between intraspecific lineages, which would be expected if pseudogenes with relaxed selection were responsible for the observed divergence. We conclude, therefore, that pseudogenes have not affected our data for the *scabrinodis* group and its relatives.

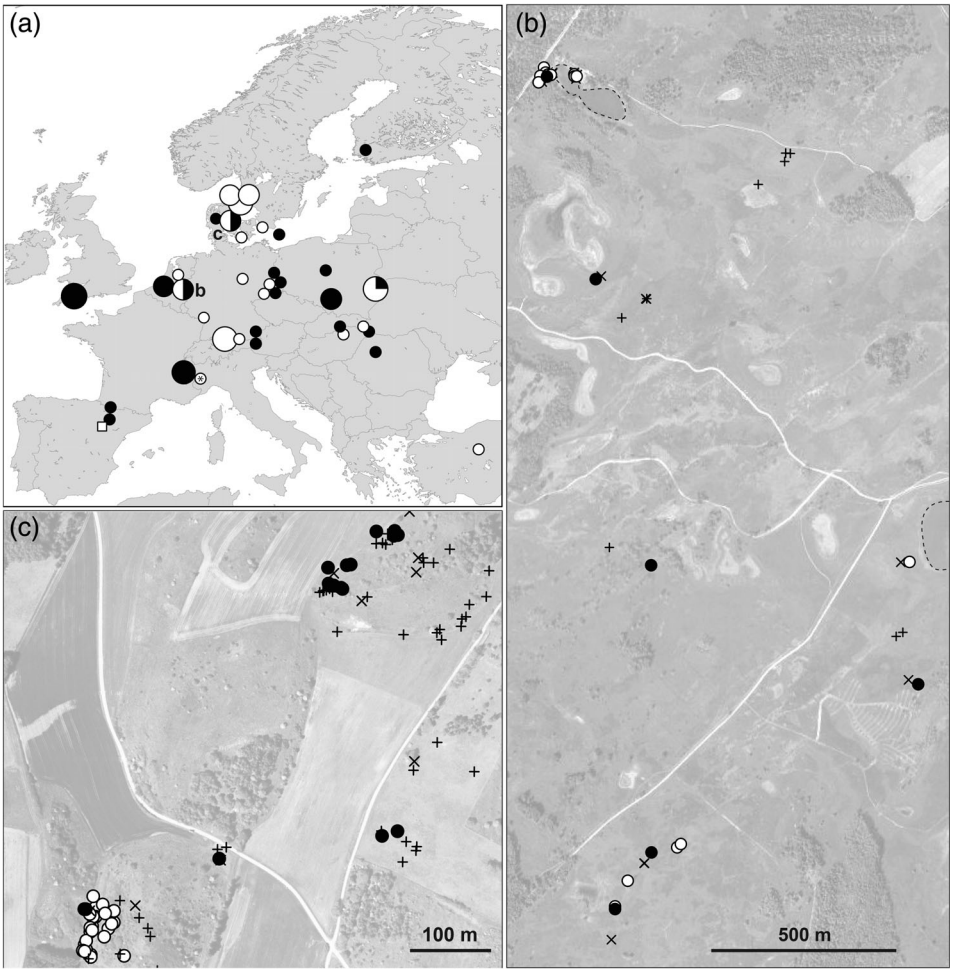

Fig. 3. Distribution of *M. scabrinodis* lineages A (filled circles) and B (open circles). (a) Geographical distribution of 57 investigated *M. scabrinodis* locations in Europe. The area of each symbol is proportional to the number of local samples included in our study. Lineages A and B co-occurred at three sites (Denmark, The Netherlands and Poland), where the proportion of each lineage is shown as a pie chart. The Italian *M. scabrinodis* specimen is marked with an asterisk, and the closely related *M. aloba* as a square. (b) Detailed map showing the distribution of colonies of the two haplotypes from the sympatric site in Denmark. (c) The sympatric site in the Netherlands. Two small lakes are shown with dashed outlines. Colonies of other *Myrmica* species are marked as + (xerothermophilic species) and × (xerothermophobic). Aerial photographs © Google Maps, 2009.

Discussion

The aim of the present investigation was to evaluate the potential presence of genetically differentiated cryptic species or ecotypes within the common European ant species *M. scabrinodis*. Using molecular phylogenetic methods based on mitochondrial DNA we found two highly divergent paraphyletic lineages within *M. scabrinodis* consistent with earlier suggestions that cryptic species or ecotypes might well occur, either in association with habitat (Elmes *et al*., 1994, 1998; Radchenko & Elmes, 2010) or with latitude or longitude (Elmes & Clarke, 2005). Although our sampling did not cover all of Europe evenly, it was extensive enough to conclude that both forms are largely sympatric at the European scale (Fig. 3a), although it might be that lineage B is underrepresented west of the river Rhine. The Danish site that we investigated in more detail supported the suggestion that the two lineages tend to be found in different habitats: lineage A in drier grassland and lineage B in bogs and at the edge of lakes (Fig. 3b), although the pattern for the site studied in the Netherlands was less clear, as habitat boundaries were less distinct, lineage B was still mainly found in wet areas (Fig. 3c). The inferred phylogenetic relationships for the overall *scabrinodis* complex further showed several inconsistencies with established taxonomy based on morphology and we discovered that a number of specimens had mtDNA sequences that diverged so much from the clade to which they had been taxonomically assigned that they are likely to be new cryptic species. The sections below will expand on each of these issues.

Novel taxonomic insights from mtDNA data

Overall, the inferred phylogenetic relationships are generally consistent with previously proposed species groups and species

Table 3. Restriction enzyme digestion sites of Dde-I and Msp-I in relation to Cytochrome B haplotypes in the intraspecific *M. scabrinodis* lineages A and B.

	170 bp----------181 bp	317 bp----------328 bp
Lineage A		
scabrinodis haplotype 1	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 2	~CCT..G.GAA~	~ACC....CAA~
scabrinodis haplotype 3	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 4	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 5	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 6	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 7	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 8	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 9	~CCT..G.GAA~	~ACT....CAA~
scabrinodis haplotype 10	~CCT..G.GAA~	~ACT....CAA~
Lineage B		
scabrinodis haplotype 11	~CC.....GAA~	~ACT..T.CAA~
scabrinodis haplotype 12	~CC.....GAA~	~ACT..T.CAA~
scabrinodis haplotype 13	~CC.....GAA~	~ACT..T.CAA~
scabrinodis haplotype 14	~CC.....GAA~	~ACT..T.CAA~
scabrinodis haplotype 15	~CC.....GAA~	~ACT..T.CAA~
scabrinodis haplotype 16	~CC.....GAA~	~ACT..T.CAA~
scabrinodis haplotype 18	~CC.....GAA~	~ACT..T.CAA~
scabrinodis haplotype 17	~CCTC...GAA~	~ACT..T.CAA~
scabrinodis haplotype 19	~CCT..G.GAA~	~ACT..T.CAA~
aloba haplotype 20	~CCT....GGA~	~ACT..T.CAA~
Msp-I		---ccgg---
Dde-I	--ctnag---	

complexes (Radchenko & Elmes, 2010), but *M. curvithorax*, *M. constricta*, *M. tulinae* and to some extent, *M. hellenica*, provided interesting exceptions. Paraphyletic relationships among samples that would morphologically be identified within single species such as *M. scabrinodis*, *M. tulinae* and *M. gallienii* are based on levels of sequence divergence in the range of 5–8%, i.e. differences that are normally observed between species within the same genus (Smith *et al*., 2005; Hajibabaei *et al*., 2006). While some of these cases could be due to hybridisation, we consider this unlikely as a general explanation (see below) and will therefore concentrate our discussion on the cryptic species scenario.

The Italian *M. scabrinodis* clearly belongs in the B lineage of *M scabrinodis*, but seems to be differentiated as a basal branch together with *M. scabrinodis* specimens from Hungary and Turkey (Cyt-B only) and *M. aloba.* Sequence divergence of the Italian specimen from the rest of the B lineage (≈2.5% Cyt-B, ≈1.6% Total) seems sufficient to justify separate species status (Fig. 2), but whether this population in fact represent the same gene pool as the Hungarian population and/or *M. aloba* remains to be further investigated. The Turkish *M. tulinae* are also highly divergent in their Cyt-B haplotypes (Fig. 2). *Myrmica tulinae* is traditionally positioned in the *scabrinodis* complex, based on male morphology (Elmes *et al*., 2002; Radchenko & Elmes, 2010) but the *M. tulinae* paratype material clusters with the *sabuleti* complex, while a worker from a neighbouring nest has a highly divergent haplotype (Fig. 2), closer to that of Turkish specimens of *M. hellenica* (Supporting Information Fig. S2), while a worker from eastern Turkey shares the most common haplotype of *M. scabrinodis* lineage B (Fig. 2). This pattern of distribution across the haplotype network suggests that the morphological characters used to identify *M. tulinae* may not reflect a common phylogenetic history but may instead reflect rare hybridisation (c.f. Bagherian Yazdi *et al*., 2012)

Towards the base of the tree (Fig. 1), the mismatch of *M. constricta* (which clusters in the *specioides* complex rather than in the expected *rugulosa* complex) also requires further investigation. This specimen is collected from a well-known population of this species, so misidentification is highly unlikely (K. Vepsäläinan, pers. commun., supporting the possibility of introgression or morphological convergence. The *specioides* complex may harbour several cryptic species, but, except for *M. hellenica* and *M. curvithorax,* we see only very low levels of divergence (0.5–1.5%) among the specimens of this complex, so further work would be of interest. *Myrmica curvithorax*, a putative representative of the *specioides* complex clustered in the *bergi* complex, where it is also distinct from the two investigated *M. gallienii* specimens. There is no doubt that *M. curvithorax* is a distinct species, but the deep nodes in the *bergi* complex, levels of sequence divergence of around 8% and paraphyly with *M. bergi* (Fig. 1) indicate that the two *M. gallienii* specimens from Poland and Germany are also likely to represent different species. The dry and saline habitat of the German material corresponds with the habitat description of the *M. gallienii* synonyms *M. jacobsoni* Kutter, 1963 and *M. limanica* Karavaiev, 1934 and it would thus be interesting to compare the investigated material to the type specimens of these synonyms.

Two specimens of *M. hellenica* from Turkey (with identical Cyt-B and COI sequences) also did not cluster in the expected

rugulosa complex, but instead with one of the *M. tulinae* specimens (Fig. 1) as a sister-clade to the *specioides* complex. The monophyly of each of these species complexes have well supported posterior probabilities, but their mutual relationships remain unclear, quite possibly because they all arose at roughly the same time (see below). This also illustrates that adding a few specimens from Turkey may give rise to new ambiguities rather than clarifying the European phylogenetic relationships, so that a much larger study with more complete taxon sampling and more analysed genes would be needed to clarify the entire Palaearctic phylogeny of these *Myrmica* species complexes. The position of *M. divergens* (from Mongolia) further underlines this point.

A contrasting pattern emerged for *M. lonae*, which was recently resurrected as a species separate from *M. sabuleti* (Seifert, 2000), but where our mtDNA data could not find any evidence to support this. No specific *M. lonae* haplotypes were found in the four sequenced specimens (Figs. 1 and 2), which suggests that they share the same gene pool as *M. sabuleti*, as most of the samples were from sympatric populations in The Netherlands and Hungary, and the remaining GenBank sequence came from Finland where only *M. lonae* has been recorded (Seifert, 2000). The similarity between *M. lonae* and *M. sabuleti* is remarkable, because all specimens shared the most abundant Cyt-B *M. sabuleti* haplotype (Fig. 3), whereas established closely related species (*M. hirsuta*, *M. spinosior*, *M. vandeli*) were differentiated with good bootstrap support. The *M. lonae* phenotype is associated with colder and wetter habitats than typical *M. sabuleti* (Elmes *et al*., 1998; Seifert, 2000; Radchenko & Elmes, 2010; JRE, personal observation), suggesting that this may be an ecotype of *M. sabuleti.* This interpretation is also supported by the fact that *M. lonae* and *M. sabuleti* have identical cuticular hydrocarbon profiles, while other *Myrmica* species examined can be readily separated based on surface chemistry (Guillem, 2014). Given this overall pattern within the *M. sabuleti* complex, we hypothesise that the Spanish specimen of *M. sabuleti* may represent yet another cryptic species (the long branch towards the right in Fig. 2).

Reconstructing the evolutionary history of the M. scabrinodis lineages and their close relatives

Glacial isolation and post-glacial recolonisation patterns have often led to high levels of differentiation between conspecific lineages that became isolated in different glacial refugia. These differentiations are expected to always be detectable when a sufficient number of neutral DNA markers are analysed, but not necessarily with morphological measures. This is particularly true for the ants, which are notoriously poor in secondary sexual characters (Boomsma *et al*., 2005). Several earlier cases are known where non-overlapping allele or haplotype distributions have been decisive for separating cryptic sympatric ant species, and where diagnostic morphological characters were obtained only after a more explicit comparison of genetic lineages had been made (Boomsma *et al*., 1990; Schultz *et al*., 1998, 2002; Seifert, 2009). As far as glacial isolation has given rise to new (sub)species, they can generally be distinguished by genetic discontinuities and, often, morphological differentiation associated with hybridisation (suture) zones where lineages that recolonised from different glacial refugia re-established contact (Pusch *et al*., 2006). Another pattern of variation between extant (sub)species may be detectable when comparing the recolonizing lineages with those that stayed behind in the refugia (e.g. Vila *et al*., 2005; Schlick-Steiner *et al*., 2007). Considerations such as this suggest that the distribution of *M. aloba* and *M. spinosior* as endemics within the Iberian Peninsula (and possibly several other newly described *Myrmica* from other refugia, such as those described from Turkey by Elmes *et al*., 2002), could result from allopatric speciation during glacial periods.

We hypothesise that most of the mtDNA sequence variation that we documented within the *scabrinodis-, sabuleti-* and *specioides*-species complexes dates back to interglacial periods, as they generally reflect divergences in the range of 1.25–2.75% (similar levels of divergence (≈2%) of mitochondrial protein coding sequence in *Drosophila* and other species have been found to be equivalent to ≈1 000 000 years; Hewitt, 2000). The upper limit of this range coincides with species-level differences, as comparable levels of sequence divergence were observed between *M. spinosior, M. hirsuta* and *M. aloba* and their core species complex relatives. These differences are probably due to allopatric isolation during a glacial maximum in different refugia in the Iberian Peninsula, Italy, the Balkans and possibly further east (c.f. Previšić *et al*., 2009). The nodes in the *bergi* complex are comparable to the level of sequence divergence observed among species complexes and are deep enough that they may derive from early glaciation events or may even originate prior to the Pleistocene (see below). Much of the variation that we detected in the *M. sabuleti* complex appears to suggest an Iberian refugium, with *M. spinosior* and a likely second (cryptic) species representing relict populations that stayed behind on the Iberian Peninsula, while Central Europe was colonised from yet another glacial refugium.

The observed sequence divergence between the A and B lineages of the *scabrinodis* complex, the main target of our study, is 2–3 times higher than that observed within species complexes, and could even predate the glacial periods as we tentatively suggested for the *bergi* complex. Lineage A shows little variation (Figs. 1 and 2) and is the only one observed SW of the Rhine, which together with the observation of this lineage in the Pyrenean region suggests an Iberian refugium during the last glaciation and rapid colonisation of NE Europe while maintaining high dispersal rates and substantial panmixia. While we have not yet had the opportunity to check the *M. scabrinodis* voucher material for the morphology associated with the recently described cryptic species *M. martini* (Seifert *et al*., 2014), all samples in our analysis that were collected in the area from which this species was described (the Pyrenees and Western Alps) belong to lineage A, with no greater differentiation than other samples in the lineage. The B lineage has a more Eastern distribution and also probably rapidly recolonised Central and Northern Europe from an Eastern refugium, while the Turkish and Balkan lineages remained in their refugia. Genetically distinct southern taxa in Spain (*M. aloba*), and Italy (Italian *M. scabrinodis*) suggest that the divergence between the A and B lineages predated the glaciation that created these refugia.

The characteristics of the A and B lineages of *M. scabrinodis* leave us with an interesting enigma. Based on their mtDNA sequence divergence, each of them should qualify to be considered as species of comparable age to the *specioides* and *sabuleti* complexes, because the sequence divergence shown in Fig. 1 suggests that they originated in the same relatively short period. The problem is, however, that so far no diagnostic morphological characters have been obtained for these lineages, with a slight overall difference in body size as the only possible exception (Sándor Csősz, personal communication). This indicates that further studies of this dichotomy will be highly rewarding, in particular if they could be combined with analyses of gene flow at nuclear markers. It is possible that the mtDNA variation is not selectively neutral but represents metabolic adaptations to different habitats, so that nuclear gene-flow between the lineages remains a possibility, in which case cryptic species status would be questionable. Explicit studies of differences in colony kin structure are also likely to be rewarding.

More extensive sampling in Mediterranean areas may reveal that lineage A also has some genetically differentiated representatives with narrow and isolated distributions, similar to that found for lineage B. Whether or not this will be found, it is clear that both lineages have a major branch (possibly the only one in clade A) of very good dispersers that independently re-colonised most of Europe and are now largely sympatric. This should imply that both have males and gynes with well-developed flight muscles and relatively monogynous colonies, relative to some other *Myrmica* species (Elmes & Keller, 1993). As the extent of polygyny across *Myrmica* species and forms is associated with habitat patchiness (Seppä *et al*., 1995) and negatively correlated with dispersal (Pedersen & Boomsma, 1999b), we might expect that the more isolated southern representatives have more polygynous colonies.

Are cryptic Myrmica *species ecologically segregated?*

Myrmica scabrinodis lineage A and B can be found both in *Sphagnum* bogs and lake margins at the wet and cold extreme of the overall habitat gradient across which species of European *Myrmica* ants distribute themselves, and in the drier grassland habitats neighbouring the typical habitats of *M. sabuleti* and *M. specioides* at the warmer and drier end of the range (Elmes *et al*., 1998). However, at a local scale, we found apparent, but variable, habitat segregation in sympatric populations in Denmark and The Netherlands. In both populations, we generally found lineage B colonies in the coldest and wettest part of the habitat, but with much more overlap in the Dutch population than in the Danish population (Fig. 3b,c).

Although this habitat segregation can be discerned with knowledge about mtDNA sequences, the substantial overlap may also explain that these A and B lineages have not been recognised earlier, for example, in the morphological investigation in relation to habitat preferences of *M. scabrinodis* and the synonym *M. rugulosoides* (Seifert, 1984). Many years of field observations by Graham Elmes and co-workers have consistently indicated that *M. scabrinodis* has several ecotypes, or even cryptic species, with different behaviour and habitat preferences (Elmes *et al*., 1994, 1998; Radchenko & Elmes, 2010). Elmes (1974) and Elmes & Wardlaw (1982a, b) also reported variation in queen number within *M. scabrinodis* correlated with vegetation and habitat temperature, with colonies in warmer drier habitats having more queens. However, if higher queen number is associated with lower dispersal and more genetically viscous populations (Seppä, 1996), then we would expect to see greater genetic differentiation in lineage A rather than lineage B if the pattern of queen numbers also reflected habitat differentiation between these two lineages, which is the opposite of the gross pattern that we observed (Figs. 1 and 2).

In light of the highly divergent paraphyletic mitochondrial lineages within *M. scabrinodis*, and the highly variable ecology and behaviour of this species (Elmes & Wardlaw, 1982a; Elmes *et al*., 1998; Radchenko & Elmes, 2010) it seems likely that further cryptic species remain to be discovered, even in the central and Northern European range (Seifert *et al*., 2009). However, we also caution that we have very little data on nuclear gene flow in the *scabrinodis* complex (Seppä, 1996), and that hybridisation and introgression of nuclear genes need to be investigated. If such examples would be found they would reduce the number of (cryptic) species, and would shift the emphasis to possible mtDNA adaptations related to habitat, as indicated above. Our present results therefore provide explicit mtDNA support to the inferences by Elmes *et al*. (1994, 1998), but also indicate that a considerable amount of further work will be needed to fully clarify the ecological aspects of these findings.

Conservation implications for Myrmica *ants as hosts of* Maculinea *butterflies*

The clarification of cryptic species within *M. scabrinodis* is of direct conservation relevance because *M. scabrinodis* is the only *Myrmica* species to be recorded as the host of all recognised European species of socially parasitic *Maculinea* butterflies (Tartally *et al*., 2019; see also the other papers in this special edition), and is one of the major hosts of *Maculinea alcon* (Denis et Schiffermüller, 1775) and *Maculinea teleius* (Bergsträsser, 1779) (Tartally *et al*., 2019). It has been hypothesised that such unknown genetic variation across host populations could possibly explain host shifts, in particular in the hygrophilous form of *M. alcon* (*M. alcon H*; Tartally *et al*., 2019), throughout its European range (Elmes & Clarke, 2005), where *M. scabrinodis* is not used as a host in the north of the butterfly's range, despite the apparent abundance of this ant species (Tartally *et al*., 2019). Our present data do not give support to a North–South gradient involving different *M. scabrinodis*-like species in Europe, so that there is no simple explanation for *M. scabrinodis* only being used as a host by *M. alcon H* south of ca. 52° N latitude. However, this hypothesis would remain interesting if it could be shown that one lineage is a more suitable host for *M. alcon H,* and that this lineage is more common in the wet habitats that overlap with the *M. alcon H* host plant *Gentiana pneumonanthe* (Linneaus, 1753) in Central and Southern Europe, as the difference between the Danish and Dutch populations might suggest. We expect that if such differences will be found they will be gradual rather than abrupt, as a few of the *M. scabrinodis* samples that we examined belonged to colonies with confirmed *M.*

alcon infections and belonged to both the A and B lineages. *Maculinea teleius* generally has a more Eastern European distribution than *M. alcon.* As *M. scabrinodis* lineage B seems underrepresented west of the River Rhine, it would be of interest to compare host suitability of the A and B lineage for these other *Maculinea* social parasites as well. In any case, the presence of cryptic species among the hosts of *Maculinea* butterflies would likely increase the number of 'evolutionarily significant conservation units' for these endangered butterflies (Casacci *et al*., 2014), as well as potentially complicating reintroduction programs when there is local adaptation to particular hosts (Nash *et al*., 2008; Tartally *et al*., 2019). *Myrmica scabrinodis* has also been recorded as the main host of the rare syrphid fly *Microdon myrmicae* (Bonelli *et al*., 2011), so similar considerations may apply for this system, although the habitat range of this fly is less well known than that of the *Maculinea* butterflies.

At the opposite end of the spectrum, the lack of clear differentiation that we find between *M. lonae* and *M. sabuleti* also has implications for the conservation of *Maculinea arion* (Linnaeus, 1758), which has been recorded as using both ants as hosts (Sielezniew *et al*., 2010; Tartally *et al*., 2019), and where there is an apparent switch from *M. sabuleti* as the main host to exclusive use of *M. lonae* as a host in the northern part of the butterfly's range (Tartally *et al*., 2019). If these two ants are not separate species, then it may ease the conservation of this species in northern areas.

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Conflict of Interest

The authors declare no conflict of interest.

Supporting information

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

Table S1 Summary of samples used, their collection locations and GenBank accession numbers.

Table S2: Details of variability and model selection for the COI and Cyt-B datasets.

Figure S1: Consensus phylogenetic tree based on COI sequences.

Figure S2: Consensus phylogenetic tree based on Cyt-B sequences.

Figure S3: Rates of synonymous and non-synonymous substitution in different clades.

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