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Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny

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Abstract

This study examines phylogenetic relationships among six species of the *Formica rufa* group ants (*F. polyctena*, *F. rufa*, *F. lugubris*, *F. paralugubris*, *F. aquilonia*, and *F. pratensis*). The phylogeny based on a 2051 bp fragment of mtDNA including cyt *b*, $tRNA^{Ser}$, and ND1 genes supports the division of the group into three major clusters: one with the species *F. polyctena* and *F. rufa*, one with *F. aquilonia*, *F. lugubris*, and *F. paralugubris*, and the third one with *F. pratensis*. The interspecific divergence estimates (mean $0.98 \pm 0.15\%$ for the main phylogenetic groups) imply that radiation took place during the Pleistocene. Comparison of the divergence estimates among the *F. rufa* group species with divergence estimates among other closely related species of insects suggests that speciation in the group was relatively fast, and the mitochondrial lineages of *F. polyctena* and *F. rufa* have not fully separated. The haplotype tree shows also signs of transfer of mtDNA between species through hybridisation. The distribution of polygyny (multiple queens per nest) along the branches of the tree indicates that the social type characterised by highly polygynous societies and large colonial networks, has originated at least three times. The species *F. aquilonia* and *F. paralugubris* that build such large supercolonies, cluster tightly together with very little nucleotide variation, suggesting that this type of social organisation could be a factor promoting speciation in the ants.

Keywords: Ants; *Formica*; Introgression; mtDNA; Polygyny; Speciation; Pleistocene; Phylogeny

1. Introduction

Pleistocene environmental changes have been suggested as important factors promoting (Mayr, 1970) or preventing speciation (Bennett, 1997). According to the Pleistocene speciation hypothesis, separation in different glacial refugia generated intraspecific divergence, and isolated gene pools were protected from mixing by hybrid zones during interglacials leading to allopatric speciation (Hewitt, 1996). The Pleistocene speciation hypothesis, initially based on biogeography of vertebrates (Mayr, 1970), remains highly controversial. Molecular systematic studies in birds showed no increase in speciation during the Pleistocene (Zink and Slowinski, 1995) and implied that speciation duration normally exceeds the Pleistocene (about two million years (Myr) before present (BP), Klicka and Zink, 1997). However, this conclusion has been challenged by reinterpretation of mitochondrial DNA (mtDNA) phylogenetic patterns in birds and mammals suggesting that the Pleistocene conditions did play an important role in completing speciation (Avise and Walker, 1998; Avise et al., 1998). The opposite view is that Pleistocene environmental changes inhibited allopatric speciation by repeatedly altering species distributions and thus prevented accumulation of evolutionary changes (Zink and Slowinski, 1995). This view is generally supported by fossil records (Bennett, 1997). The Pleistocene speciation hypothesis can be evaluated by reconstructing phylogeny and estimating species divergence time, which is expected to be less than two million years under this hypothesis.

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The *Formica rufa* group, belonging to the subgenus *Formica* s.str., includes several species of mound-building red wood ants. These morphologically similar species inhabit, often sympatrically, woodlands throughout Eurasia. Taxonomy of the red wood ants has been unstable mainly because of morphological similarity and ability to hybridise and to form mixed colonies (cf. Czechowski, 1996). After revisions by Yarrow (1955) and Betrem (1960), eight European species were included in the *F. rufa* group: *F. rufa* L., *F. polyctena* Foerst., *F. lugubris* Zett., *F. aquilonia* Yarrow, *F. pratensis* Retz., *F. nigricans* Emery, *F. truncorum* Fabr., and *F. uralensis* Ruszky. However, the latter two species have been excluded from the *F. rufa* group (Dlussky, 1967), and there are additional species related to *F. truncorum*, e.g., *F. frontalis*. Furthermore, *F. nigricans* has been combined with *F. pratensis* (Dlussky, 1967; Seifert, 1992). A new species *F. paralugubris* was morphologically described recently (Seifert, 1996) after an allozyme study (Pamilo et al., 1992) revealed that two sympatric types of *F. lugubris* in Switzerland represented different gene pools. Currently, the *F. rufa* group is thus regarded as six different species: *F. rufa*, *F. polyctena*, *F. lugubris*, *F. paralugubris*, *F. aquilonia*, and *F. pratensis*.

Despite morphological similarity, the *F. rufa* group species have different types of social organisation. *F. polyctena*, *F. aquilonia*, and *F. paralugubris* are obligatorily highly polygynous species (with multiple-queen colonies) often forming large networks of interconnected nests (Chapuisat et al., 1997; Crozier and Pamilo, 1996, pp. 114–115). *F. rufa* and *F. pratensis* are mainly monogynous (with single-queen colonies) though polygynous nests have been recorded for both species (Kutter, 1977). *F. lugubris* is polygynous on the British Isles and Switzerland and mainly monogynous in Ireland and Fennoscandia (Crozier and Pamilo, 1996, p. 114). The type of social organisation is associated with dispersal behaviour of newly mated females: females from monogynous colonies disperse by flight and found new nests independently while those from polygynous colonies establish new nests with the help of workers in close neighbourhood (Keller, 1991; Rosengren and Pamilo, 1983). Limited dispersal of females from polygynous societies can lead to strong differentiation between populations and possibly to speciation if male dispersal is also restricted (Gyllenstrand, 2002; Pamilo and Rosengren, 1984; Seppä and Pamilo, 1995). This scenario can be tested by examining whether ants that build extremely polygynous societies and large colonial networks (as the P type of *Solenopsis invicta*; Ross and Fletcher, 1985) split into separate sister species, or whether they are separately derived from monogynous or less polygynous ancestral types.

In this study, we reconstruct the mtDNA phylogeny of the *F. rufa* group sampled on a large geographic scale. The phylogenetic reconstruction is used for three purposes. First, we want to reveal the phylogenetic relationships and timing of divergence among the morphologically recognised species in order to evaluate the possible speciation effect of the Pleistocene environmental changes, and to clarify the taxonomy of the *F. rufa* group. During the Pleistocene glaciations, forest was restricted into a number of refugial areas in Eurasia (cf. Bennett, 1997) and separation into different glacial refugia could initiate speciation in the *F. rufa* group strongly associated with forest environment. Second, we use mtDNA phylogeny to infer evolution of social organisation and its possible effect on speciation rate. Third, we preliminarily assess the phylogeographic structure within each morphologically defined species in order to evaluate the applicability of mtDNA variation for studying intraspecific phylogeography in the red woods ants.

2. Materials and methods

2.1. Sampling and molecular techniques

A total of 44 individuals including all six species of the *F. rufa* group were sampled from different localities in Eurasia over most of their distribution (Fig. 1, Table 1). One individual of *F. truncorum* and two individuals of *F. frontalis* were used as outgroups representing the same subgenus *Formica* s.str. All samples were stored in −70 °C or in 70% ethanol until DNA extraction. Total genomic DNA was extracted from only the head and alitrank of single individuals with the DNeasy Tissue Kit (Qiagen).

A 4-kb mitochondrial fragment was amplified from two samples using ND4 and ND1 primers (Jermiin and Crozier, 1994) and then sequenced in order to design specific primers for the *F. rufa* group and to choose a fragment with high level of variation for further analysis. These 4-kb sequences were aligned with that of the honeybee (Crozier and Crozier, 1993) to confirm that the target region of the mtDNA was amplified. For the present study, we used a continuous 2-kb fragment including 1047 bp of the 5′-end of the cytochrome *b* gene (cyt *b*), 57 bp of the intergenic region I, 74 bp of the transfer RNA with a UCN anticodon for serine ($tRNA^{Ser}$), 20 bp of the intergenic region II, and 853 bp of the 3′-end of the NADH dehydrogenase 1 (ND1). Polymerase chain reaction (PCR) and sequencing of this fragment were performed with the primers CB1, CB2, CB3, tR^{S}, ND1 (Jermiin and Crozier, 1994) and with five primers designed by using the Oligo Primer Analysis Software v. 6.45 (Table 2). PCR was carried out in 25 μL volumes containing 1× PCR buffer, 2.0 mM $MgCl_2$, 0.4 μg/μL BSA, 0.2 mM dNTPs (MBI Fermentas), 0.4 μM of each primer and 2.0 U *Taq* polymerase (Fermentas). A program for the amplification in a thermal cycler was used as follows: 3 min at 94 °C, 35 cycles of

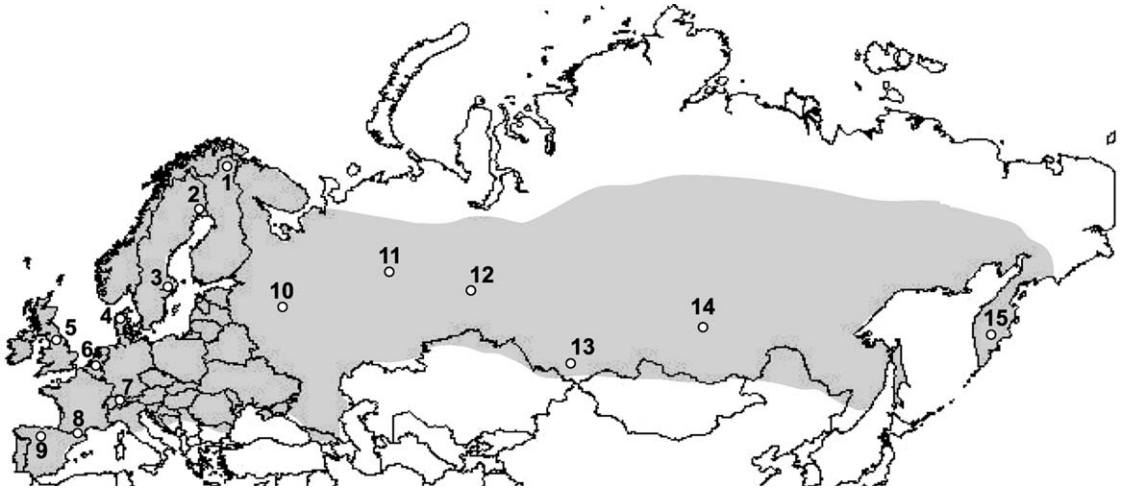

Fig. 1. Map showing the sampling localities and species distribution of the *Formica rufa* group (Dlussky, 1967).

Table 1
Distribution of mtDNA haplotypes of the *Formica rufa* group species

Locality	*F. lugubris*	*F. paralugubris*	*F. aquilonia*	*F. pratensis*	*F. polyctena*	*F. rufa*
1			Q			
2			P			
3	E		P	U	W	AA
4					Z	AA
5	M, N		P			
6						AA
7	L	O, O				
8	H, J, K			I, I		
9						AC
10	E		R			
11					AB	
12	G		P	S	O, Y	AA
13	D		P	T	W, Y	AD
14	A, C		B, P	V	X	
15	F					

Localities refer to Fig. 1, haplotypes refer to Figs. 2 and 3.

Table 2
Oligonucleotide primers for amplification and sequencing of the cytochrome *b* region in the *Formica rufa* group

Primer	Sequences	Positions[a]
CB-11059	5′-CTATAATGAATCTACCCTCACC-3′	11,059–11,180→
CB-11449	5′-GTAATTACAGTTGCTCCT-3′	←11,431–11,449
CB-12190	5′-ATTTTTTATTTAGTTAATGA-3′	12,190–12,209П
ND1-12400	5′-AACGCATTCGAGGTAATAAA-3′	12,400–12,419П
ND1-12931	5′-TATTTTTTGAATTATTCCAT-3′	←12,912–12,931[b]

[a] Positions of primers are given according to the *Apis mellifera* mitochondrial genome (Crozier and Crozier, 1993). The 3′-ends of primers are indicated by arrows.

[b] The position of the primer ND1-12931 is given according to the position of the primer ND1 (Jermiin and Crozier, 1994) because ND1 sequences of *A. mellifera* and *F. rufa* are difficult to align.

30 s at 92 °C, 30 s at 45 °C, 1–2 min at 68 °C, and 10 min at 72 °C.

Successful PCR products were cleaned with the QIAquick Gel Extraction Kit (Qiagen) and then manually sequenced with the Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit according to the manufacturer's specification (USB). Sequencing was also performed on an Applied Biosystems 377 automated DNA sequencer using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). In total, 2051 bp were scored in 47 individuals (EMBL Accession Nos. AY488759–AY488791).

2.2. Data analysis

Sequence variation and the substitution pattern of the 2-kb mtDNA fragment were analysed using the program MEGA v. 2 (Kumar et al., 1993). Sequence divergence was estimated using the Jukes–Cantor method as the overall differences were small and the Jukes–Cantor method presents the simplest model with a small total variance (Nei and Kumar, 2000, p. 112). The results remained similar when we calculated the pairwise distances taking into account transitions and transversions separately or the nucleotide frequencies. The neighbour-joining (NJ) tree was constructed with the MEGA program. Since the examined mitochondrial fragment covered both coding and non-coding regions and the general level of variation was very low we analysed the segment by treating each nucleotide site equally and included both transitions and transversions into analysis. A median-joining haplotype network was constructed with the Network 3.1.1.1 program (Bandelt et al., 1999). Nucleotide diversity (π) within species was calculated using the program DnaSP v. 3.50 (Rozas and Rozas, 1999).

3. Results

3.1. Nucleotide composition and sequence variation

We analysed a 2051 bp fragment of mtDNA in 44 *F. rufa* group specimens, two *F. frontalis*, and one *F. truncorum*. The fragment covered three genes and two intergenic regions (Table 3). The sequence examined was highly AT-biased, similarly to the honeybee mitochondrial genome (Crozier and Crozier, 1993): on average 40.4% of the nucleotides were A, 38.7% T, 13.5% C, and 7.4% G. The analysis showed that the AT content in the cyt *b* gene (76%) was lower than in the other regions (81–95%). AT content at the third codon position in the protein encoding genes was 88.2% which was similar to that in the honeybee mitochondrial genome (92.1%, Crozier and Crozier, 1993). The second position was least AT-biased (73.1%) similarly to the honeybee mtDNA (76.4%).

We found 33 different haplotypes among the 47 *Formica* specimens. Over the entire 2020 bp region, excluding sites with alignment gaps and missing data, 70 nucleotide positions were variable with 47 parsimony informative polymorphic sites. Cyt *b* showed the highest level of variation among the four regions examined with a high proportion of non-synonymous substitutions (Table 3). Substitutions in the protein-encoding genes were most abundant at the third positions (87%) and least abundant at the second positions (1%).

3.2. Phylogenetic relationships among species

The NJ tree rooted to the closest species *F. truncorum* and *F. frontalis* (Fig. 2) showed that the *F. rufa* group formed a tight cluster that was subdivided into three main clades with strong bootsrap supports: IA—*lugubris/paralugubris/aquilonia*, IB—*pratensis*, and II—*polyctena/rufa*. Within the clade IA, *F. paralugubris* and *F. aquilonia* formed a monophyletic group strongly supported by bootstrap. Three haplotypes (B—*F. aquilonia* from locality 1, I—*F. pratensis* from two sites in locality 8, and O—*F. polyctena* from locality 12, Figs. 1 and 2, Table 1) clustered in clades of morphologically different species. These samples were morphometrically rechecked (Seifert, personal communication). The discordance between morphology and mtDNA phylogeny most probably indicates interspecific hybridisation and introgression.

The median-joining network was manually modified by taking away seven pairs of direct and backward substitutions from the branches leading to the clades IA and II and placing them as single substitutions on the branch leading to the IB clade (Fig. 3). The *F. aquilonia* haplotypes (P, Q and R) showed a star-like internal topology with the most common and geographically widespread haplotype P in the centre. The most widely spread *F. rufa* haplotype AA had a similar central position in a star-like topology of the clade II. It is noteworthy that all the *F. polyctena* haplotypes could be derived from this haplotype with one or two nucleotide changes (excluding the haplotype O which most likely indicated hybridisation).

3.3. Interspecific divergence, timing of speciation, and evolution of social organisation

The net divergence estimates between the main phylogenetic group (I vs II and IA vs IB, Fig. 2) were $d_{\text{I–II}} = 0.97 \pm 0.21\%$ and $d_{\text{IA–IB}} = 0.83 \pm 0.22\%$. The average net

Table 3
Polymorphism among 44 sequences of the *Formica rufa* group (values for all 47 sequences including the outgroups are shown in parentheses)

mtDNA region	Total # sites	# Polymorphic sites	# Non-synonymous substitutions
Cytochrome *b*	1047	42 (56)	6 (9)
$tRNA^{Ser}$	74	1 (2)	—
ND1	853	25 (36)	2 (5)
Intergenic regions I and II	77	2 (3)	—
Total	2051	70 (97)	8 (14)

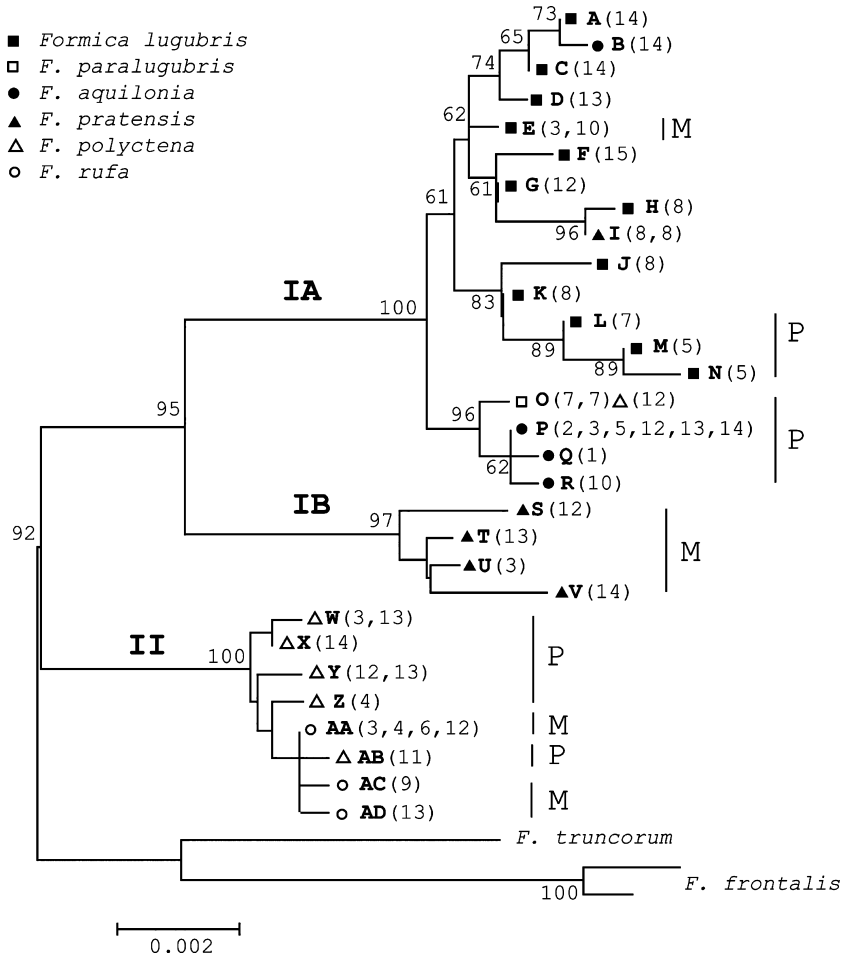

Fig. 2. Neighbour-joining phylogenetic tree of 30 mtDNA haplotypes of the *Formica rufa* group species. The tree is rooted using *F. truncorum* and two *F. frontalis* sequences. Numbers in parentheses indicate localities for one specimen of a particular haplotype and refer to Fig. 1 and Table 1. Bootstrap percentages with values over 60 are shown for nodes. Monogyny is indicated on the tree as M, polygyny as P.

divergence estimate for the main phylogenetic groups IA, IB, and II is $0.98 \pm 0.15\%$. Within the clade IA, the net divergence estimate between *F. lugubris* and *F. paralugubris*/*F. aquilonia* was $0.20 \pm 0.09\%$.

The approximate time of divergence between the phylogenetic groups and species can be estimated from the net divergence estimates and the divergence rate. Divergence rates estimated for mtDNA in closely related insect taxa vary from 0.4–1% per Myr for ND1 in *Carabus* beetles (Prüser and Mossakowski, 1998) to 5% per Myr for COI in *Hegeter* beetles (Juan et al., 1996). In the absence of mtDNA calibration rates for ants, we use the divergence rate of 2% per Myr as estimated for *Drosophila* (DeSalle et al., 1987) and *Pimelia* beetles (Juan et al., 1995) and close to the conventional arthropod mtDNA rate of 2.3% pairwise divergence per Myr (Brower, 1994). Assuming the divergence rate of 2% per Myr, the time of divergence among the main phylogenetic groups (IA, IB, and II) in the *F. rufa* group is about 490 thousand years (kyr) BP, and between *F. lugubris* and *F. paralugubris*/*aquilonia* about 100 kyr BP. Despite the imprecision of these time estimates, they imply speciation duration considerably less than 2 Myr. This conclusion is still held under the slowest rate of 0.4% per Myr suggested for some insect taxa (Prüser and Mossakowski, 1998) and thus the most conservative rate for the Pleistocene speciation hypothesis.

The social type with high polygyny and formation of colonial networks was represented in all the phylogenetic clades except in IB: *F. lugubris* from England and Switzerland (haplotypes L, M, and N in the clade IA), *F. paralugubris* and *F. aquilonia* (haplotypes O, P, Q, and R in the clade IA), and *F. polyctena* (haplotypes W, X, Y, Z, and AB in the clade II.

3.4. Intraspecific mtDNA diversity and phylogeographic structure

The estimates of intraspecific nucleotide diversity varied among the species from $0.29 \pm 0.08\%$ in *F. lugubris* to 0% in *F. paralugubris* (Fig. 4). Individuals with haplotypes affiliated to a different morphological species

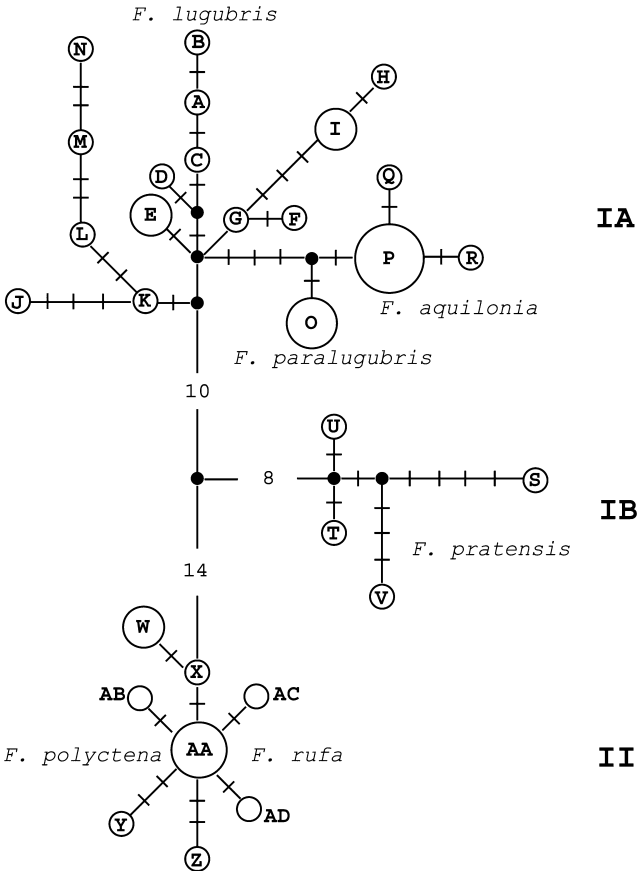

Fig. 3. Medium-joining network showing phylogenetic relationships between haplotypes of the *Formica rufa* group species. Circled areas are approximately proportional to the number of individuals bearing a particular haplotype. Branch lengths are proportional to the number of mutations involved between haplotypes. Mutations are indicated as ticks. Haplotypes and clades refer to Fig. 2 and Table 1. Numbers indicate numbers of changes along the branches.

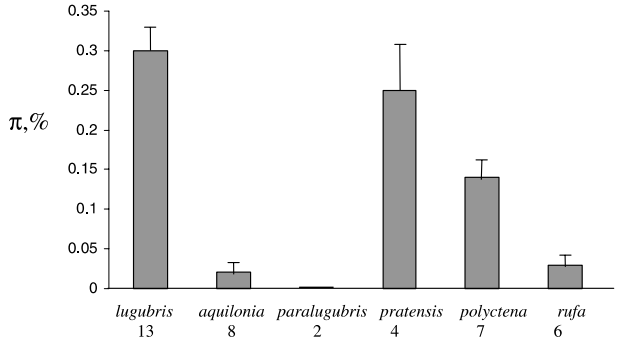

Fig. 4. Nucleotide diversity estimates for the six *Formica rufa* group species. Bands indicate the range (means + SE), numbers of haplotypes included in the analysis are shown under species names.

clade (B, I, and O, Fig. 2) were excluded from the analysis to eliminate the effect of interspecific hybridisation. Lack of variation in *F. paralugubris* might be a result of the small sample size (two individuals from different localities in Switzerland). There was no evident association between the type of social organisation and nucleotide diversity (π). The highly polygynous *F. polyctena* and *F. aquilonia* had $\pi = 0.14 \pm 0.04\%$ and $0.02 \pm 0.02\%$, and the mostly monogynous *F. pratensis* and *F. rufa* had $\pi = 0.25 \pm 0.11\%$ and $0.03 \pm 0.03\%$, respectively.

Despite the extensive geographic sampling, no phylogeographic structure was detected within species. The only association between the genealogies and geographic distribution of the haplotypes was found in *F. lugubris* (Figs. 1 and 2). The eastern group included haplotypes (A–D) from two rather distant Siberian localities (13 and 14). The western group included haplotypes (J–N) from Pyrenees (8), Switzerland (7), and Britain (5).

4. Discussion

4.1. Phylogenetic relationships among species

The mtDNA phylogeny shows that the six *F. rufa* group species form a very close group compared to the species used as outgroup: *F. truncorum* and *F. frontalis*. The clade of *F. polyctena*/*F. rufa* is basal within this group in the rooted phylogenetic tree and *F. pratensis* is clearly distinct from all the other species. The branching pattern does not support the suggestion based on morphological differences that *F. pratensis* diverged before the separation of the other *F. rufa* group species (Seifert, personal communication) and agrees better with the earlier allozyme results (Pamilo et al., 1979) in associating *F. pratensis* with the species *F. lugubris* and *F. aquilonia*. The species *F. rufa* and *F. polyctena* belong to one undivided monophyletic group and this implies recent divergence and incomplete lineage sorting between them. The lack of hiatus in the mtDNA phylogeny is consistent with the occurrence of frequent hybridisation between *F. rufa* and *F. polyctena* in nature (Czechowski, 1996; Seifert, 1991). Although *F. rufa* and *F. polyctena* are not distinct as regards mtDNA, we do not suggest that their taxonomic status should be revised as there are pronounced differences in morphological, social, and population characteristics (Collingwood, 1979). The species *F. paralugubris* that was only recently described morphologically (Seifert, 1996) is phylogenetically close to *F. aquilonia*. So far *F. paralugubris* is known only from the Alps and the Jura mountains in Switzerland and adjacent regions. This species is morphologically similar to *F. lugubris*, but the allozyme study (Pamilo et al., 1992) and behavioural experiments based on workers' discrimination against the sexual pupae of an alien type (Rosengren et al., 1994) showed that *F. lugubris* and *F. paralugubris* represent different gene pools. In Switzerland, all three species *F. lugubris*, *F. aquilonia*, and *F. paralugubris* are highly polygynous and differentiated at a set of allozyme and microsatellite

loci (Chapuisat et al., 1997; Pamilo et al., 1992). It is possible that *F. paralugubris* has arisen from hybridisation between *F. lugubris* and *F. aquilonia* and consequent isolation of a highly polygynous population for a long time. Thus the mtDNA phylogeny generally supports the division of the *F. rufa* group into distinct species suggested on the morphological basis. However, the lack of phylogenetic hiatus between morphologically distinct *F. rufa* and *F. polyctena* shows that the correspondence between the mtDNA phylogeny and morphology is not complete.

It has been shown that different species of the *F. rufa* group can hybridise and produce workers with intermediate phenotypes (Czechowski, 1996; Seifert, 1991). In this study, we selected only individuals with species specific morphological characteristics that showed no hybrid traits. However, the haplotypes affiliated to different morphological species clades most probably indicate three hybridisation events in the past generations. One *F. aquilonia* from locality 14 in Siberia and two *F. pratensis* from locality 8 in the Pyrenees had haplotypes that clustered together with *F. lugubris* from the same localities (Figs. 1 and 2). One *F. polyctena* from locality 12 in the Urals had the same haplotype as *F. paralugubris.* It is noteworthy that *F. paralugubris* has so far not been recorded outside Central Europe. It seems less likely that the observations would result from segregation of ancestral polymorphism as the differences between the species pairs are otherwise clear (although not large). Genetic and morphological evidence for interspecific hybridisation indicate that speciation is not completed in the *F. rufa* group. Nevertheless, the good correspondence between the mtDNA phylogeny and morphological distinctions among mostly sympatric species implies considerable extent of reproductive isolation maintaining integrity of the species.

4.2. Evolution of social organisation

The distribution of the social types in the phylogenetic tree shows that the transition between monogyny and a very high level of polygyny has taken place more than once during the evolutionary time. This result agrees with the general conclusion that polygyny has multiple origins (Ross and Carpenter, 1991; Ward, 1989). It has been shown that polygyny can arise from monogyny through adoption of new queens in different ecological (Herbers, 1993; Rosengren and Pamilo, 1983) and physiological conditions (Sundström, 1993). Although many ant species show either monogyny or polygyny, some species have intraspecific social polymorphism where colonies are monogynous in some populations and polygynous in others (Ross and Fletcher, 1985; cf. Komene et al., 1999; Lindström et al., 1996). Three species in the *F. rufa* group, *F. polyctena*, *F. aquilonia*, and *F. paralugubris*, are highly polygynous (with tens or even hundreds of breeding females per colony), and often form large colonies with interconnected nests (Chapuisat et al., 1997; Collingwood, 1979). *F. rufa* is a monogynous species as well as *F. pratensis* (Collingwood, 1979) although Swedish and Belgium populations of *F. rufa* were found to be slightly and highly polygynous, respectively (Gyllenstrand, 2002, E. Van Walsum, personal communications), and *F. pratensis* was shown to have a few reproductive queens in colonies (Pirk et al., 2001). *F. lugubris* populations in Finland are monogynous and those in Switzerland and England are polygynous (Gyllenstrand, 2002; Pamilo et al., 1992). The mtDNA phylogeny has two clades with fixed high level of polygyny, namely that of, *F. paralugubris*/*F. aquilonia* and the one with *F. lugubris* from Switzerland and England. Unfortunately the level of polygyny in many of the *F. lugubris* populations included in this study is unknown. The clade of *F. polyctena*/*F. rufa* includes both types of social organisation associated with very low sequence divergence. These results give no strong phylogenetic evidence supporting the importance of polygyny for speciation, except perhaps for *F. aquilonia* and *F. paralugubris* both of which build large supercolonies and show little sequence variation.

4.3. Pleistocene speciation hypothesis and phylogeography

A comparison of interspecific divergence in the *F. rufa* group with estimates from other groups of closely related insects shows relatively low divergence among the *F. rufa* group. The maximum pairwise divergence between the haplotypes of 2.2% in the *F. rufa* group is rather within the intraspecific divergence range for other insects, for example 2.9–5.2% for *Carabus* beetles (Prüser and Mossakowski, 1998), 1.9–7.4% for *Brachyderes* beetles (Emerson et al., 2000), maximum of 4.4% for *Talitropsis* crickets (Trewick, 2000), maximum of 6.5% for *Maoricicada* cicada (Buckley et al., 2001). However, *Nesotes* beetles from Canary Islands showed recent divergence and lower estimates: 0.6–1.7% within clades with one to three species (Rees et al., 2001). These comparisons suggest that speciation in the *F. rufa* group was relatively fast.

The divergence estimates of the *F. rufa* group species suggest that speciation took place during the Pleistocene, even under the slowest and most conservative divergence rate of 0.4% per Myr. Vicariant separation into different forest refugia during the Pleistocene glaciations might have been important for speciation in the *F. rufa* group. However, the main radiation in this group predated the last glaciation (110 kyr BP, Anderson and Borns, 1997). There is no data to suggest specific geographic setting for refugial hypothesis concerning speciation in the *F. rufa* group species distributed sympatrically today. Results of this study give one specific example suggesting importance of refugial

separation for speciation in the *F. rufa* group ants. Under the average divergence rate of 2% per Myr, time of divergence between the two northern most species of the group, *F. lugubris* and *F. aquilonia*, roughly corresponds to duration of the last glaciation (about 100 kyr BP). Furthermore, *F. aquilonia* clade demonstrates typical star-like internal phylogeny (Fig. 3) with the most common haplotype (P) distributed from England to the Lake Baikal in the centre surrounded by two unique haplotypes (Q and R). The star-like phylogeny and low diversity in *F. aquilonia* provide evidence for a recent bottleneck event followed by population expansion (Slatkin and Hudson, 1991). Postbottleneck time can be estimated from nucleotide diversity divided by the divergence rate (Rogers and Jorde, 1995) as about 10 kyr. While this time estimate is not significantly different from zero it is close to the beginning of postglacial colonisation in the Holocene (Bennett, 1997). These findings suggest that the clade leading to *F. aquilonia* and *F.paralugubris* could have diverged from *F. lugubris* in isolation and through a bottleneck event in a separate forest refugium.

Lack of intraspecific phylogeographic structure on continental scale in most species of the group is even more unexpected as most other insects with few exceptions demonstrated pronounced phylogeographic structure (cf. Avise, 2000). The clades of *F. rufa*/*F. polyctena* and *F. aquilonia* demonstrate star-like internal phylogenies with the central haplotypes being distributed over the most of Eurasia, and with low nucleotide diversity. This pattern gives evidence for relatively recent bottleneck events followed by population expansion (Slatkin and Hudson, 1991). It is reasonable to assume that both groups went through a bottleneck surviving glaciation in forest refugia and colonised most of the Eurasia during the Holocene. Low variation after refugial bottlenecks and short time in mutational units after colonisation can explain the lack of phylogeographic structure in these species. It is also worth while to examine whether one mitochondrial variant could have hitchhiked along with the spreading CI-inducing symbiont, decreasing within-species mtDNA polymorphism, as the endosymbiotic *Wolbachia* bacteria are common in the *F. rufa* group (Anu Sirviö, unpublished).

Limited phylogeographic structure in the most variable *F. lugubris* could indicate mixing of different gene pools during postglacial colonisation from different forest refugia. A population level study with larger sample sizes is required to reveal the geographic structure and historical relationships among populations in this species.

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