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The many ways to delimit species: hairs, genes and surface chemistry

Perttu SEPPÄ, Heikki HELANTERÄ, Kalevi TRONTTI, Pekka PUNTTILA, Anton CHERNENKO, Stephen J. MARTIN & Liselotte SUNDSTRÖM

Abstract

Species identification forms the basis for understanding the diversity of the living world, but it is also a prerequisite for understanding many evolutionary patterns and processes. The most promising approach for correctly delimiting and identifying species is to integrate many types of information in the same study. Our aim was to test how cuticular hydrocarbons, traditional morphometrics, genetic polymorphisms in nuclear markers (allozymes and DNA microsatellites) and DNA barcoding (partial mitochondrial COI gene) perform in delimiting species. As an example, we used two closely related *Formica* ants, *F. fusca* and *F. lemani*, sampled from a sympatric population in the northern part of their distribution. Morphological characters vary and overlap in different parts of their distribution areas, but cuticular hydrocarbons include a strong taxonomic signal and our aim is to test the degree to which morphological and genetic data correspond to the chemical data. In the morphological analysis, species were best separated by the combined number of hairs on pronotum and mesonotum, but individual workers overlapped in hair numbers, as previously noted by several authors. Nests of the two species were separated but not clustered according to species in a Principal Component Analysis made on nuclear genetic data. However, model-based Bayesian clustering resulted in perfect separation of the species and gave no indication of hybridization. Furthermore, *F. lemani* and *F. fusca* did not share any mitochondrial haplotypes, and the species were perfectly separated in a phylogenetic tree. We conclude that *F. fusca* and *F. lemani* are valid species that can be separated in our study area relatively well with all methods employed. However, the unusually small genetic differentiation in nuclear markers (F_{ST} = 0.12) shows that they are closely related, and occasional hybridization between *F. fusca* and *F. lemani* cannot be ruled out.

Key words: Allozymes, cuticular hydrocarbons, DNA barcoding, DNA microsatellites, *Formica*, morphology, species delimitation.

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Dr. Perttu Seppä (contact author), Dr. Kalevi Trontti, M.Sc. Anton Chernenko & Prof. Dr. Liselotte Sundström, Department of Biological Sciences, University of Helsinki, PO Box 65, FI-00014 University of Helsinki, Finland. E-mail: perttu.seppa@helsinki.fi

Dr. Heikki Helanterä, Department of Biological Sciences, University of Helsinki, PO Box 65, FI-00014 University of Helsinki, Finland; Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK.

Dr. Pekka Punttila, Finnish Environment Institute, PO Box 140, FI-00251 Helsinki, Finland.

Dr. Stephen J. Martin, Department of Animal and Plant Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK.

Introduction

Categorization of species into entities that can be recognized and distinguished from each other is a natural way for us humans to perceive and understand the world around us. Linné championed this by introducing the binomial nomenclature and nested hierarchy for all known species, but a logical follow-up of the emergence of evolutionary biology in the 19th century was to start studying how species emerge, how they change in time and how they are related to each other. This has lead to new problems in defining species, and now we have a plethora of different ways to define when two populations have diverged enough so that they should be considered new species (WILKINS 2003). Correct identification of species remains a challenge, however. It not only lays the basis for understanding the diversity of the living world and how it emerged, but it is also a prerequisite for understanding many evolutionary patterns and processes (SITES & MARSHALL 2003).

The traditional way to distinguish species has been to use external morphology. However, morphology sometimes conveys a false signal of the phylogenetic status of the units under study, as local adaptation may result in morphological change without simultaneous speciation, or conversely, morphological characters may be under strong stabilizing selection and speciation may occur without any notable morphological change (COLBORN & al. 2001, SÁEZ & al. 2003, BAKER & BRADLEY 2006, HOFFMAN & al. 2010). During the last few decades, molecular markers have been used for resolving the evolutionary histories of species as

well as identifying new ones and establishing reproductive isolation between units of interest (HILLIS & al. 1996). Most recently, sequencing of a standardized part of mtDNA, so called DNA barcoding, has been suggested as a standard solution for species identification (HEBERT & al. 2003). Besides morphology and genetics, other types of characters can also be used for delimiting species. These include acoustic signals in birds (ISLER & al. 1998) and amphibians (ANGULO & REICHLE 2008), composition of the chemical compounds synthesized by various plant species (KIM & al. 2000, GE & al. 2008, LOVE & al. 2009), the ecological niche species occupy (WIENS & GRAHAM 2005, ROSS & al. 2010) or surface chemistry (cuticular hydrocarbons, CHC) in ants (MARTIN & al. 2008a, b) and bumblebees (MARTIN & al. 2010).

Despite their abundance and ecological importance, ants are not particularly species-rich (WILSON 1990, 1992), but even in well-studied areas such as Europe, new species are still continuously described (e.g., SEIFERT 1996a, 1997, 2000). A good example of a problematic ant taxonomy is the genus *Formica* (see VEPSÄLÄINEN & PISARSKI 1981), where recent speciation (GOROPASHNAYA & al. 2004), hybridization between differentiated lineages (SEIFERT 1999, SEIFERT & GOROPASHNAYA 2004, SORVARI 2006, KULMUNI & al. 2010, SEIFERT & al. 2010), and queens of different species potentially breeding in the same nests (CZECHOWSKI & RADCHENKO 2006, KORCZYNSKA & al. 2010) may make species identification a nightmare when using traditional morphological characters only (VEPSÄLÄINEN & PISARSKI 1981). Morphological characters are usually studied from worker material, and intra-nidal variation in the characters studied, such as the number of hairs in different body parts, can be extensive (e.g., SEIFERT 2003), making identification of genetically independent lineages difficult.

Formica fusca LINNAEUS, 1758 and *F. lemani* BONDROIT, 1917 are common palearctic ants, which inhabit both dry and wet, open and semi-open habitats, such as meadows, woodland edges and peat bogs (COLLINGWOOD 1979, CZECHOWSKI & al. 2002). In Northern Europe, they are typical pioneering species in the early successional stages of coniferous boreal forests (PUNTTILA & al. 1991). Their distributions overlap considerably in Europe, but *F. lemani* predominates at higher latitudes and altitudes than *F. fusca* (Fig. 1; COLLINGWOOD 1979, CZECHOWSKI & al. 2002). Both species are facultatively polygynous (HANNONEN & al. 2004, BARGUM & al. 2007; L. Sundström, H. Helanterä & A. Chernenko, unpubl.). Roughly half of the *F. fusca* nests have more than a single queen, the typical number being two to three, although nests with over 100 queens are occasionally found (HANNONEN & al. 2004; L. Sundström, H. Helanterä & A. Chernenko, unpubl.). This is reflected as a moderate to high relatedness among nestmate workers (HANNONEN & al. 2004, BARGUM & al. 2007). Data on queen numbers in *F. lemani* are scanty, but our own observations and relatedness patterns suggest that *F. lemani* is slightly less polygynous than *F. fusca* on average (GARDNER & al. 2007, SEPPÄ & al. 2009).

Formica fusca and *F. lemani* are usually distinguished by examining the number of standing hairs in different body parts, *F. fusca* being generally less hairy (Appendix 1, as digital supplementary material to this article, at the journal's web pages). The cut-off values for these characters vary with authors, and sometimes also for a single author

Fig. 1: Approximate distributions of *Formica fusca* and *F. lemani* in Europe (adapted from CZECHOWSKI & al. 2002). The distribution of *F. fusca* is indicated with dark shading, *F. lemani* with light shading, and sympatric areas with intermediate shading. The sampling sites are: 1. Hyytiälä; 2. Hathersage; 3. Thetford; 4. Exeter and 5. Kendal.

(Appendix 1), possibly reflecting geographic variation in hairiness. For instance, the number of hairs on the promesonotum of a *F. fusca* worker is given as zero by most authors, but individuals with up to three (YARROW 1954) or four (KUTTER 1977) promesonotal hairs have also been accepted as *F. fusca*. On the other hand, all authors agree that *F. lemani* is more hairy, yet most individuals in some southeast European populations are reported as hairless (DLUSSKY & PISARSKI 1971). Because of the many similarities in their biology, *F. fusca* and *F. lemani* have been regarded as closely related sister species (e.g., MARTIN & al. 2008a), but phylogenetic studies on the genus *Formica* have not addressed this particular question (SAMESHIMA & al. 1999, GOROPASHNAYA 2003).

The aim of our study was to address the problem of species delimitation, with the black ants *Formica fusca* and *F. lemani* as an example. Our work was motivated by the ambiguous morphological details given by various authors when identifying them (Appendix 1) and the practical needs in our own studies in boreal forests on the northern edge of their distribution. Thus, our aim is not to conduct a thorough taxonomical examination. Instead, we tested how surface chemistry, traditional morphometrics, and genetic polymorphisms in nuclear and mitochondrial markers perform in delimiting species, and whether the different methods convey the same information about the species identity of the nests. MARTIN & al. (2008a, b) showed that *F. fusca* and *F. lemani* can be distinguished solely based on their CHC profiles, irrespective of habitat or population of origin. Consequently, we decided to use surface chemistry as a baseline for species delimitation, and cross-referenced it to nest clusters identified from morphological and genetic data. We assume that *F. fusca* and *F. lemani* are valid

species, and that con-specific nests cluster together irrespective of their population of origin.

Material and methods

Sampling: Our main data are based on worker samples of *Formica fusca* and *F. lemani* collected in 2007 from 48 nests at one recently clear-cut site close to the Hyytiälä Forest Research Station in Central Finland. For comparative purposes, we sampled *F. fusca* workers also from three locations in the UK (Thetford, Norfolk n = 8; Exeter, Devon n = 3; Kendal, Cumbria n = 1), and *F. lemani* workers from 17 nests near Hathersage, Derbyshire (Fig. 1). These latter *F. fusca* samples were combined in the genetic analysis as a single population.

Surface chemistry: We assessed CHC profiles of one to two workers (total 90) from 46 nests sampled in the Hyytiälä population. In addition, we used available data from three to five workers from the nests sampled from the UK populations as a reference. Detailed laboratory methods are described in MARTIN & al. (2008a), but briefly, a hexane extract of each ant was analyzed on a gas chromatograph connected to a mass spectrometer. CHCs were characterized by their diagnostic ions and Kovats indices. The peak area of each compound on the total ion chromatogram was then measured, and used in the subsequent analysis. The relative amounts of each compound reported as the number of ions contained beneath the peak was tabulated for each ant. The proportion of each compound was calculated based on the total amount ions and the mean value derived for each nest. The mean proportion was then transformed using the method of AITCHISON (1986) before conducting a Principal Components Analysis (PCA). Separation of nests and populations was illustrated by plotting the scores of the two first principal components against each other.

Morphometrics: Five workers from each Hyytiälä nest were measured for nine morphological characters (Appendix 2, as digital supplementary material to this article, at the journal's web pages) following EICHHORN (1972) and SEIFERT (1996a, b, 1997, 2003). A PCA was run on the variable characters, separately for all individual workers and for the colony averages calculated from the five workers measured for each colony. The analyses were based on correlation matrices with the data centred and standardized for the morphological characters used in the analyses, and run in CANOCO (TER BRAAK & ŠMILAUER 1998).

Allozyme and DNA microsatellite genotyping: We genotyped eight workers from each nest in Hyytiälä (range 7 - 15, total 499) for allozyme variation at four loci (for details, see SEPPÄ 1992, and Appendix 3, as digital supplementary material to this article, at the journal's web pages), and one to four workers from each nest in Hyytiälä (total 180), Hathersage (41) and in Thetford, Exeter and Kendal (total 31) at ten DNA microsatellite loci (for details, see SEPPÄ & al. 2009, and Appendix 3). Genetic variation at the allozyme and DNA microsatellite loci was described by the number of alleles (n_{ALL}), estimating the allelic richness (R_S, EL MOUSADIK & PETIT 1996) and the expected heterozygosities (H_E) for each locus.

We described genetic population structure using a PCA, where both the most common allele at each locus and rare alleles observed only in a single nest were omitted. Separation of nests and populations was illustrated by plotting

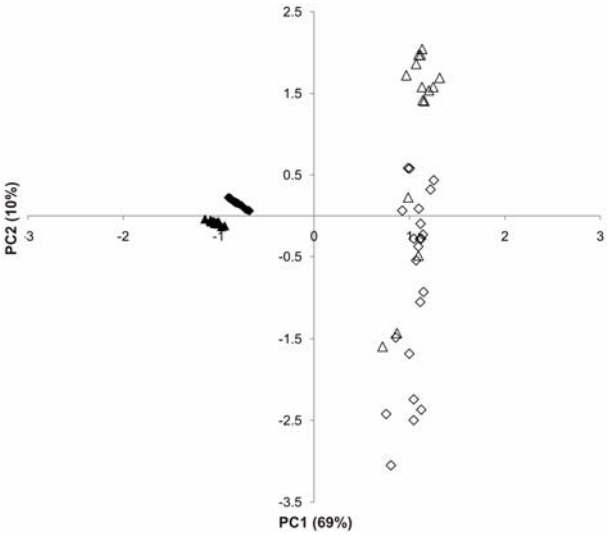

Fig. 2: Sample scores of nests in the ordination space formed by the two first principal components in the chemical data. In the analysis, seventeen *Formica fusca* and twenty-one *F. lemani* nests previously sampled and analysed were added from the same UK populations. The open and filled symbols are *F. fusca* and *F. lemani*, respectively, diamonds are Finnish and triangles UK samples.

the sample scores in the ordination space formed by the two first principal components. We also used a model-based Bayesian clustering and admixture method (BAPS v. 5.3; CORANDER & MARTTINEN 2006) for the DNA microsatellite data. The software also builds a neighbor-joining tree (SAITOU & NEI 1987) for the clusters constructed. The BAPS analysis was made at the individual level, using a single randomly sampled individual from each nest, and repeating the procedure four times. We allowed the software to freely find the optimal number of clusters in the data. Finally, we also estimated differentiation between populations and species by using Analysis of Molecular Variance (AMOVA, EXCOFFIER & al. 1992), which calculates F_{ST} estimates for each predefined level of hierarchy. For AMOVA, we used GENALEX (PEAKALL & SMOUSE 2006), and determined the significant deviation of F_{ST} from zero by permuting the data 9999 times.

mtDNA sequencing for DNA barcoding: We sequenced a 611 bp region of the mitochondrial COI gene from one worker from six *Formica fusca* and ten *F. lemani* nests in Hyytiälä. The nests were chosen to represent evenly the space defined by the two first principal components (Fig. 5). PCR primers used in the amplification (F: ACTAGGATCTCCAGACATAGC, R: GCTCGTGTATCAACATCTAA) were designed from NCBI GenBank sequences of *F. fusca* (AB010925) and *F. lemani* (AB019425). PCR reactions were done with the Phusion PCR kit (Finnzymes) using 10 pmol both primer and 20 - 50 ng of DNA template. PCR profile followed the kit protocol with annealing temperature at 55°C. Amplification products were purified and sequenced using the primers above with BigDye v.1.1. sequencing kit (Applied Biosystems). Sequences were obtained with MegaBACE 1000 sequencer (GE), assembled with SEQUENCHER 4.1 (Gene Codes), and aligned with CLUSTAL (THOMPSON & al. 1994).

We calculated the sequence divergence (JUKES & CANTOR 1969), and constructed a neighbor-joining tree (SAITOU

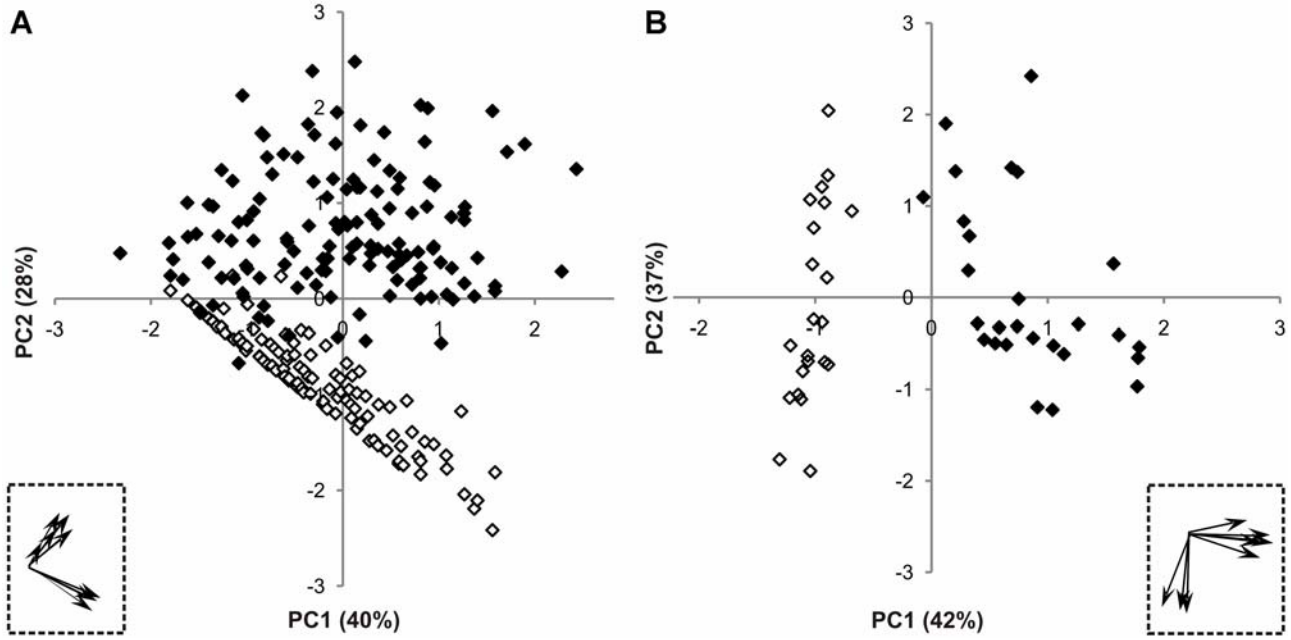

Fig. 3: Sample scores of individual workers (A) and colony means (B) in the ordination space formed by the two first principal components in the morphological data. The open and filled diamonds are *Formica fusca* and *F. lemani*, respectively. Factor loadings of the variables (morphological characteristics) are shown as vectors in insets in both panels. Vectors towards top right (panel A) and right (panel B) are associated with hairiness, and vectors towards bottom right (panel A) and bottom left (panel B) with body size.

& NEI 1987) from Kimura 2-parameter distances (KIMURA 1980) with MEGA version 4 (TAMURA & al. 2007). Tree confidence was tested by bootstrapping 1000 times. In the analysis, the *F. fusca* and *F. lemani* sequences used for primer design (see above) were included as a comparison, and a *Formica exsecta* sequence also obtained from GenBank (AB103364) was used as an outgroup. Sequence divergences were calculated from the sequence data for all pairs of haplotypes. Finally, we estimated differentiation between species with ARLEQUIN v. 3.1 (EXCOFFIER & al. 2005).

Results

Surface chemistry: The CHC profiles of *Formica fusca* and *F. lemani* are very distinct since both species possess unique compounds. As a result, the CHC profiles formed two clearly separated clusters in the ordination space delineated by the two first principal components (Fig. 2). The reference nests from UK were also closely associated with the Hyytiälä nests, suggesting that all Hyytiälä nests could be clearly assigned to either *F. fusca* or *F. lemani*. This confirms the previous result by MARTIN & al. (2008a) and justifies the use of surface chemistry as a baseline for species delimitation. In *F. lemani*, the UK and Finnish samples were clearly separated from each other, although they clustered very close to each other in the ordination. By contrast, *F. fusca* showed more variation than *F. lemani* in both populations (approximately along the PC1), and populations also overlapped in *F. fusca* (Fig. 2).

Morphometrics: Head capsule and scapus length indicated that individual *Formica fusca* workers were significantly larger than those of *F. lemani* (Appendix 2). At the nest level, however, only scapus length differed significantly between the species. *Formica lemani* workers had significantly more hairs on the pronotum, mesonotum, and in the margins of all three legs / femora than *F. fusca* both at the individual and the nest levels (Appendix 2).

When analysed at the individual level, the size of the individuals increased from the top left to the bottom right in the PCA ordination space formed by the two first principal components, whereas the hairiness gradient ran from the bottom left to the top right (Fig. 3A). Thus, *F. fusca* and *F. lemani* were quite clearly separated in the ordination space for large workers, but less so for small ones. In the PCA on the colony averages (Fig. 3B), the colonies of the two species were clearly separated from each other also when the average size of the workers in a colony was small. To illustrate the difference between the species in the morphological data, we calculated the frequency distribution of the combined number of hairs on the pro- and mesonotum (i.e., promesonotum). At the individual level (Fig. 4A), the number of hairs overlapped. Fifty (18%) and nine (3.3%) individuals had one or two promesonotal hairs, respectively, and three of them were *F. lemani* in both classes. At the nest level (Fig. 4B), separation of the species was clear, with the maximum in *F. fusca* nests being 1.0 ± 1.0 (arithmetic mean ± standard deviation) and the minimum in *F. lemani* nests 2.8 ± 1.48 promesonotal hairs on average.

Identification of genetic clusters: We found no evidence for linkage disequilibrium in any of the allozyme or DNA microsatellite locus pairs (SEPPÄ & al. 2009) and all loci were retained in the analysis. In both species, the Hyytiälä populations tended to be genetically more diverse compared to the UK populations and similarly, *Formica lemani* populations tended to be genetically more diverse than sympatric *F. fusca* populations (Appendices 3 and 4, as digital supplementary material to this article, at the journal's web pages). In the PCA on allozyme data, the species

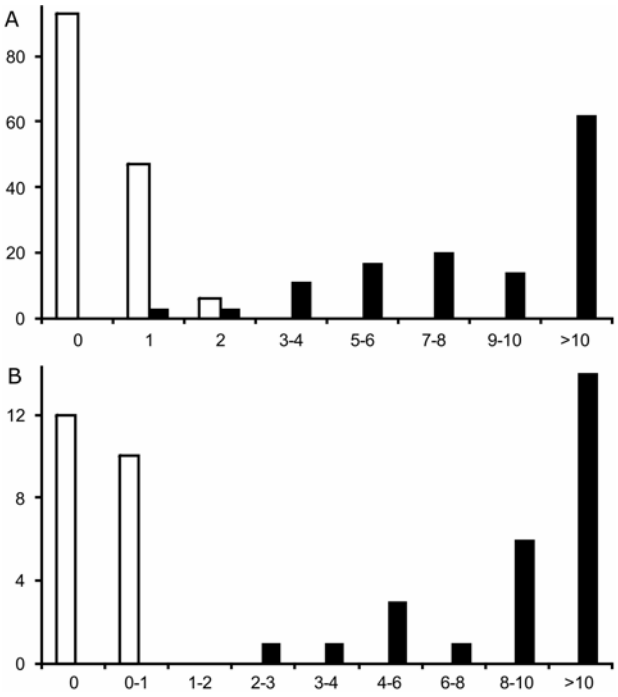

Fig. 4: Frequency distribution of the number of hairs in individual ants (A) and nests (B). Open and closed bars are *Formica fusca* and *F. lemani*, respectively.

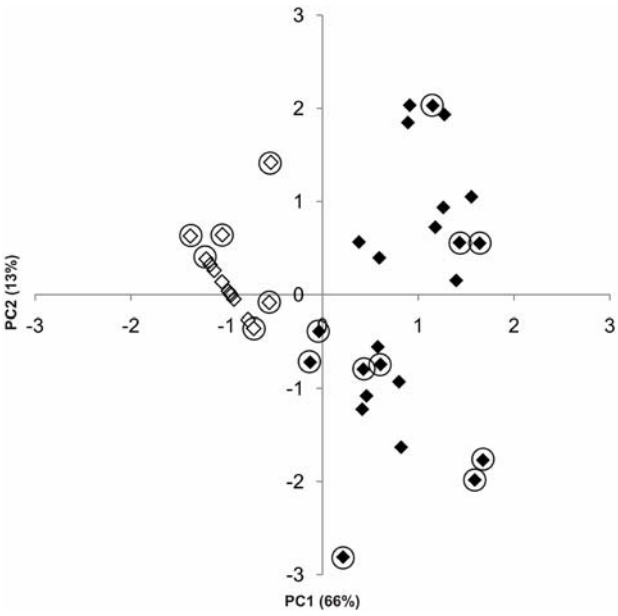

Fig. 5: Sample scores of nests in the ordination space formed by the two first principal components in the allozyme data from Hyytiälä. The open and filled symbols are *Formica fusca* and *F. lemani*, respectively, identified with the CHC analysis. The nests chosen for the DNA barcoding analysis are circled.

were separated in the ordination space formed by the two first principal components, but the distribution of sample scores was more or less continuous instead of forming discrete groups, and two species did not overlap (Fig. 5). In the PCA on the DNA microsatellite data, both the species and the con-specific Finnish and UK populations were separated in the ordination space, but the distribution of sample scores was again continuous rather than discrete (Fig. 6).

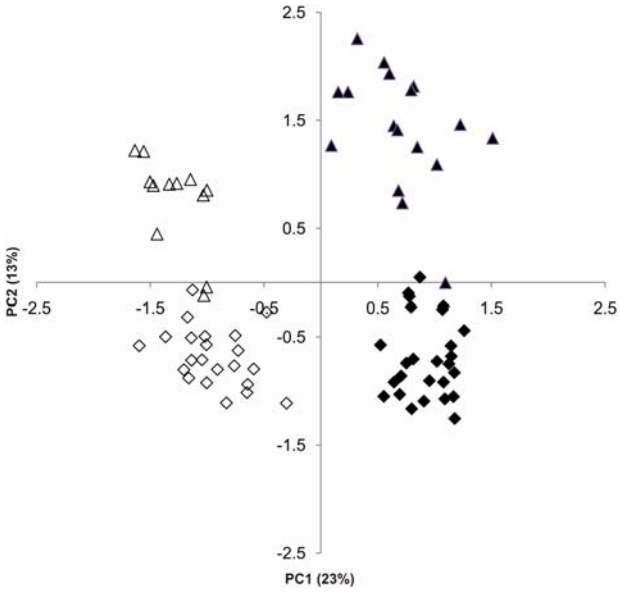

Fig. 6: Sample scores of nests in the ordination space formed by the two first principal components in the DNA microsatellite data. The open and filled symbols are *Formica fusca* and *F. lemani*, respectively, identified with the CHC analysis; diamonds are Finnish and triangles UK samples.

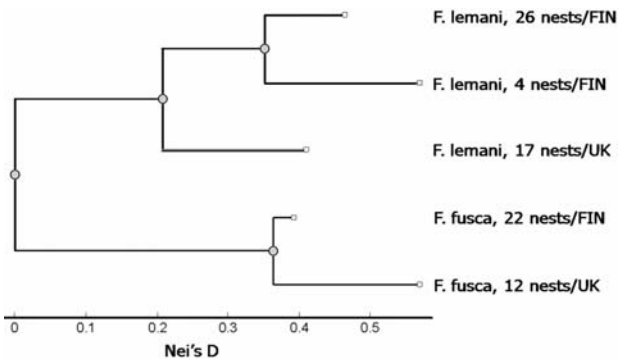

Fig. 7: An example of a neighbor-joining tree of *Formica fusca* and *F. lemani* nest clusters identified by Bayesian clustering. The species identity is based on the CHC analysis.

The optimal number of genetic clusters found in four different tries of Bayesian clustering was five (3 tries) or six (1 try). The analysis always separated individual *Formica fusca* and *F. lemani* nests to distinct clusters, and the different con-specific clusters always grouped together in a neighbor-joining tree (Fig. 7). There was also an expected split between geographically isolated con-specific populations, but a small number of Hyytiälä nests were separated as its own cluster in *F. lemani* (3 tries) or in both species (1 try), and up to three nests were also occasionally clustered with nests from a population they were not sampled from. Finally, one *F. lemani* nest sampled from UK showed admixture of equal genetic contributions from *F. lemani* populations from both UK and Finland.

For the allozyme data from Hyytiälä, an AMOVA with nests as the bottom and the species as the top level of the hierarchy showed that between-species variation accounted

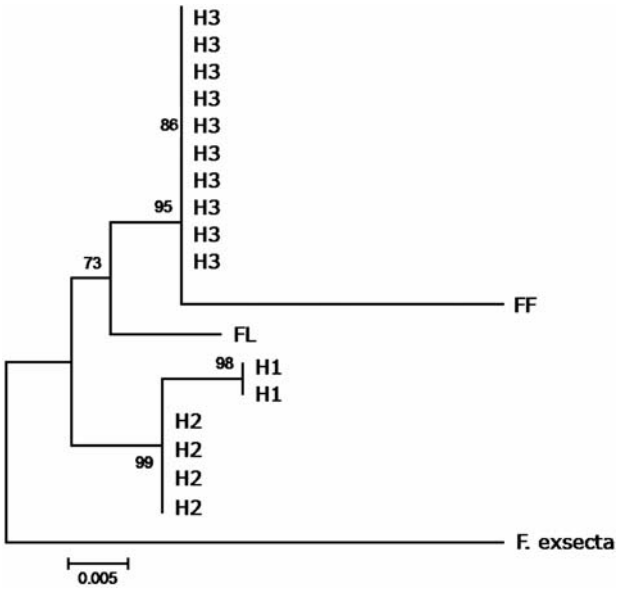

Fig. 8: Neighbor-joining tree based on the partial COI sequence. H1 and H2 are the *Formica fusca* haplotypes and H3 is the *F. lemani* haplotype found in the Hyytiälä population. Sequences obtained from GenBank that were added in the analysis are FF (*F. fusca*, AB010925), FL (*F. lemani*, AB019425) and *F. exsecta* (outgroup, AB103364).

for 35% of the total genetic variation in the data, and that the genetic differentiation between them was high and significantly greater than zero (F_{ST} = 0.35, $p < 0.001$). For the DNA microsatellite data, an AMOVA with populations in Hyytiälä and the UK as the bottom level, and species as the top level of the hierarchy showed that between-species variation accounted for 12%, and between-population variation accounted for 15% of the total genetic variation in the data. The genetic differentiation was significant at both levels (between species: F_{ST} = 0.12, $p < 0.001$; between con-specific populations: F_{ST} = 0.17, $p < 0.001$). When sympatric Hyytiälä populations were analysed separately, between-species variation accounted for 25% of the total genetic variation, with an F_{ST} = 0.25 ($p < 0.001$).

mtDNA sequencing: We identified three haplotypes in the Hyytiälä population, two in *Formica fusca* (two and four individuals of each) one in *F. lemani* (ten individuals). In addition, the *F. fusca* and *F. lemani* sequences obtained from GenBank had unique haplotypes. Sequence divergence between the *F. fusca* haplotypes in Hyytiälä was 0.65%, and the inter-specific sequence divergence among *F. fusca* and *F. lemani* haplotypes was 1.96% on average. In the neighbor-joining tree, the Finnish *F. fusca* and *F. lemani* sequences clustered separately with a strong bootstrap support (Fig. 8). As the species did not share any haplotypes in these populations, the genetic differentiation in the mitochondrial genome approached unity ($F_{ST\text{-}mt}$ = 0.93, $p < 0.001$). Divergence between the *F. lemani* haplotype from Hyytiälä and the one obtained from GenBank was also small (0.98%), but the divergence between our *F. fusca* haplotypes and the one obtained from GenBank was substantially larger, 4.91% on average. Indeed, the latter *F. fusca* sequence clustered in the neighbor-joining tree as a sister group to the Hyytiälä *F. lemani* sequences with strong bootstrap support, and the *F. lemani* sequence obtained from GenBank clustered as a sister group to both of these (Fig. 8).

Discussion

The motivation for this study was primarily pragmatic, to provide guidance in species delimitation for students working with ant species closely resembling each other. This includes ourselves, as ants of the subgenus *Serviformica* are an important model in our research (e.g., HANNONEN 2002, HELANTERÄ 2004, BARGUM 2007, SEPPÄ & al. 2009), but we hope that our integrated approach will prove useful for others working with similar problems as well. Indeed, here we showed that *Formica fusca* and *F. lemani* are entities that can be distinguished positively by surface chemistry and DNA barcoding, but that they are still closely related species as reflected in the minor genetic and morphological differentiation between them. We must note, however, that our main sampling was geographically limited, and the characters found to separate *F. fusca* and *F. lemani* may well be idiosyncratic to our study area. Our results support the currently emerging consensus that the most promising approach for correct species delimitation involves combining many types of information in the same study (SITES & MARSHALL 2003, 2004, SEIFERT 2009, ROSS & al. 2010, SCHLICK-STEINER & al. 2010).

Morphological separation of *F. fusca* and *F. lemani*: The number of hairs on pronotum, mesonotum and femora distinguish *Formica fusca* and *F. lemani* in most identification keys (Appendix 1). Our data support their use, as the best hair characteristics separating the two species in the PCA were (in this order) the numbers of hairs on pronotum, femur of the foreleg and mesonotum. However, these characters overlap and are not always sufficient for positively distinguishing *F. fusca* and *F. lemani* from single individuals. On the other hand, nest averages based on five workers did not overlap for the number of hairs on pronotum and mesonotum, and the overlap was negligible for the femur of the foreleg. Thus, the two first characters were clearly sufficient for separating the species at the nest level, but not at the individual level.

When workers were large, even single individuals allowed identification of nests. This supports the suggestions of authors who emphasize that large workers should be selected for scrutiny to reliably distinguish closely related species in the genus *Formica* (e.g., DOUWES 1979, 1995, SEIFERT 1991, 2007). However, large workers are not always readily available. For instance, incipient nests tend to have small-sized workers. This should be compensated by studying few to several individuals from each nest to reach a similar level of confidence in identification as with large-sized workers, which has also been suggested repeatedly (e.g., COLLINGWOOD 1979, DOUWES 1979, 1995, SEIFERT 1991, 2007). Furthermore, data commonly collected for ecological studies by using pitfall traps are composed of individual workers of random size and unknown colony origin.

Genetic separation of *Formica fusca* and *F. lemani*. – Nuclear markers: The genetic analysis separated *Formica fusca* and *F. lemani*, but at the same time showed that they are closely related entities. The PCA analysis on nuclear data showed that *F. fusca* and *F. lemani* nests neither overlapped nor formed well-separated clusters. In the allozyme data, *F. lemani* nests in Hyytiälä showed much greater dispersion than those of *F. fusca*, probably reflecting the greater genetic variation in *F. lemani* compared to *F. fusca*. In the DNA microsatellite data, Hyytiälä and UK nests of both *F. fusca* and *F. lemani* separated well ac-

cording to their populations of origin, showing that populations were genetically differentiated.

Model-based Bayesian clustering (BAPS) confirmed the PCA result. BAPS always clustered *Formica fusca* and *F. lemani* nests separately, and con-specific clusters were also closely associated in the neighbor-joining tree. Nests with the same geographic origin did not always cluster together, however, and the *F. lemani* population in Hyytiälä was genetically heterogeneous. One larger and one smaller genetic cluster were identified, but individual nests were not consistently assigned to the same clusters in different tries. Some *F. lemani* nests from the UK were also occasionally clustered among the Finnish ones, indicating smaller genetic differentiation between con-specific *F. lemani* populations than the PCA suggested. The admixture analysis suggested that one *F. lemani* nest sampled from the UK was a first generation hybrid between Finnish and UK *F. lemani*. However, this is unlikely given the large geographic distance between the populations.

Comparing F_{ST} estimates based on different markers and study systems is not straightforward as the amount of genetic variation affects the maximum F_{ST} at a locus. Thus comparisons of results based on different systems should be based on standardized data (HEDRICK 2005). This is probably the reason why differentiation between *Formica fusca* and *F. lemani* in the DNA microsatellite data was smaller than between con-specific populations. The distribution of genetic variation across the units studied, with both species having many private alleles, boosted the amount of genetic variation when the analysis was made on the species level and subsequently reduced the maximum value F_{ST} can reach in each locus.

Bearing this in mind, genetic differentiation between *Formica fusca* and *F. lemani* in DNA microsatellite markers (F_{ST} = 0.12) is rather small compared to DNA microsatellite studies on other ants. *Formica aquilonia, F. lugubris* and *F. paralugubris* are closely related red wood ant species (GOROPASHNAYA & al. 2004). Differentiation between them is at the same level as between *F. fusca* and *F. lemani*, while differentiation between them and other *Formica rufa* group species was much larger (medians of pairwise estimates: F_{ST} = 0.16 and F_{ST} = 0.30, respectively; BERNASCONI & al. 2011). *Formica rufa* and *F. polyctena* are another pair of closely related *Formica* ants (GOROPASHNAYA & al. 2004, SEIFERT & al. 2010), with similar differentiation as between *F. fusca* and *F. lemani* (median F_{ST} = 0.16, GYLLENSTRAND & al. 2004). On the other hand, differentiation between *Myrmica rubra* and its social parasite and closest relative *M. microrubra* is somewhat larger (F_{ST} = 0.18; STEINER & al. 2006, VEPSÄLÄINEN & al. 2009; see below concerning the species status of *M. microrubra*), as is the differentiation between *Solenopsis saevissima* populations probably including several so far undescribed cryptic species (F_{ST} = 0.23, ROSS & al. 2010). So, it seems clear that *F. fusca* and *F. lemani* are closely related, and genetic divergence between them is unusually small when measured from nuclear data.

Genetic separation of *Formica fusca* and *F. lemani.* – DNA barcoding: Sequencing of a partial mitochondrial COI gene identified *Formica fusca* and *F. lemani* as separate groups in the Hyytiälä population, as they did not share any haplotypes. Intra-specific sequence divergence in *F. fusca* (0.65%) was similar to other groups studied (e.g., butterflies: 0.43%; HEBERT & al. 2010), but the average inter-specific divergence between *F. fusca* and *F. lemani* (1.96%) was only modest (e.g., butterflies: 7.7%, HEBERT & al. 2010). Interestingly, the *F. fusca* and *F. lemani* sequences obtained from GenBank (SAMESHIMA & al. 1999) and added as a comparison to our phylogenetic analysis did not cluster with our Finnish samples as expected. The *F. lemani* sequence from Japan clustered as a sister group with Finnish *F. lemani*, but *F. fusca* sampled from Poland and Italy also clustered with strong support with Finnish *F. lemani* rather than Finnish *F. fusca*. Chemical analysis of *F. lemani* and *F. fusca* samples from several regions (S.J. Martin, unpubl.) found that species misidentification is common. This highlights the importance of our integrated approach when identifying species with unclear morphological clues.

Because of the smaller effective population size in mitochondrial compared to nuclear genome, mitochondrial differentiation is expected to exceed nuclear differentiation. None the less, nuclear differentiation between *Formica fusca* and *F. lemani* was relatively small and mitochondrial differentiation exceeded it manifold. This may be due to missing some haplotypes because the mitochondrial data was based on a single population, whereas the nuclear data encompassed several populations and two regions. In addition, a higher rate of back-mutations in microsatellite sequences compared to mitochondrial sequences may compound these effects. However, low differentiation at nuclear loci, combined with clear separation in mitochondrial haplotypes may also indicate the presence of male-mediated gene flow between the species. If so, the greater nuclear diversity and higher number of private alleles in *F. lemani* suggests this gene flow would be mediated mainly by *F. fusca* males mating with *F. lemani* females. Nevertheless, it cannot be frequent as no intra-specific first generation hybrids were found in the admixture analysis. Finally, the lack of shared haplotypes in sympatric populations indicates that adoption of hetero-specific queens and thus hybridization between *F. fusca* and *F. lemani* via mixed nests does not occur, as suggested in other *Formica* species (CZECHOWSKI & RADCHENKO 2006, KORCZYNSKA & al. 2010). Indeed, *F. fusca* and *F. lemani* both reject hetero-specific eggs and are strongly aggressive towards adults of other species (A. Chernenko, H. Helanterä, L. Sundström & S. Martin, unpubl.), so adoption of hetero-specific queens is unlikely.

Integrated approaches to delimit ant species: Surprisingly few attempts have been made to combine morphological and genetic methods towards an integrated approach in delimiting ant species. So far, the most common approach has been to combine morphological and mitochondrial data, usually from the COI gene. STEINER & al. (2005) and SCHLICK-STEINER & al. (2006) identified and demarcated phylogenetic entities in the genus *Tetramorium*, including several cryptic species, and used them to clarify their biogeography and distribution patterns. BERNASCONI & al. (2010) developed a restriction fragment length polymorphism method for discriminating the wood ants *Formica lugubris* and *F. paralugubris* based on mitochondrial polymorphisms, and corroborated this with DNA microsatellites. Furthermore, BERNASCONI & al. (2010) were able to identify and separate a new sibling species from *F. lugubris* with this approach. A combination of morphological and DNA microsatellite data was used by SEIFERT & al.

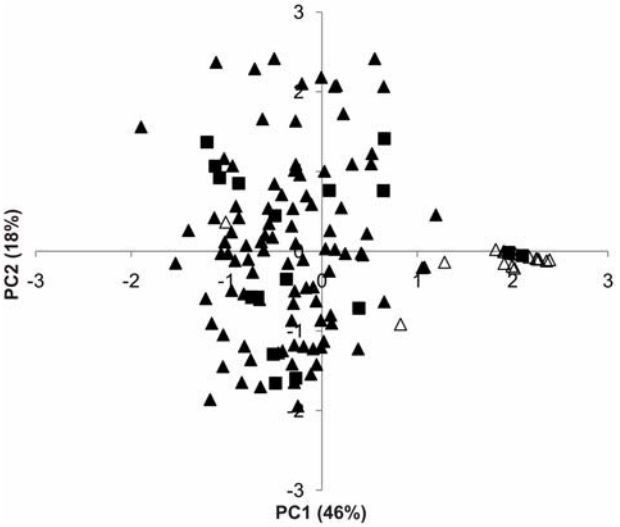

Fig. 9: Sample scores of nests in the ordination space formed by the two first principal components in the allozyme data (from SEPPÄ & al. 2009) used to test the genetic identification method. The open and filled triangles are *Formica fusca* and *F. lemani*, respectively, identified with morphometrics. The closed squares are nests not identified with morphometrics.

(2010) to separate the wood ants *F. rufa* and *F. polyctena*, and identify hybrid populations between the two species.

The power of an integrated analysis increases when both mitochondrial and nuclear markers are used in combination with morphological data. Based on morphology, allozyme markers and mitochondrial data, ROSS & SHOEMAKER (2005) showed that *Solenopsis invicta* and *S. richteri* were reproductively isolated in their native range in South America, although they hybridize extensively in the introduced areas in USA. STEINER & al. (2006) studied morphology and genetic variation in DNA microsatellites and both nuclear and mitochondrial sequences, and suggested that the inquiline *Myrmica microrubra* (see SEIFERT 1993) is not reproductively isolated from its host *M. rubra* and does not deserve species status (but see VEPSÄLÄINEN & al. 2009). Finally, by combining morphological characters, allozyme and DNA microsatellite markers, mitochondrial sequences and ecological-niche modeling, ROSS & al. (2010) showed that *Solenopsis saevissima* comprises several previously unrecognized species, and that genetic differentiation between populations previously identified as *S. saevissima* has been influenced by hybridization with other sympatric or parapatric *Solenopsis* species.

Testing species delimitation – combining morphometrics and nuclear genetic data: To test our species delimitation approach, we applied a combination of morphometrics and allozyme markers on a larger dataset (SEPPÄ & al. 2009). This data set encompassed *Formica fusca* and *F. lemani* workers from a total of 146 nests at six sites near Hyytiälä and Seitseminen National Park (Ikaalinen) in 1991 and 1993 (see SEPPÄ & al. 2009 for details of sampling). The initial species identification of these samples was made by assessing the number of hairs on the promesonotum at five sites and calculating a colony average based on one to eight workers (124 nests, median 2 workers / nest), following SEIFERT (1996b). The nests with at most one hair were classified as *F. fusca*, and those with more than one as *F. lemani*. This kind of identification probably reflects well routine species identification in laboratories not specializing in detailed morphological analysis. No initial species identification was made at the sixth site, but these samples were included in the genetic analysis to demonstrate the power of using only allozymes to delimit the species.

Ten workers from each nest (range 2 - 20, total 1482 workers) were genotyped for allozyme variation in six loci (SEPPÄ & al. 2009). In the PCA, *Formica fusca* and *F. lemani* as well as the unidentified nests were separated in the ordination space determined by the two first principal components. As above, separation was not categorical and a few of the nests were placed among the nests of the other species (Fig. 9). Particularly, one nest identified as *F. fusca* was deeply nested within the main cluster of *F. lemani* nests in the PCA. Whether the measured individuals in this nest were particularly small is not known. In AMOVA with species as the top and populations as the bottom level of the hierarchy, the amount of genetic variation allocated to the species level was 32% and genetic differentiation among the species was highly significant ($F_{ST} = 0.33$, $p < 0.001$). The amount of genetic variation at the population level was small (2%), but differentiation between populations was still significantly greater than zero ($F_{ST} = 0.026$; $p < 0.001$).

Are there species-specific characters that delimit species, and how useful are they? Despite their close phylogenetic relationship, all classes of characters used in this study separated *Formica fusca* and *F. lemani* relatively well. Consequently, all classes must include also species-specific characters, and it is largely up to the investigators and the facilities available which method they prefer to use. Cuticular hydrocarbons were the most accurate way to delimit *F. fusca* and *F. lemani*, as their CHC profiles are very distinct despite them being sister species. Methylalkanes and especially the C25 dimethylalkanes dominate in *F. fusca*, whereas they are almost absent in *F. lemani*. By contrast 9Z-alkenes are common in *F. lemani*, but absent in *F. fusca* (MARTIN & al. 2008a, b). Similarly, the CHC profiles of nests identified as *F. japonica* based on their morphology were clustered into four profoundly different types, suggesting that it comprises several species (AKINO & al. 2002). Social insects use CHC compounds as recognition cues (VAN ZWEDEN & D'ETTORRE 2010). Unlike morphological or neutral genetic markers (e.g., DNA microsatellites), large differences in the CHC profiles between *F. fusca* and *F. lemani* may have arisen due to strong disruptive selection, possibly in the form of sexual selection to avoid inter-specific matings. Yet, many other closely related *Formica* species have similar surface chemistry (MARTIN & al. 2008a) showing no signs of disruptive selection.

Another method to separate *Formica fusca* and *F. lemani* unambiguously in our limited sampling was DNA barcoding, sequencing of a partial mitochondrial COI gene. *Formica fusca* and *F. lemani* did not share any haplotypes, and in a phylogenetic tree, they were placed separately with strong support. Separation of *F. fusca* and *F. lemani* based on genotype-frequency data was not as clear as with sequencing or surface chemistry. In the DNA microsatellite data, the species shared about half of a total of 99 alleles, with more private alleles in *F. lemani* than in *F. fusca* (30

vs. 19). There were strong allele frequency differences between the species at most loci, the most common allele being the same in both species only in one locus. In the allozyme loci, *F. fusca* and *F. lemani* shared thirteen of the twenty-one alleles. *Formica lemani* had more private alleles than *F. fusca* (8 vs. 2) and there were also strong allele-frequency differences between the species in some loci. Most morphological characters studied separated *F. fusca* and *F. lemani* significantly, even though all of them overlapped at individual level. The best separation was obtained with the combination of hairs on pronotum and mesonotum.

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