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Genetic polymorphism in "mixed" colonies of wood ants (Hymenoptera: Formicidae) in southern Finland and its possible origin

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Abstract. Wood ant colonies that appear to consist of individuals representing different species are described in several previous papers. The present study is the first to elucidate the genetic basis of the spectacular morphological variability observed within such colonies. Two seemingly mixed colonies (FM-1 and FM-2) from southern Finland were investigated. On the basis of the morphology of their workers these colonies were comprised of individuals with phenotypes typical of *Formica rufa* L., *F. polyctena* Först., and *F. aquilonia* Yarr. The sequence of an mtDNA fragment (5' end of the cytochrome b gene) was used to examine the phylogenetic relationships among haplotypes of workers from homogeneous colonies of different wood ant species and the two supposedly mixed colonies, and to sort the individuals within the colonies into matrilines. Six microsatellite loci were used to analyse the genetic differentiation between colonies and among workers within colonies, and to detect putative hybrids. The results show that, independently of their phenotype, workers from the "mixed" colonies were genetically more similar to other individuals in their colony than to those in the homogeneous *F. rufa*, *F. polyctena* or *F. aquilonia* colonies. However, while colony FM-1 consisted of offspring of the same queen or more likely several maternally related queens, colony FM-2 consisted of the offspring of at least four unrelated queens. The data suggest hybridisation between *F. polyctena* × *F. aquilonia* and *F. polyctena* × *F. rufa* (and possibly subsequent mating between these hybrids) as the most probable mechanism leading to the existence of these two colonies, which implies that the hybrids are fertile. This study shows that colonies of wood ant hybrids can arise spontaneously and persist under natural conditions. The results also revealed that even some morphologically homogeneous colonies are genetically heterogeneous. In the case of closely related, morphologically similar species that interbreed, morphology can be a bad predictor of genetic differences between individuals.

INTRODUCTION

Wood ants, i.e. representatives of the subgenus *Formica* s. str. in general, and particularly members of the *F. rufa*-group, although well studied (see e.g. Cotti, 1963; Beretta Boera, 1979; Gösswald, 1989; Czechowski, 1996), still pose taxonomic problems and often puzzle field researchers. The *F. rufa*-group sensu Dlussky (1967) originally included four species of wood ants: *F. rufa* L., *F. polyctena* Först., *F. aquilonia* Yarr., and *F. lugubris* Zett. *Formica paralugubris* Seifert, a subsequently described sibling species of *F. lugubris* (Seifert, 1996a), is now accepted as a member of the same group. A quarter of a century ago, Vepsäläinen & Pisarski (1981) called the *F. rufa*-group "a taxonomic enfant terrible" among wood ants and described the state of its taxonomy as "chaos before order". So far these vivid expressions have lost none of their relevance.

Not infrequently, colonies of wood ants are found in which the species status is practically indeterminable based on the commonly accepted morphological criteria (see e.g. Yarrow, 1955; Dlussky, 1967; Kutter, 1977; Collingwood, 1979; Douwes, 1981; Seifert, 1996b; Czechowski et al., 2002). Workers from the same nest can exhibit intermediate species characters or appear to represent different species. In addition, the appearance of workers within a colony may change over time and samples taken from the same nest in different years may be classified differently (Pamilo & Vepsäläinen, 1977; Vepsäläinen & Pisarski, 1981; Czechowski, 1996). Recent investigations have cast some light on this conundrum. On one hand, there is now some evidence supporting the possibility of interspecific hybridisation within the *F. rufa*-group (e.g. Seifert, 1991, 1999; Czechowski, 1996; Goropashnaya et al., 2004; Gyllenstrand et al., 2004; Seifert & Goropashnaya, 2004; Sorvari, 2006), while on the other hand, there are data that reveal the occurrence of temporary social parasitism within the subgenus *Formica* s. str., i.e. colonies that are taken over by heterospecific queens (see e.g. Pisarski & Czechowski, 1994; Czechowski, 1996). The latter phenomenon would result in temporarily mixed colonies.

Nevertheless, not all cases encountered in the field seem to be the result of one of the above processes. Some

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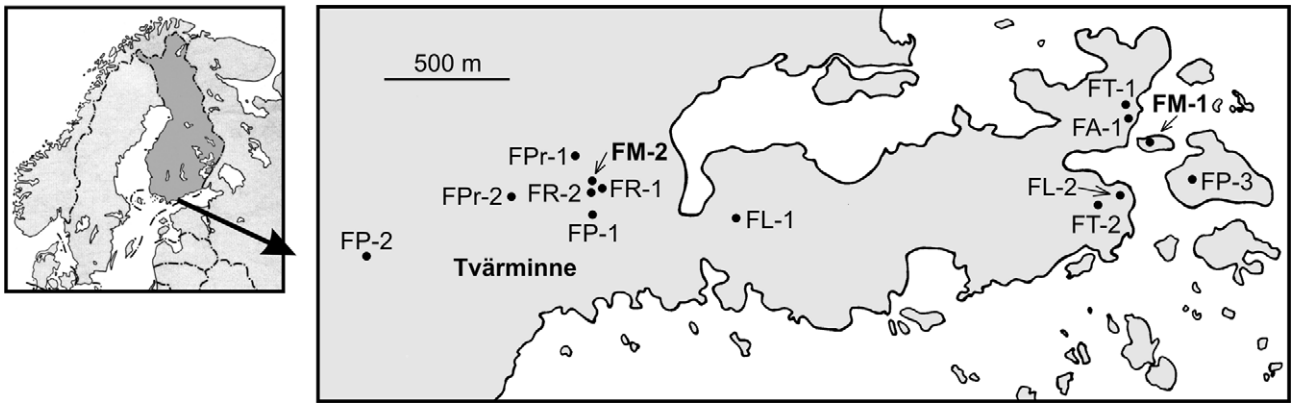

Fig. 1. Maps showing the location and the distribution of the *Formica* colonies studied. FM-1 – "mixed" colony 1; FM-2 – "mixed" colony 2; FR-1 and FR-2 – colonies 1 and 2 of *F. rufa*; FP-1, FP-2, and FP-3 – colonies 1, 2, and 3 of *F. polyctena*; FA-1 – colony of *F. aquilonia*; FL-1 and FL-2 – colonies 1 and 2 of *F. lugubris*; FPr-1 and FPr-2 – colonies 1 and 2 of *F. pratensis*; FT-1 and FT-2 – colonies 1 and 2 of *F. truncorum*.

colonies seem to permanently consist of individuals of different species. Recently, two such supposedly polygynous colonies were observed in southern Finland. Their visibly mixed nature was revealed by an examination of the morphology of the workers and, in one of these cases, also of the young sexuals (males and alate queens) (Czechowski & Radchenko, 2006). The aim of the present study was to characterise the genetic differentiation in these mysterious colonies and determine the origin of their spectacular morphological heterogeneity and to show whether morphology is really a good predictor of genetic differences in ants under discussion.

MATERIAL AND METHODS

Study area and ant sampling

The region of the Hanko Peninsula in southern Finland (Fig. 1) is well known for the unusually frequent and often unaccountable inter- and intracolonial variability in wood ants of the *F. rufa*-group (see Czechowski, 1996). From this region, and more specifically, from the Tvärminne archipelago and the vicinity of Tvärminne village, Czechowski & Radchenko (2006) reported two colonies, FM-1 and FM-2, which, based on the morphology of workers (and in the case of FM-1 also the alate queens and males), were recognised as tri-species mixed colonies of *Formica aquilonia*, *F. polyctena*, and *F. rufa*. The prevailing phenotypes were typical of *F. polyctena* in FM-1 and *F. rufa* in FM-2. The mixed nature of FM-1 persisted much longer than the maximum longevity of workers. This was supported by an analysis of samples taken from the same nest in successive years from 2002 to 2008. During this period the proportions of the "species" remained relatively stable (65–84% *F. polyctena*, 14–31% *F. aquilonia*, 1–7% *F. rufa* workers). The details of the morphology of the individuals as well as "species" composition of FM-2 are in Czechowski & Radchenko (2006).

Workers from these two "mixed" colonies were subjected to genetic analyses. Nest samples were taken for this purpose in 2004. At this time, the proportions of the phenotypes *rufa* : *polyctena* : *aquilonia* in colonies FM-1 and FM-2 were 4% : 65% : 31% and 58% : 38% : 4%, respectively (Czechowski & Radchenko, 2006). Representatives of each phenotype were selected from the nest samples (n > 100 for each colony), their morphology investigated (see Czechowski & Radchenko, 2006) and then preserved in 96% ethanol. Individuals were classified into morphological groups corresponding to the different species based on commonly accepted key criteria, especially the presence, numbers, and appearance of standing hairs on the occipital margin of the head, the ventral surface of the head, and the pilosity on the dorsal part of the alitrunk (see e.g. Dlussky, 1967; Dlussky & Pisarski, 1971; Collingwood, 1979; Seifert, 1996b; Czechowski et al., 2002). Specimens that were morphologically typical of a particular species were chosen for genetic analysis. In the case of the *F. polyctena* and *F. rufa* phenotypes, which were represented by a continuous transition from those with the typical external appearance of *F. polyctena* to that of *F. rufa*, "typical of a particular species" meant individuals that were either hairless or very hairy. The *aquilonia* phenotype was homogeneous and included workers that corresponded completely to the phenotype of this species (see Czechowski & Radchenko, 2006).

We also collected samples from nests of "pure" colonies of *F. rufa* (2 nests), *F. polyctena* (3 nests), and *F. aquilonia* (1 nest), which were present in the same region and could be a potential source of individuals for the mixed colonies. Additionally, nest samples of other wood ant species occurring in the area: *F. lugubris* (2 nests), *F. pratensis* Retz. (2 nests), and *F. truncorum* F. (2 nests), were included in the analysis. They were used to further support the hypothesis that the mixing/hybridisation phenomenon studied involved only three species: *F. rufa*, *F. polyctena*, and *F. aquilonia*. They also served as outgroups in the phylogenetic analyses.

The colonies with workers morphologically typical of a given species and also morphologically homogeneous within the colony were selected from among numerous wood ant colonies present in the area. All the 14 colonies studied, both the "mixed" ones and those sampled for comparison, were situated quite close to each other. The greatest distance between the nests was ca. 3.5 km (Fig. 1).

A total of 72 workers were used in the analysis: 5 FM-1R, 5 FM-1P, 5 FM-1A ("mixed" colony FM-1: phenotypes of *F. rufa*, *F. polyctena*, and *F. aquilonia*, respectively), 6 FM-2R, 6 FM-2P, 5 FM-2A ("mixed" colony FM-2: phenotypes of *F. rufa*, *F. polyctena*, and *F. aquilonia*), 5 FR-1, 5 FR-2 (colonies 1 and 2 of *F. rufa*), 5 FP-1, 5 FP-2, 7 FP-3 (colonies 1, 2, and 3 of *F. polyctena*), 5 FA-1 (colony of *F. aquilonia*), 2 FL-1, 1 FL-2 (colonies 1 and 2 of *F. lugubris*), 2 FPr-1, 1 FPr-2 (colonies 1 and 2 of *F. pratensis*), 1 FT-1 and 1 FT-2 (colonies 1 and 2 of *F. truncorum*). For a map of the relative locations of the colonies studied see Fig. 1.

Methods

Total genomic DNA was extracted from the body of each individuals using the DNeasy Tissue Kit (Qiagen Gmbh, Hilden, Germany). To reduce the likelihood of contamination with prey DNA from the alimentary tract, abdomens were removed before the genomic DNA was extracted. Two kinds of molecular markers were chosen for the genetic analyses: (1) a fragment of mitochondrial DNA (mtDNA) and (2) six microsatellite loci of nuclear DNA. The mtDNA fragment was used to analyse phylogenetic relationships among haplotypes of individuals from the "mixed" colonies and the colonies of different species, and to sort the individuals from the "mixed" colonies into matrilines. Microsatellite loci were used to analyse genetic differentiation between colonies and among workers within colonies, and detect putative hybrids.

The mtDNA fragment including the 5' end of the cytochrome b gene was amplified by PCR using primers CB-11059 and CB-11449 (Goropashnaya et al., 2004). The reaction was performed in a total volume of 50 µl containing 2 µl of template DNA, 2 µl of each primer (10 pmol), 250 mM KCl, 7.5 mM $MgCl_2$, 300 mM Tris (pH 8.4), 10 mM dNTPs (Sigma-Aldrich, St. Louis, MO, USA), and 2 U RED*Taq* Polymerase (Sigma-Aldrich, St. Louis, MO, USA). The reaction conditions for the amplification were: 2 min at 95°C, then 35 cycles of 15 s at 94°C, 20 s at 48°C, 1 min at 72°C, followed by 5 min at 72°C. Amplified products were purified using the QIAquick PCR Purification Kit (Qiagen Gmbh, Hilden, Germany) and then sequenced using the individual PCR primers with a CEQ™ 8000 (Beckman Coulter, Fullerton, CA, USA) automated DNA sequencer. In total 341 base pairs were scored for each of the 71 individuals. For one out of the 72 samples sequencing was unsuccessful.

The analysis of mtDNA variability was performed using the 71 sequences obtained in this study plus 41 orthologous *Formica* sequences from the NCBI database: 7 *F. rufa* (FR), 9 *F. polyctena* (FP), 10 *F. aquilonia* (FA), 5 *F. lugubris* (FL), 2 *F. pratensis* (FPr), 2 *F. truncorum* (FT), 2 *F. paralugubris* (FPl), 2 *F. frontalis* Santschi [FFr; an Iberian endemic species related to *F. truncorum* (see Tinaut & Martínez, 1998)], and 2 *F.* (*Serviformica*) *candida* F. Smith (FCa). The sequences were retrieved and compared using BLAST, aligned using Clustal W (Thompson et al., 1994), and then the alignment was checked visually.

Phylogenetic relationships among the identified haplotypes were analysed using PAUP (Swofford, 1998). To choose the most appropriate model of sequence evolution, a hierarchical likelihood ratio test implemented using the program Modeltest (Posada & Crandall, 1998) was performed. Based on the model HKY+Γ with the parameters estimated by Modeltest, a maximum likelihood tree was constructed. In addition, neighbour joining and minimum evolution trees were constructed, based on the model HKY+Γ and maximum likelihood distances estimated by Modeltest. All these trees were constructed using the program PAUP and the heuristic search algorithm. PAUP was also used to construct maximum parsimony trees. All phylogenetic trees were rooted by sequences of *F. candida*. Confidence in the estimated relationships was determined using the bootstrap approach (Felsenstein, 1985). Bootstrap values were obtained based on 1000 replicates and using the heuristic search algorithm implemented in PAUP.

The statistical parsimony approach implemented in the software TCS (Clement et al., 2000) was used to construct a network of phylogenetic relationships among haplotypes, with six mutational steps for 95% parsimonious connection. The network was nested according to rules described by Templeton et al. (1992) and Templeton & Sing (1993).

Microsatellite variation was analysed at six loci: FE8, FE13, FE16, FE17, FE37, and FE42 (Gyllenstrand et al., 2002). PCR amplification was carried out in 20 µl volumes containing 2 µl of template DNA, 2 µl of primers, and 10 µl Multiplex PCR Kit (Qiagen Gmbh, Hilden, Germany) under reaction conditions: 15 min at 95°C, then 16 cycles of 30 s at 94°C, 90 s at 58°C, and 60 s at 72°C; 10 cycles of 30 s at 94°C, 90 s at 57°C, and 60 s at 72°C; 10 cycles of 30 s at 94°C, 90 s at 55°C, and 60 s at 72°C, followed by 30 min at 60°C. Fragment analysis was performed using a CEQ™ 8000 (Beckman Coulter, Fullerton, CA, USA) automated DNA sequencer.

The GENEPOP software package (Raymond & Rousset, 1995) was used to calculate observed heterozygosity (H_O), expected heterozygosity (H_E) (Nei, 1978), inbreeding coefficient (F_{IS}), and test for Hardy-Weinberg equilibrium and population differentiation (Goudet et al., 1996). GENEPOP was also used to calculate fixation index (F_{ST}) between pairs of phenotypes and pairs of colonies.

The assignment test and detection of first generation migrants were performed using the program GeneClass2 (Piry et al., 2004). The test was used to assign each individual to the colony and the phenotypic group to which it was genetically most similar. This allowed determination of whether "mixed" colonies constituted groups of genetically related individuals or simply assemblages of unrelated individuals from three different species. For each individual, the probability that it did not descend from the colony where it was collected was calculated using a test for detecting first generation migrants. This test allowed us to detect immigrants (i.e., individuals with a foreign genotype that were not born in a given colony) and their offspring (e.g., offspring of a local queen and a male of a different species). For each individual, the probability that it genetically fits its own phenotypic group was also calculated. This allowed us to detect individuals that were genetically more similar to individuals of a phenotype other than their own. Both the assignment test and that for detecting first generation migrants were performed using the Bayesian method of Rannala & Mountain (1997) and the Monte-Carlo re-sampling algorithm of Paetkau et al. (2004) with 1000 simulated individuals and a type I error rate of 0.01.

To determine whether the species of wood ants defined based on morphology are genetically well differentiated, we performed an analysis of population genetic structure based on frequencies of microsatellite alleles using software Structure 2.0 (Pritchard et al., 2000). This test was performed (1) without any prior information about individuals and (2) with individuals grouped according to their phenotype. We ran the analyses with a burn-in period of 10,000 MCMC replications and 100,000 replications after burn-in, and repeated each analysis three times. We used a model with population admixture and independent allele frequencies among populations.

RESULTS

Among the 71 sequenced samples, 10 mtDNA haplotypes (GenBank accession numbers: FJ623067, FJ623068, FJ623069, FJ623070, FJ623071, FJ623072, FJ623073, FJ623074, FJ623075, and FJ623076) were identified (Table 1). The analysed sequence (the 5' end of the cytochrome b gene) comprised 341 base pairs, which contained 9 (3%) polymorphic sites. No insertions/deletions were found. The haplotypes differed in 1–9 base pairs. The average nucleotide composition was: 41.4% T, 32.1% A, 14.6% C, and 11.9% G. In most colonies only one haplotype was found, but in colony FP-1 and "mixed" colony FM-2 there were two and four

TABLE 1. Frequency of mtDNA haplotypes (% of individuals with a given haplotype) in colonies and phenotypic groups within mixed *Formica* colonies; n – group size. Phenotype codes: FM-1 – "mixed" colony 1; FM-2 – "mixed" colony 2; FR-1 and FR-2 – colonies 1 and 2 of *F. rufa*; FP-1, FP-2, and FP-3 – colonies 1, 2, and 3 of *F. polyctena*; FA-1 – colony of *F. aquilonia*. For details, see last paragraph of Study area and ant sampling.

Colony, phenotype	n	Haplotypes									
		H1	H2	H3	H4	H5	H6	H7	H8	H9	H10
FM-1R	5									100	
FM-1P	5									100	
FM-1A	5									100	
FM-2R	6			100							
FM-2P	6		66.67		16.67		16.67				
FM-2A	5			100							
FR-1	5	100									
FR-2	5	100									
FP-1	5		20								80
FP-2	5									100	
FP-3	6									100	
FA-1	5								100		
FL-1	2								100		
FL-2	1	100									
FPr-1	2					100					
FPr-2	1					100					
FT-1	1							100			
FT-2	1							100			

haplotypes, respectively. All individuals from "mixed" colony FM-1, independent of the phenotype, had the same haplotype (H9) as that of *F. polyctena* colonies FP-2 and FP-3. In colony FM-2, workers with phenotypes of *F. rufa* and *F. aquilonia* had the same haplotype (H3), but workers of the *F. polyctena* phenotype had three other haplotypes (H2, H4, and H6), two of them unique (Table 1, Fig. 3).

The network of phylogenetic relationships between haplotypes revealed three main clades (Fig. 2). The first clade (2-1) contained the haplotypes that were found in 17 individuals of *F. rufa*, 8 of *F. polyctena*, 2 of *F. lugubris*, and all 17 workers from "mixed" colony FM-2. Haplotypes of individuals from colony FM-2 showed strong phylogenetic similarity to a haplotype of individuals of *F. rufa* from which they differed by at most three mutational steps. The second clade (2-2) grouped haplotypes of 4 individuals of *F. truncorum*, 4 of *F. pratensis*, and 2 of *F. frontalis*. The third clade (2-3) contained haplotypes of 17 individuals of *F. polyctena*, 15 of *F. aquilonia*, 6 of *F. lugubris*, 2 of *F. paralugubris*, 1 of *F. pratensis*, and all 15 individuals from "mixed" colony FM-1 (Fig. 2).

Analysis of phylogenetic trees gave similar results. All constructed trees shared a similar topology and confirmed the subdivision of haplotypes of *F. rufa*, *F. polyctena*, and *F. aquilonia* species into two clades (2-1 and 2-3), while species that were in the central clade of the haplotype network (2-2) took a position outside clades 2-1 and 2-3 in all trees (Fig. 3).

We compared allele frequencies at microsatellite loci in two "mixed" colonies with allele frequencies in pure colonies of *F. rufa*, *F. polyctena*, and *F. aquilonia* (allele frequencies in these groups are given in the Appendix). The number of individuals sampled with the phenotypes of *F. lugubris*, *F. pratensis* or *F. truncorum* was very small (1-2 per colony), and these samples were used only for comparison with *F. rufa*, *F. polyctena*, and *F. aquilonia*.

In both "mixed" colonies, the observed heterozygosity was lower than expected heterozygosity, the inbreeding coefficient F_{IS} was high (Table 2) and Hardy-Weinberg test showed a significant heterozygote deficit ($P < 0.05$ in each case). A significant heterozygote deficit and high inbreeding coefficient were recorded for pure colonies of *F. polyctena*, but not pure colonies of two other species (Table 2). A heterozygote deficit and high inbreeding coefficient may reflect inbreeding or multiple gene pools (possibly different species) building up a colony (Wahlund's effect). The test for population differentiation showed a highly significant differentiation of allele frequencies among two "mixed" colonies and pure colonies of *F. rufa*, *F. polyctena*, and *F. aquilonia* ($P < 0.00001$).

Based on allele frequencies at microsatellite loci, we estimated genetic distances, expressed as pairwise F_{ST}, between ants grouped according to phenotypes and according to colonies. For phenotypic comparison, ants from both "mixed" and homogeneous colonies were grouped together according to phenotypes of *F. rufa*, *F. polyctena*, and *F. aquilonia*. The genetic distance between *F. rufa*, *F. polyctena*, and *F. aquilonia* phenotypes was relatively small and ranged from 0.06 to 0.08 (Table 3). In contrast, the genetic distance between colonies of the same phenotype was relatively high (0.11–0.29) with values comparable to the genetic dis-

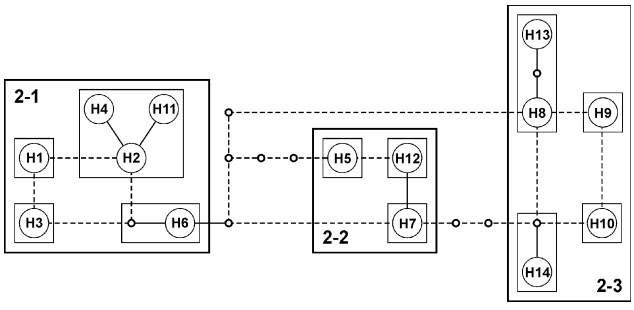

Fig. 2. Statistical parsimony network of the phylogenetic relationships among the *Formica* mtDNA haplotypes. Circles with numbers represent haplotypes. Each line between two haplotypes represents a single mutational change. Small circles represent interior nodes in the network that were not present in the sample, i.e. these are inferred intermediate haplotypes between two nearest neighbour haplotypes in the network that differed by two or more mutations. Thin line rectangles indicate haplotypes grouped in 1-step clades. Thick line rectangles indicate 1-step clades grouped in 2-step clades. The haplotypes of *F. candida* were not included in the network because they were connected with the haplotypes of other species by more than 6 mutational steps, which constituted the threshold of the 95% parsimonious connection.

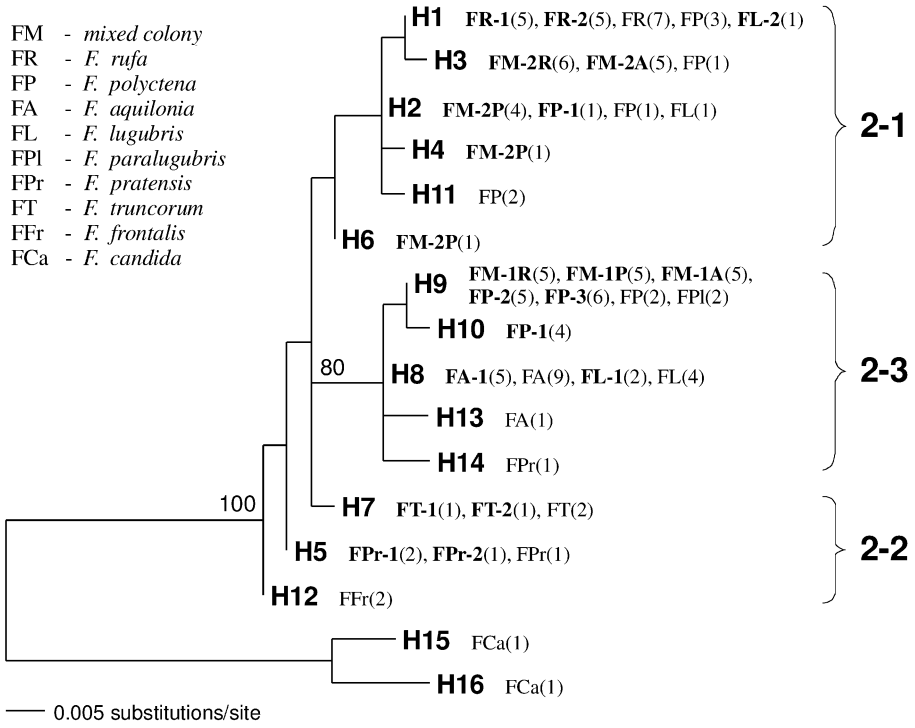

Fig. 3. Maximum likelihood tree generated from 16 mtDNA haplotypes found in the *Formica* colonies tested (indicated in bold; for abbreviations, see last paragraph of Methods) and orthologous sequences from NCBI database. The number of individuals with a given haplotype is shown in parentheses. Bootstrap values over 50% are shown for nodes. The numbering of the clades is the same as in Fig. 2.

tance between colonies of different phenotypes (0.13–0.47) (Table 4).

In the case of "mixed" colony FM-2, the genetic distance to the geographically closest *F. rufa* colonies was especially small (FM-2 to FR-2: 0.05, geographic distance 50 m; FM-2 to FR-1: 0.08, geographic distance 40 m). However, the genetic distance between colony FM-2 and the geographically most distant (ca. 2680 m) *F. polyctena* colony FP-3 was also small: 0.08. In the case of "mixed" colony FM-1 the shortest genetic distance (0.10) was to the nearby (ca. 270 m) *F. polyctena* colony FP-3 (Table 4).

The assignment test performed based on microsatellite data using GeneClass2 assigned all individuals (except one from colony FM-1) to the colony from which they originated. Workers within all the colonies tested, including the "mixed" ones, were more genetically similar to their own colony than to any of the others. Average values of assignment of individuals from particular colonies to their own colony and all other colonies are given in Table 5. All individuals (except one from colony FP-3) originating from phenotypically homogeneous colonies were assigned with the highest probability to a priori groups consisting of individuals of the same morphological phenotype. In "mixed" colony FM-1, all workers with the *F. rufa* phenotype were assigned with the highest probability to the group of individuals with the *F. polyctena* phenotype. In colony FM-2, workers with the *F. rufa* phenotype were assigned either to the group of individuals with the *F. rufa* phenotype or the *F. aquilonia* phenotype. Similarly, workers with the *F. aquilonia* phenotype from colony FM-2 were assigned either to the group of individuals with the *F. rufa* phenotype or the *F. aquilonia* phenotype. Other individuals from "mixed" colonies were assigned to groups consisting of individuals sharing the same morphological phenotype. Average values of assignment of individuals of different phenotypes to a priori groups consisting of individuals of one particular phenotype are given in Table 6.

TABLE 2. Observed heterozygosity (H_O), expected heterozygosity (H_E) and inbreeding coefficient (F_{IS}) of "mixed" colonies and "pure" colonies of the three species, calculated based on microsatellite data. *P* – the *P*-value of the Hardy-Weinberg equilibrium test.

Colony, species	H_O	H_E	F_{IS}	*P*
FM-1	0.466667	0.546428	0.1460	0.0500
FM-2	0.460784	0.554338	0.1835	0.0002
F. rufa	0.516667	0.521296	0.0089	0.1473
F. polyctena	0.509804	0.652267	0.2184	0.0000
F. aquilonia	0.733333	0.475000	–0.5439	0.9908

TABLE 3. Genetic distance (F_{ST}) between pairs of *Formica* phenotypes, calculated based on microsatellite data.

Phenotype	*F. rufa*	*F. polyctena*	*F. aquilonia*	*F. lugubris*	*F. pratensis*
F. polyctena	0.0828				
F. aquilonia	0.0766	0.0636			
F. lugubris	0.1538	0.1288	0.0761		
F. pratensis	0.2405	0.2356	0.1366	0.1604	
F. truncorum	0.0970	0.1024	0.0690	0.1214	0.1080

The test to detect first generation migrants based on allele frequencies at microsatellite loci was performed in two ways: detection of migrants from an alien colony and detection of individuals genetically more similar to individuals of a phenotype other than their own. The test also assigned offspring of migrants to the population from which one or both parents originated, so it could detect workers descending from a male or queen of an alien genotype. Six cases of "migrants", i.e. mis-assigned individuals ($P \leq 0.01$), between colonies were found (two in colony FM-2 and one in each of colonies FM-1, FR-2, FP-1, and FP-3) and six between phenotypes (two in colony FM-2 and one in each of colonies FM-1, FR-2, FP-2, and FP-3). In general, the test for the detection of first generation migrants assigned individuals in a similar way to the assignment test. In colony FM-1, all individuals with the *F. rufa* phenotype were assigned with the highest probability to the a priori group consisting of individuals with the *F. polyctena* phenotype, and three workers with the *F. polyctena* phenotype were assigned to the group of individuals with the *F. rufa* phenotype. In colony FM-2, all individuals with the *F. aquilonia* phenotype were assigned with the highest probability to the group of individuals with the *F. rufa* phenotype.

The STRUCTURE analysis performed without any prior information about individuals did not indicate any clear population genetic structure. The test based on phenotype gave the most probable population structure consistent with the phenotypic groups. However, two workers from colony FM-1 with the *F. rufa* phenotype were assigned to the *F. polyctena* phenotype and one worker from colony FM-2 with the *F. aquilonia* phenotype was assigned to the *F. rufa* phenotype. For 7 individuals with the *F. rufa* phenotype (including 5 from "mixed" colony FM-1), 8 with *F. polyctena* (1 from "mixed" colony FM-2), and 3 with *F. aquilonia* (all from FM-2), the probability of assignment to the phenotype in concordance with their morphology was lower than 0.9.

DISCUSSION

Analyses of mitochondrial and nuclear DNA confirmed the close phylogenetic relationship between the wood ant species *F. rufa*, *F. polyctena*, and *F. aquilonia*. This result reflects the strong morphological and behavioural similarity of these species (see e.g. Dlussky, 1967; Dlussky & Pisarski, 1971; Seifert, 1996b; Czechowski et al., 2002) and corroborates the results of earlier genetic studies (Goropashnaya et al., 2003, 2004). However, the analysis of mtDNA haplotypes indicated relationships among these species different to those established previously. According to Goropashnaya et al. (2003, 2004), *F. rufa* and *F. polyctena* form a monophyletic group distinct from *F. aquilonia*. In the present study, clade 2-1 corresponded to *F. rufa* and *F. polyctena*, clade 2-2 to *F. truncorum*, *F. pratensis*, and *F. frontalis*, and clade 2-3 to *F. aquilonia* and *F. polyctena*. Therefore, *F. rufa* and *F. aquilonia* at this southern Finnish location appeared to be clearly distinct with regard to their mtDNA haplotypes, while *F. polyctena* at this location had mtDNA haplotypes that were phylogenetically related to those of local *F. aquilonia* and *F. rufa*. *F. polyctena* also showed the highest genetic polymorphism in both nuclear and mitochondrial DNA of the three species studied.

Most of the *F. rufa*-group species are able to interbreed under natural conditions and individuals of intermediate phenotypes between species are frequently observed (Seifert, 1991, 1999; Czechowski, 1993, 1996; Czechowski & Douwes, 1996; Goropashnaya et al., 2004; Sorvari, 2006). Unusually hairy individuals of *F. polyctena* reported from continental Europe are generally thought to be hybrids of *F. polyctena* and *F. rufa* (Seifert, 1991, 1996b; Czechowski & Douwes, 1996). On the other hand, Sorvari (2006) considers the hairy morphs of *F. polyctena* from central and southern Finland to be hybrids of *F. polyctena* and *F. aquilonia*. Genetic data confirm the ability of *F. rufa*, *F. polyctena*, and *F. aquilonia* to cross-breed among themselves and with other

TABLE 4. Genetic distance (F_{ST}) between pairs of *Formica* colonies, calculated based on microsatellite data.

Colony	FM-1	FM-2	FR-1	FR-2	FP-1	FP-2	FP-3	FA-1	FL-1
FM-2	0.1137								
FR-1	0.2633	0.0757							
FR-2	0.1125	0.0528	0.1303						
FP-1	0.1731	0.1774	0.3527	0.2277					
FP-2	0.2367	0.3366	0.4745	0.3475	0.2708				
FP-3	0.1028	0.0769	0.1716	0.1255	0.1097	0.2920			
FA-1	0.2407	0.2448	0.4159	0.3024	0.1967	0.3575	0.2306		
FL-1	0.2079	0.1601	0.3225	0.2388	0.3170	0.3918	0.0723	0.2832	
FPr-1	0.3489	0.2102	0.3160	0.2863	0.3395	0.5281	0.1430	0.3636	0.1538

TABLE 5. Average values of assignment of individuals from particular colonies (rows) to all colonies from which more than one individual was sampled. The highest values are indicated in bold. These results were obtained based on microsatellite data using the program GeneClass.

Colony	FM-1	FM-2	FR-1	FR-2	FP-1	FP-2	FP-3	FA-1	FL-1	FPr-1
FM-1	**0.55**	0.10	0.00	0.08	0.04	0.00	0.10	0.00	0.01	0.00
FM-2	0.02	**0.51**	0.03	0.06	0.01	0.00	0.10	0.00	0.02	0.00
FR-1	0.00	0.15	**0.70**	0.07	0.00	0.00	0.04	0.00	0.00	0.00
FR-2	0.09	0.06	0.01	**0.59**	0.01	0.00	0.03	0.00	0.04	0.00
FP-1	0.01	0.03	0.00	0.01	**0.71**	0.00	0.20	0.01	0.00	0.00
FP-2	0.01	0.00	0.00	0.00	0.07	**0.64**	0.02	0.00	0.00	0.00
FP-3	0.00	0.03	0.00	0.01	0.02	0.00	**0.68**	0.00	0.03	0.00
FA-1	0.00	0.01	0.00	0.00	0.09	0.00	0.00	**0.83**	0.02	0.00
FL-1	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	**0.96**	0.10
FPr-1	0.00	0.00	0.00	0.00	0.06	0.00	0.11	0.00	0.52	**0.92**

Formica s. str. species (Goropashnaya et al., 2004; Gyllenstrand et al., 2004; Seifert & Goropashnaya, 2004). The incomplete reproductive isolation of these species can be explained by their relatively recent speciation, which took place during the Pleistocene (Pamilo et al., 1979; Vepsäläinen & Pisarski, 1981; Collingwood & Agosti, 1986; Goropashnaya et al., 2004). Nevertheless, the frequency of hybrids is strongly differentiated geographically and may be very high locally (Seifert, 1991, 1999; Seifert & Goropashnaya, 2004). Particularly noticeable is the morphological ambiguity of workers of the *F. rufa*-group species, particularly *F. polyctena* and *F. aquilonia*, often observed in northern Europe (Gösswald et al., 1965; Rosengren, 1977a, b; Collingwood, 1979; Douwes, 1979; Pamilo et al., 1979, 1992; Vepsäläinen & Pisarski, 1981), which suggests that very frequent hybridisation may occur between these species. The region of southern Finland investigated in the present study is particularly well known to myrmecologists for the unusually frequent and often inexplicable variability among *F. rufa*-group species, represented there by *F. rufa*, *F. polyctena*, *F. aquilonia*, and *F. lugubris* (see e.g. Czechowski, 1996). The remarkable morphological spectrum seen here suggests hybridisation within the local wood ant species and/or the existence of multi-species colonies (e.g. Pamilo & Vepsäläinen, 1977; Pamilo et al., 1979).

Analyses of mitochondrial and nuclear DNA did not confirm the hypothesis that workers exhibiting different phenotypes belonging to the same "mixed" colonies constitute a conglomeration of individuals of three different species, as suggested in a previous paper based on morphological key features (Czechowski & Radchenko, 2006). All but one of the workers investigated from the two "mixed" colonies were assigned with the highest probability to the colony from which they originated. Therefore, independently of their phenotype, workers from the "mixed" colonies were genetically more similar to workers in their own colony than to those in any of the homogeneous *F. rufa*, *F. polyctena*, or *F. aquilonia* colonies. In other words, individuals from the same "mixed" colony, even those of different phenotypes, were evidently related.

In "mixed" colony FM-1 the *F. polyctena* phenotype prevailed, that of *F. aquilonia* was less frequent, while the number of individuals with the *F. rufa* phenotype was very low. All individuals from this colony had the same

TABLE 6. Average values of assignment of individuals of different phenotypes (rows) to a priori groups consisting of individuals with one particular phenotype: *F. rufa*, *F. polyctena*, *F. aquilonia*, *F. lugubris*, *F. pratensis* or *F. truncorum*. The highest values (and second highest values if > 0.5) are indicated in bold. These results were obtained based on microsatellite data using the program GeneClass.

Phenotype	*F. rufa*	*F. polyctena*	*F. aquilonia*	*F. lugubris*	*F. pratensis*	*F. truncorum*
FM-1R	0.33	**0.60**	0.00	0.01	0.00	0.04
FM-2R	**0.67**	0.10	**0.53**	0.02	0.00	0.15
FR-1	**0.65**	0.06	0.05	0.02	0.00	0.09
FR-2	**0.46**	0.04	0.25	0.16	0.00	0.09
FM-1P	0.49	**0.65**	0.02	0.01	0.00	0.07
FM-2P	0.02	**0.37**	0.11	0.06	0.00	0.01
FP-1	0.00	**0.49**	0.01	0.00	0.00	0.01
FP-2	0.01	**0.32**	0.00	0.00	0.00	0.02
FP-3	0.00	**0.23**	0.00	0.04	0.00	0.03
FM-1A	0.01	0.04	**0.66**	0.05	0.00	0.01
FM-2A	**0.61**	0.09	**0.62**	0.05	0.00	0.31
FA-1	0.00	0.06	**0.59**	0.04	0.00	0.01
FL-1 + FL-2	0.09	0.05	0.16	**0.91**	0.02	0.07
FPr-1 + FPr-2	0.00	0.05	0.16	0.29	**0.77**	0.23
FT-1 + FT-2	0.02	0.15	0.05	0.10	0.00	**0.99**

haplotype. Therefore, the workers could be the offspring of one queen (possibly mated with males of different species or with hybrid males) or of several queens that were maternal kin. Nevertheless, the explosive growth of this colony (see Czechowski & Radchenko, 2006) strongly suggests that FM-1 enlarged its pool of fertilised queens at some point, perhaps after the death of the foundress. It is known that orphaned (queenless) wood ant colonies are capable of adopting new queens, making their continued existence possible (Pisarski & Czechowski, 1994). Indeed, their workers can even take the initiative to actively recruit new queens (see Czechowski, 1994).

It is very unlikely that colony FM-1, which was probably three years old at the time, was able to produce its own sexuals, so the most reliable explanation is that this colony adopted some foreign queens. They may have been derived from a polydomus *F. polyctena* colony on a neighbouring island. This colony included the nest FP-3 (see Fig. 1), which possessed the same mtDNA haplotype as colony FM-1. The small genetic distance between colonies FM-1 and FP-3 supports this possibility.

The haplotype of colony FM-1 clustered in the clade of *F. polyctena* and *F. aquilonia* to which no *F. rufa* haplotype was affiliated. All workers of the *F. rufa* phenotype were assigned to the *F. polyctena* phenotype based on their microsatellite genotype. Thus, in colony FM-1 the individuals with the *F. rufa* phenotype were genetically closer to *F. polyctena* than to *F. rufa*. Hybrid ants can have phenotypes intermediate between those of the parental species (Pearson, 1983; Seifert, 1984, 1991, 1999) or a phenotype typical of one of the parents (Seifert & Goropashnaya, 2004). Therefore, the genetic data did not allow us to reject the possibility that in colony FM-1 the workers with the *F. rufa* phenotype descended from queens that were *F. polyctena* × *F. aquilonia* hybrids. The colony FM-1 could also contain both *F. polyctena* × *F. aquilonia* and *F. polyctena* × *F. rufa* hybrid queens, or alternatively the queens were second generation hybrids *F. polyctena* × *F. aquilonia* × *F. rufa*. The colony produced males with *F. aquilonia* and *F. polyctena*/*F. rufa* phenotypes (males of the latter two species are morphologically indistinguishable). During the nuptial flight, cases of copulation were observed on the FM-1 mound surface (W. Czechowski, unpubl.), which indicates that the local queens might have been fertilised by their brothers and/or nephews. This indicates high inbreeding and suggests a potential mechanism by which the high incidence of hybrids is maintained in this colony.

In the second "mixed" colony, FM-2, the *F. rufa* phenotype prevailed, that of *F. polyctena* was represented by fewer individuals, while the incidence of the *F. aquilonia* phenotype was very low. Four haplotypes occurred in this colony, which indicated that it consisted of individuals descending from at least four unrelated queens. Despite this multiple origin, the results of the assignment test suggest that these individuals were more related to each other than to individuals from other colonies belonging to their respective phenotypic groups. This suggests that all these individuals were related in the paternal line, e.g. as a result of copulation of queens with males from the same colony.

All haplotypes that occurred in colony FM-2 clustered in the clade of *F. rufa* and *F. polyctena*, to which no *F. aquilonia* haplotype was affiliated. All workers with the *F. aquilonia* phenotype had the same haplotype as the workers with the *F. rufa* phenotype. Most of the workers with the *F. polyctena* phenotype had the same haplotype as that occurring in the nearby *F. polyctena* colony FP-1. In FM-2, some individuals with the *F. aquilonia* phenotype were assigned to the *F. rufa* phenotype and some individuals with the *F. rufa* phenotype to the *F. aquilonia* phenotype based on their microsatellite genotypes. Therefore, in colony FM-2 the workers with the *F. aquilonia* phenotype were genetically closer to *F. rufa* than to *F. aquilonia*. Topographically, "mixed" colony FM-2 was situated very close to the *F. rufa* colonies FR-1 and FR-2 and the genetic distance between them was also very small. This suggests that in colony FM-2 the workers with the *F. aquilonia* phenotype could have descended from queens that were *F. rufa* × *F. polyctena* hybrids. Nevertheless, it is very unlikely that *F. polyctena* × *F. rufa* hybrid workers could exhibit the more hairy phenotype of *F. aquilonia*. Probably colony FM-2 (as well as FM-1) contained both *F. polyctena* × *F. aquilonia* and *F. polyctena* × *F. rufa* hybrid queens, or the queens were *F. polyctena* × *F. aquilonia* × *F. rufa* hybrids.

The cases of "mixed" colonies FM-1 and FM-2 clearly differed; colony FM-1 consisted of individuals that were the offspring of the same queen or (more probably) several queens related maternally, while colony FM-2 consisted of individuals that were descendents of at least four unrelated queens. However, the most likely mechanism leading to the existence of both these colonies was the same: hybridisation between the species of the *F. rufa*-group leading to the existence of fertile(!) hybrids. This finding adds strong support to the hypothesis that speciation is not complete in the *F. rufa*-group (Goropashnaya et al., 2004). *F. polyctena*, *F. aquilonia*, and *F. rufa* are not reciprocally monophyletic with regard to mtDNA haplotypes, which may be due to incomplete lineage sorting. Hybridisation followed by introgression of mtDNA haplotypes may provide an alternative explanation of this fact. It is already known that many species of ants readily interbreed and produce viable and fertile offspring under laboratory conditions (Buschinger, 2001). The present study demonstrates that wood ant colonies composed of hybrids can exist permanently, or at least for many years, under natural conditions, and that these hybrids may morphologically resemble specific representatives of the *F. rufa*-group (*F. rufa*, *F. polyctena*, *F. aquilonia*) or more or less intermediate forms (*F. rufa*/*F. polyctena*).

Fertility of hybrids between *F. rufa*-group species, which is strongly suggested by the results of this preliminary study and the likely mechanism of appearance of these hybrids, discussed here in the light of the findings of this and a previous study (Czechowski & Radchenko, 2006), underlie the phenomenon of "mixed" colonies. The above hypothesis requires step-by-step factual verifi-

cation, a task that is undoubtedly complex and time consuming. Further research on hybridisation in wood ants and other ant genera, carried out on larger sample sizes, is required to determine the mechanisms underlying this phenomenon and assess the frequency, circumstances and determinants of its occurrence.

In all the colonies studied, both "mixed" and homogeneous, only individuals with species-specific morphological features, i.e. those manifesting no intermediate traits, were analysed. However, genetic differences between workers were not always associated with differences in their morphology. The ants of the same phenotype with different haplotypes and individuals of the same haplotype but with different phenotypes were found both in homogenous and heterogeneous colonies. Analysis of nuclear DNA showed that workers more genetically similar to individuals of a phenotype different from their own (probably hybrids) occurred not only in the "mixed" colonies, but also in colonies that were morphologically recognised as "pure" *F. polyctena* or *F. rufa*. This finding indicates the possible presence of some admixture of cross-mated or even hybrid queens in queen pools of the polygynous, apparently homospecific colonies of these species.

Seifert & Goropashnaya (2004) point out that mitochondrial DNA studies may frequently result in the misidentification of wood ants. They suggest that in this species group, in which there is a very high frequency of interspecific hybridisation, nuclear DNA conformed better than mtDNA with the morphological identification of species. However, our study shows incongruence between morphological and both mitochondrial and nuclear DNA data.

Our results reveal that the identification of species of closely related and frequently interbreeding species of ants based solely on genetic analysis may lead to confusing results. Caution is needed, particularly when the genetic analyses are based on small samples, a short DNA sequence or a small number of microsatellite loci. On the other hand, the species status of some colonies is indeterminable based on morphological criteria. In such cases a combination of genetic, morphological, and ecological studies is recommended.

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Appendix. Microsatellite frequencies for "mixed" colonies and the *Formica* phenotypic groups studied. FM-1 – "mixed" colony 1; FM-2 – "mixed" colony 2; FR – *F. rufa*; FP – *F. polyctena*; FA – *F. aquilonia*; FL – *F. lugubris*; FPr – *F. pratensis*; FT – *F. truncorum*; N_A – number of alleles at a given locus; N_Σ – total numbers of alleles found at all the loci tested; n – number of individuals; c – number of colonies.

Locus	Allele[a]	FM-1 n = 15 c = 1	FM-2 n = 17 c = 1	FR n = 10 c = 2	FP n = 12 c = 3	FA n = 5 c = 1	FL n = 3 c = 2	FPr n = 3 c = 2	FT n = 2 c = 2
FE8	298	–	–	0.050	–	–	–	–	–
	308	–	–	–	0.118	0.100	–	–	–
	310	0.933	0.529	0.350	0.588	0.500	0.500	–	–
	312	0.033	0.412	0.600	0.294	0.400	0.500	1.000	1.000
	318	–	0.059	–	–	–	–	–	–
	322	0.033	–	–	–	–	–	–	–
	N_A	3	3	3	3	3	2	1	1
FE13	190	–	0.088	–	0.147	–	0.333	0.167	–
	193	0.533	0.353	0.500	0.235	–	–	–	0.500
	196	0.167	0.088	–	0.118	0.900	0.333	0.667	0.500
	199	0.167	0.294	0.500	0.088	–	0.333	–	–
	202	0.133	0.176	–	0.412	0.100	–	0.167	–
	N_A	4	5	2	5	2	3	3	2
FE16	155	–	–	–	0.088	–	–	–	0.500
	157	–	0.176	–	0.029	0.500	–	–	–
	159	–	–	–	0.118	–	0.500	0.667	0.250
	161	0.200	0.294	–	0.029	–	–	0.333	0.250
	163	–	–	–	0.265	–	–	–	–
	165	–	–	–	0.088	–	–	–	–
	167	0.133	–	0.300	–	–	–	–	–
	169	–	–	0.100	–	0.500	0.500	–	–
	171	0.533	0.029	0.100	0.235	–	–	–	–
	173	0.133	0.088	–	0.088	–	–	–	–
	175	–	0.294	0.050	0.029	–	–	–	–
	177	–	0.118	0.200	0.029	–	–	–	–
	179	–	–	0.250	–	–	–	–	–
	N_A	4	6	6	10	2	2	2	3
FE17	113	–	–	–	0.059	–	–	–	–
	117	–	–	–	0.059	–	–	–	–
	119	0.500	0.500	0.400	0.471	0.500	0.333	–	0.500
	121	0.333	0.471	0.500	0.265	–	0.500	0.500	0.500
	123	0.167	0.029	–	0.029	0.500	0.167	0.500	–
	125	–	–	0.050	0.118	–	–	–	–
	127	–	–	0.050	–	–	–	–	–
	N_A	3	3	4	6	2	3	2	2
FE37	113	–	0.029	–	0.029	–	–	–	–
	117	0.267	0.118	–	0.059	0.200	–	0.333	–
	119	0.433	0.441	0.500	0.735	0.700	–	0.333	0.500
	121	0.267	0.412	0.400	0.088	–	0.833	0.167	0.500
	123	–	–	–	–	0.100	0.167	0.167	–
	125	0.033	–	0.050	0.059	–	–	–	–
	127	–	–	0.050	–	–	–	–	–
	135	–	–	–	0.029	–	–	–	–
	N_A	4	4	4	6	3	2	4	2
FE42	261	0.633	0.971	1.000	0.441	0.500	0.667	1.000	0.500
	263	0.367	0.029		0.529	0.500	0.333	–	0.500
	265	–	–		0.029	–	–	–	–
	N_A	2	2	1	3	2	2	1	2
N_Σ		20	23	20	33	14	14	13	12

[a] Numbers indicate the number of base pairs in each allele.