Myrmecological News	14	37-47	Vienna, January 2011

Molecular taxonomy of the *Formica rufa* group (red wood ants) (Hymenoptera: Formicidae): a new cryptic species in the Swiss Alps?

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Abstract

Because of their beneficial impact on forest ecosystems, European red wood ants (*Formica rufa* group) are protected by law in many European countries and are considered to be among the most reliable bioindicators of forest stability. However, their taxonomy has been much debated and, unfortunately, it is too often neglected. This happens mainly because the morphology-based method for species delimitation requires lots of time and experience. We therefore employed 9 microsatellite loci and mitochondrial DNA (COI gene) to verify the power of genetic markers for red wood ant species delimitation and to investigate the cryptic diversity of these ants within the Eastern Swiss Alps. We analyzed 83 nests belonging to all red wood ant species that occur in the Swiss National Park area. Genetic data indicated that these species represent different genetic pools. Moreover, results showed that *Formica aquilonia* YARROW, 1955 and *F. paralugubris* SEIFERT, 1996 often hybridize within the Park, confirming that these two species are genetically very close and could have diverged only recently. Nevertheless, microsatellites also revealed that one entire population, located in the Mingèr Valley and morphologically identified as *F. lugubris* ZETTERSTEDT, 1838, is genetically different to all other analyzed *F. lugubris* populations found within the same area and to other red wood ant species. These findings, confirmed by mitochondrial DNA analyses, suggest the existence of a new cryptic species within the Eastern Swiss Alps. This putative cryptic species has been provisionally named *F. lugubris*-A2. These results have a great importance for future conservation plans, monitoring and evolutionary studies on these protected ants.

Key words: Microsatellites, *Formica rufa* group, cryptic species, species delimitation, biodiversity.

Myrmecol. News 14: 37-47 (online 24 June 2010)
ISSN 1994-4136 (print), ISSN 1997-3500 (online)

Received 8 December 2009; revision received 3 March 2010; accepted 17 March 2010

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Introduction

Ants are ecologically important and have a major role in many terrestrial ecosystems (HÖLLDOBLER & WILSON 1990, PASSERA & ARON 2005). Considering their ubiquitous distribution, their sensitivity to environmental variables and their importance at many trophic levels, ants are considered among the most suitable species for monitoring ecosystems (UNDERWOOD & FISHER 2006). In addition, ants are often used to assess special questions in evolutionary biology like the evolution of cooperation as in the shift from single-queen colonies to multi-queen colonies (e.g., ZHU & al. 2003, GYLLENSTRAND & al. 2005) and the transformation from ordinary social life style to a social parasite (e.g., MORI & al. 2001, SAVOLAINEN & VEPSÄLÄINEN 2003). Naturally, correct species delimitation is a fundamental prerequisite in monitoring studies, as well as in ecology, evolutionary biology and conservation biology in general (SITES & MARSHALL 2003, 2004, MACE 2004).

Cryptic species, which are morphologically hardly distinguishable (BICKFORD & al. 2007), represent a major problem to correct species classification and biodiversity studies. A large number of cryptic species have been discovered in ants (LUCAS & al. 2002, ROSS & SHOEMAKER 2005, SCHLICK-STEINER & al. 2006a, b, SEIFERT 2009, ROSS & al. 2010) in which species delimitation is often based on morphologically variable worker ants. Despite considerable progress in the morphometric analysis (e.g., SEIFERT 2002), ant species with small interspecific differences and high intraspecific variation are often poorly resolved by morphological methods alone (LUCAS & al. 2002, KNADEN & al. 2005, ROSS & SHOEMAKER 2005, STEINER & al. 2005, SCHLICK-STEINER & al. 2006a, b, ROSS & al. 2010).

Molecular approaches have proved extremely useful in the delimitation of species and in monitoring the cryptic diversity in well-studied groups of organisms (e.g., LAMBERT & al. 2005, SCHWARTZ & al. 2006, BICKFORD & al. 2007, VOGLER & MONAGHAN 2007, MOREAU 2009). Some good examples also exist among ants, in which morphologically similar species may differ markedly in their mito-

chondrial DNA (SMITH & al. 2005, STEINER & al. 2005, PUSCH & al. 2006, SCHLICK-STEINER & al. 2006a, b, STEINER & al. 2006, BERNASCONI & al. 2010) or microsatellites (MACARANAS & al. 2001, GYLLENSTRAND & al. 2004, KNADEN & al. 2005, BERNASCONI & al. 2010).

The ants of the *Formica rufa* group s.str. belong to the most studied ant groups in Europe with respect to their biology and ecology (see COTTI 1963, 1995, 1996). Because of their beneficial impact on forest ecosystems, these ants are protected by law in many European countries and are considered to be among the most reliable bioindicators of forest stability (GÖSSWALD 1990). All red wood ant species are morphologically very similar and show high intraspecific variability. They are also able to hybridize in some cases (SEIFERT & GOROPASHNAYA 2004) or to form mixed colonies (SEIFERT 1991, CZECHOWSKI 1996, SEIFERT & al. 2010). Consequently, the morphological delimitation of these species can be quite complicated and the taxonomy of the group has been much debated (VEPSÄLÄINEN & PISARSKI 1981, COLLINGWOOD 1987, SEIFERT 1991). A phylogenetic study of GOROPASHNAYA & al. (2004) suggested the group to be formed by six species in Europe: *Formica rufa* LINNAEUS, 1761, *F. polyctena* FOERSTER, 1850, *F. lugubris* ZETTERSTEDT, 1838, *F. paralugubris* SEIFERT, 1996, *F. aquilonia* YARROW, 1955, and *F. pratensis* RETZIUS, 1783. However, the correct taxonomy of the group is often neglected mainly due to the lack of reliable and easy-to-use delimitation methods (e.g., BONERA 2002, but see GROPPALI & BONERA 2004, BOUDJEMA & al. 2006). The species *Formica lugubris* and *F. paralugubris* are a good example of difficult taxonomy – until 1996, they were considered conspecific (*F. lugubris*). However, alarm pheromones (CHERIX 1983), allozymes (PAMILO & al. 1992) and behaviour (ROSENGREN & CHERIX 1981, ROSENGREN & al. 1994) indicated the existence of two different *F. lugubris* types in the Swiss Jura Mountains. This finally led SEIFERT (1996a) to the description of *F. paralugubris* as a sibling species of *F. lugubris*. Our genetic studies (BERNASCONI & al. 2010) demonstrate that the two species can be reliably distinguished from each other on the basis of mitochondrial(mt)DNA-based markers and microsatellites, i.e., nuclear DNA.

Within the *Formica rufa* group ants, the species *F. rufa* and *F. polyctena* form hybrid zones in Central Europe (SEIFERT 1991, CZECHOWSKI 1996), but a genetic study comparing sympatric and allopatric populations showed that the two species form clearly separate gene pools in other regions (GYLLENSTRAND & al. 2004). Our aim here is to expand such a study to cover all the species of the *F. rufa* group in an area where the species exist in syntopy or at least close to each other. The Alpine region is a suitable place for such a study. Firstly, all *Formica rufa* group species are present in this area. Secondly, some authors have highlighted the existence of scattered ice-free areas located within the Alps or at their periphery during the last glacial maximum. Numerous Alpine species persisted and developed independently in these refugia, which are now seen as centres of Alpine species diversity and endemism (STEHLIK 2000, 2003, SCHÖNSWETTER & al. 2005, HAUBRICH & SCHMITT 2007, PARISOD & BESNARD 2007, PARISOD 2008). Our main focus is in *F. lugubris* because this species has a much higher level of mtDNA haplotype diversity than its close relatives *F. aquilonia*, *F. rufa*, *F. polyctena*, and *F. paralugubris* within Eurasia (GOROPASHNAYA & al. 2004).

Here, we present microsatellite data, (I) to test the utility of genetic markers for species delimitation, (II) to investigate cryptic diversity, and (III) to evaluate the possible role of hybridisation in the evolution of the species group. We refer to the Unified Species Concept of DE QUEIROZ (2007) and we use the genotypic cluster criterion of MALLET (1995) as the species delimitation criterion.

Methods

Sample collection: Sampling was conducted between 2005 and 2008 in the Swiss National Park (Canton of Grisons) and the surrounding area (Fig. 1). Created in 1914, this strict nature reserve offers the unique opportunity to study the evolution of wood ant populations in unmanaged forests. Moreover, all *F. rufa* group species are present in this region (DEVENOGES 1999, CHERIX & al. 2007; C. Bernasconi, unpubl.). From each nest, about 30 workers were collected at the nest surface, stored in absolute ethanol and deposited at the Museum of Zoology of Lausanne (Switzerland) as voucher specimens. Because of restrictions imposed by the Park regulations, we sampled nests mainly located along the pathways. A total of 83 nests (35 belonging to *F. lugubris*, 22 to *F. aquilonia*, 14 to *F. paralugubris*, three to *F. polyctena*, five to *F. rufa* and four to *F. pratensis*) were sampled for this study.

Morphological identification: Species identification was carefully done using the morphological criteria of SEIFERT (1996a, b, 2007) for workers and also by comparing morphological traits in workers and queens with reference material already deposited at the Museum of Zoology of Lausanne (Switzerland).

DNA extraction and microsatellite genotyping: Genomic DNA was isolated from workers using QIAamp DNA Mini Kit (Qiagen). The entire body of ants was used for DNA extraction. Eight to ten workers from each nest were analyzed using nine microsatellite loci: FL12, FL20, FL21, FL29 (CHAPUISAT 1996), and FE13, FE19, FE37, FE38, FE51 (GYLLENSTRAND & al. 2002). In total, 683 individuals were genotyped. PCR conditions were mainly as described by CHAPUISAT (1996) and GYLLENSTRAND & al. (2002) with slight modifications of the amplification conditions following optimisation by MÄKI-PETÄYS & al. (2005). Primers were labelled with HEX, NED and FAM fluorescent dyes and the amplification products were analyzed on a capillary sequencer (Applied Biosystems, Foster City, CA). Alleles were scored by length using the computer program GeneMapper.

Estimation and delimitation of genetic units: All genotypes were screened using a Bayesian admixture procedure implemented in STRUCTURE 2.2 (PRITCHARD & al. 2000, http://pritch.bsd.uchicago.edu). This model was designed to identify the unknown number of K genetic clusters of individuals, and at the same time to probabilistically assign individuals to one cluster or more than one cluster if they are genetically admixed as a result of hybridization. STRUCTURE was run with the admixture model, and 10 repetitions of 100000 iterations following a burn-in period of 20000 iterations. Other parameters have been set to default values.

We assessed population structure by comparing species that coexist in the same habitat and might be more prone

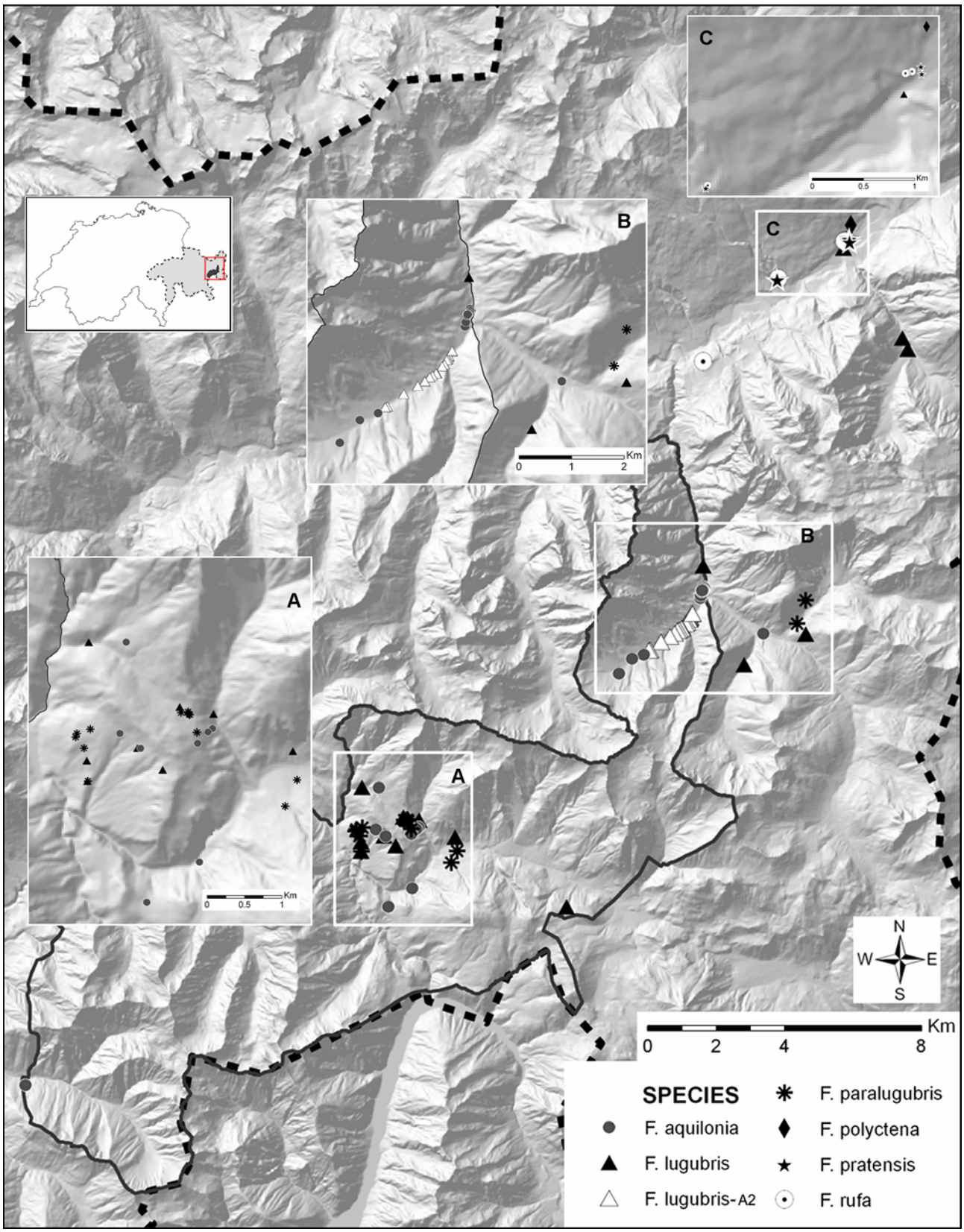

Fig. 1: Location of the analyzed nests, sampled within the Swiss National Park area. (A), (B), (C) zoom to a closer view.

to hybridize. We therefore divided the dataset in two groups. The first group contains samples belonging to species living in lowland habitat: *F. polyctena*, *F. rufa* and *F. pratensis*; nests of these species were found in syntopy in the sampling region. The second group contains species living at high altitudes and in coniferous forests in the Alps: *F. lugubris*, *F. aquilonia* and *F. paralugubris*; these species were also found in syntopy or in close vicinity within the study area. We analyzed the two groups independently by evaluating the number of clusters (K), which best fits our

Tab. 1: Genetic diversity in red wood ant species over the nine microsatellite loci. He, expected heterozygosity without bias (NEI 1978); Ho, observed heterozygosity; standard deviation in parentheses. F_{IS}, deviation from Hardy-Weinberg equilibrium; F_{ST} genetic differentiation among the nests. Both F_{IS} and F_{ST} were estimated following WEIR & COCKERHAM (1984), and all the estimates differ significantly from zero with $P < 0.001$.

Species	No. of nests	No. of alleles	He	Ho	F_{IS}	F_{ST}
F. rufa	5	28	0.383 (0.228)	0.331 (0.202)	0.150	0.274
F. polyctena	3	35	0.570 (0.092)	0.441 (0.143)	0.246	0.204
F. pratensis	4	34	0.444 (0.283)	0.381 (0.270)	0.159	0.195
F. lugubris	17	62	0.604 (0.248)	0.555 (0.230)	0.083	0.240
F. lugubris-A2	18	46	0.486 (0.226)	0.440 (0.210)	0.098	0.067
F. paralugubris	14	49	0.652 (0.145)	0.498 (0.108)	0.241	0.204
F. aquilonia	22	62	0.665 (0.152)	0.508 (0.130)	0.239	0.214
Overall	83	95				

Tab. 2: Average membership coefficient (q_{group}) of the lowland species. Each species was assigned to one group if q_{group} was ≥ 0.90, otherwise it was assigned jointly to several groups (admixture).

	Group I	Group II	Group III
F. rufa	0.01	0.99	0.00
F. polyctena	0.98	0.01	0.01
F. pratensis	0.00	0.00	0.99

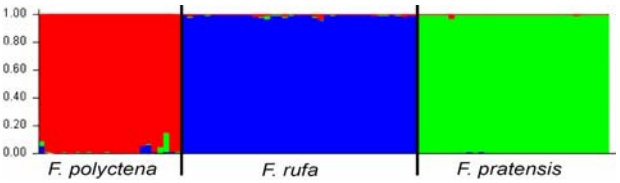

Fig. 2: Individual membership coefficients (q_{ind}) of the lowland species *F. rufa* (blue), *F. polyctena* (red) and *F. pratensis* (green) analyzed with the computer program STRUCTURE. Each individual is represented by a vertical line, which is partitioned into three coloured segments that represent the individual's estimated membership fractions in K = 3 clusters. The black lines separate individuals belonging to the same species, as identified on morphological grounds.

datasets. Posterior probability values for K (log likelihood; ln L) were estimated assigning a prior from 1 to 10. In some situations the posterior probability L may increase with K after the real value of K has been reached (EVANNO & al. 2005) and this made the inference of K difficult in our data. Therefore, we calculated the ΔK statistic, proposed by EVANNO & al. (2005). Samples were placed into the respective subpopulation based upon the highest percentage of membership (q_{ind}). Individuals with $q_{ind} \geq 0.90$ were assigned to only one cluster, whereas individuals with a proportion of membership to each cluster $q_{ind} < 0.90$ (admixed individuals) were assigned to more than one cluster. The threshold value of 0.90 was arbitrarily defined to be sure that at least 90% of the individual's genome is assigned to one cluster (MANEL & al. 2002, CEGELSKI & al. 2003).

Factorial Correspondence Analysis: A Factorial Correspondence Analysis (FCA) of individual multilocus scores was used to describe patterns of differentiation. It was computed using the software package GENETIX 4.02 (BELKHIR & al. 1996 - 2004). Individuals were considered as parts of distinct groups, according to assignment analysis performed with STRUCTURE.

Analyses of genetic variation and population structure: GENETIX was also used to calculate the allele frequencies, allele number, observed (Ho) and expected (He) heterozygosities for each species or genetic group. Deviations from Hardy-Weinberg equilibrium and the genetic structure of the K populations defined with STRUCTURE were characterised by Wright's fixation indices (WRIGHT 1943, WEIR & COCKERHAM 1984). Calculations were carried out using the program FSTAT v.2.9.4 (GOUDET 1995, http://www.unil.ch/dee/page6759_fr.html). Standard errors of F-statistics were obtained by jackknifing over nests and confidence intervals were obtained by jackknifing over loci (5000 permutations) (GOUDET 1995).

Mitochondrial DNA investigations: As the microsatellite results suggested genetic separation between *F. lugubris* and *F. lugubris*-A2, we checked whether these ants also show differences in the mtDNA haplotypes. For this we used the restriction method based on the COI gene and developed earlier to distinguish between *F. lugubris* and *F. paralugubris* (BERNASCONI & al. 2010). We thus analyzed 1 to 5 individuals from each *F. lugubris*-A2 nest.

Moreover, we conducted further analyses by sequencing the same mtDNA fragment used by GOROPASHNAYA & al. (2004) and including part of the cytochrome b gene, the intergenic region I, the transfer RNA, the intergenic region II and part of the NADH dehydrogenase 1. We then compared the *F. lugubris*-A2 sequences with those obtained by GOROPASHNAYA & al. (2004) (EMBL Accession numbers: AY488759 - AY488791). The phylogenetic tree including all sequences was constructed by using the same method (Maximum Likelihood, ML) as by GOROPASHNAYA & al. (2004).

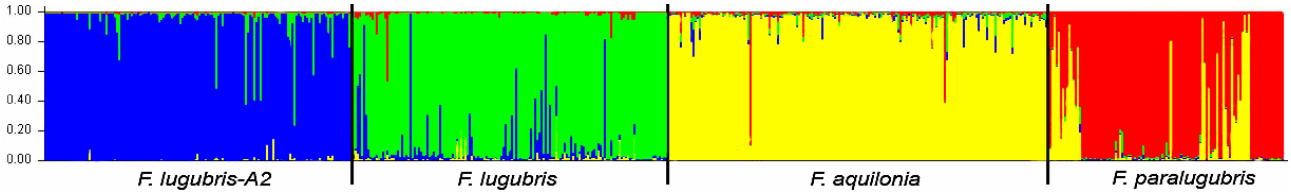

Fig. 3: Individual membership coefficients (q_{ind}) of the four species living at high altitudes and in coniferous forest in the Alps: *F. lugubris*-A2 (blue), *F. lugubris* (green), *F. aquilonia* (yellow), and *F. paralugubris* (red) analysed with the computer program STRUCTURE. Each individual is represented by a vertical line, which is partitioned into four coloured segments that represent the individual's estimated membership fractions in K = 4 clusters. The black lines separate individuals belonging to the same species, as identified on morphological grounds.

Results

Estimation and delimitation of genetic units: The Bayesian analysis (STRUCTURE) detected several genetic groups in the two data sets. On the basis of the ΔK values, there were three distinct genetic groups within the lowland cluster of *F. polyctena*, *F. rufa* and *F. pratensis*, and four groups in the other cluster including the high-altitude species *F. aquilonia*, *F. lugubris* and *F. paralugubris*. Each species was assigned to one genetic group if its average membership coefficient to that group q_{group} was ≥ 0.90, otherwise it was assigned jointly to several groups. All individuals belonging to *F. rufa*, *F. polyctena* and *F. pratensis* clustered in three separate groups, each group representing one morphologically identified species. The individual (q_{ind}) and the average membership coefficients (q_{group}) of each species to each group indicated no hybridization between these three species (Tab. 2, Fig. 2).

As mentioned above, the high-altitude samples were split into four different groups. Most of the *F. aquilonia* and *F. paralugubris* workers formed separate groups of their own. Nevertheless, the individual and average membership coefficients indicated that admixture has most probably occurred between these two species in such a way that some samples identified morphologically as *F. paralugubris* showed genetic affinity with *F. aquilonia* (Tab. 3, Fig. 3). Interestingly, all the admixed nests were located in the same restricted area.

Formica lugubris nests were divided into two distinct genetic groups: Individuals from one entire location (18 nests) in the Mingèr Valley within the Swiss National Park did not group with other *F. lugubris* individuals but formed a genetically distinct group of their own. We will refer to this population as *F. lugubris*-A2 throughout the rest of this paper (note that the term "*F. lugubris-X*", used by SEIFERT 2010, referred to the same population). A few admixed individuals were observed between *F. lugubris* and *F. lugubris*-A2 (Tab. 3, Fig. 3).

All the pairwise F_{ST} values between the seven genetic groups were significantly greater than zero (Tab. 4). The smallest F_{ST} value (0.101) was between *F. lugubris* and *F. lugubris*-A2. Similar values were also found between *F. aquilonia* and *F. paralugubris* (0.117) and between *F. aquilonia* and *F. lugubris* (0.130).

Factorial Correspondence Analysis: The FCA of the individual genotypes (Figs. 4, 5) indicated that *F. pratensis* is well separated from the rest of the species and that, although *F. pratensis*, *F. rufa* and *F. polyctena* live in syntopy, the species form clearly distinct gene pools.

Tab. 3: Average membership coefficient (q_{group}) of the four species living at high altitudes and in coniferous forests in the Alps. Each species was assigned to one group if q_{group} was ≥ 0.90, otherwise it was assigned jointly to several groups (admixture).

	Groups			
	I	**II**	**III**	**IV**
F. lugubris	0.88	0.09	0.02	0.02
F. lugubris-A2	0.05	0.93	0.01	0.01
F. paralugubris	0.02	0.02	0.94	0.03
F. aquilonia	0.01	0.01	0.19	0.79

In contrast, *F. aquilonia* and *F. paralugubris* were genetically close with the genotype distributions overlapping widely (Fig. 5); the overlapping genotypes corresponded to the putative hybrid individuals already detected by STRUCTURE. The distribution of the individual data points in the FCA also showed that the genotypes of *F. lugubris*-A2 are located marginally and outside the group formed by the species *F. lugubris*, *F. aquilonia* and *F. paralugubris*. Furthermore, there was some overlap between the distributions of the *F. lugubris*-A2 and *F. lugubris* data points. The individuals in the overlapping area corresponded to the putatively hybrid individuals detected by STRUCTURE.

Population genetic diversity and test of fit to Hardy-Weinberg: All nine microsatellite loci were polymorphic and the overall number of alleles per locus ranged from 2 to 31. All the seven genetic groups identified by STRUCTURE showed a deficiency of heterozygotes with the average F_{IS} values ranging from 0.08 to 0.25. This suggests deviations from the expected Hardy-Weinberg genotype frequencies (Tab. 1). It is, however, problematic to make a definitive statistical test because ants from the same nest are not genetically independent from each other. At least a part of the observed homozygote excess could be due to the presence of null alleles (PEMBERTON & al. 1995). This should, however, not much affect the above cluster analyses. F_{ST} values calculated between nests within each genetic group were significantly different from zero and varied from 0.067 of *F. lugubris*-A2 to 0.240 of *F. lugubris* (Tab. 1).

Mitochondrial DNA investigations: The results of the restriction analyses based on COI indicated that all the analyzed *F. lugubris*-A2 samples share the same haplotype, which is different from that observed in the other *F.*

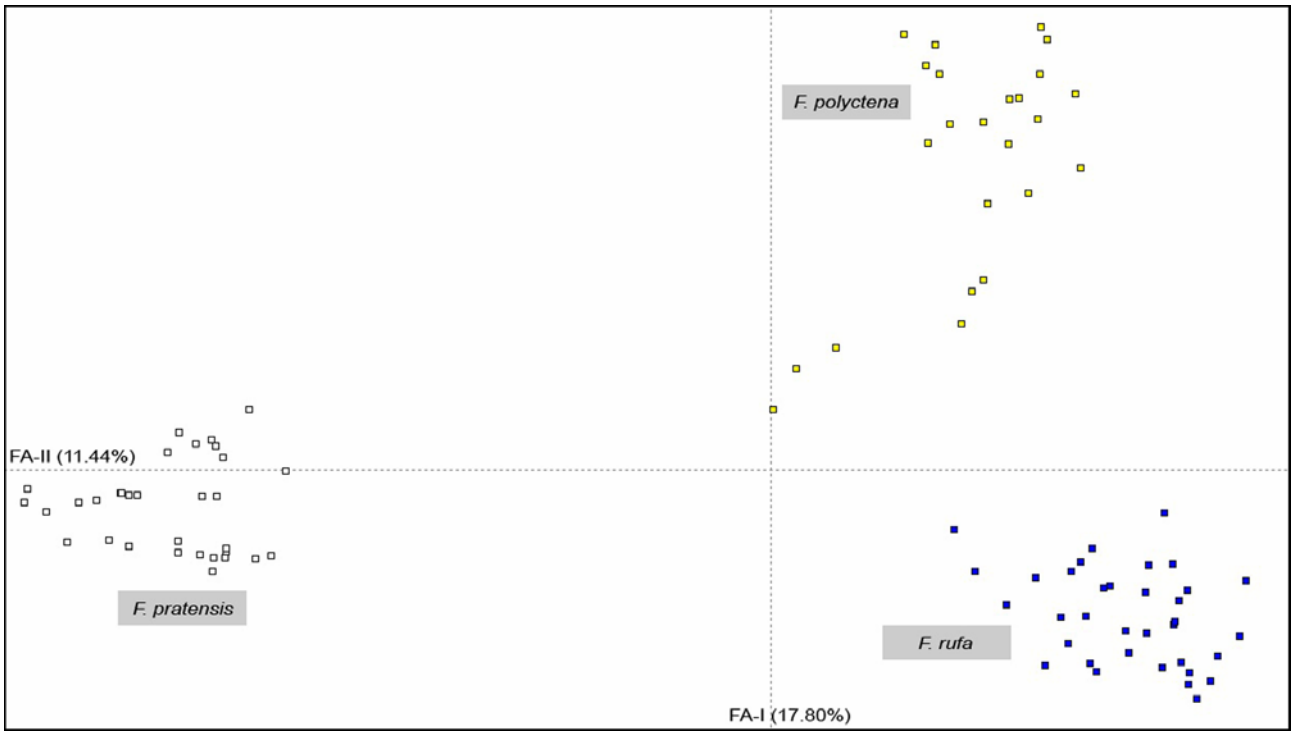

Fig. 4: Factorial Correspondence Analysis, computed using GENETIX, showing relationships among the multilocus genotypes of the three red wood ant species living at lowland: *F. rufa* (blue), *F. polyctena* (yellow) and *F. pratensis* (white). Coloured points represent the individual genotype for each sample. FA-I and FA-II are the first and second principal factors of variability.

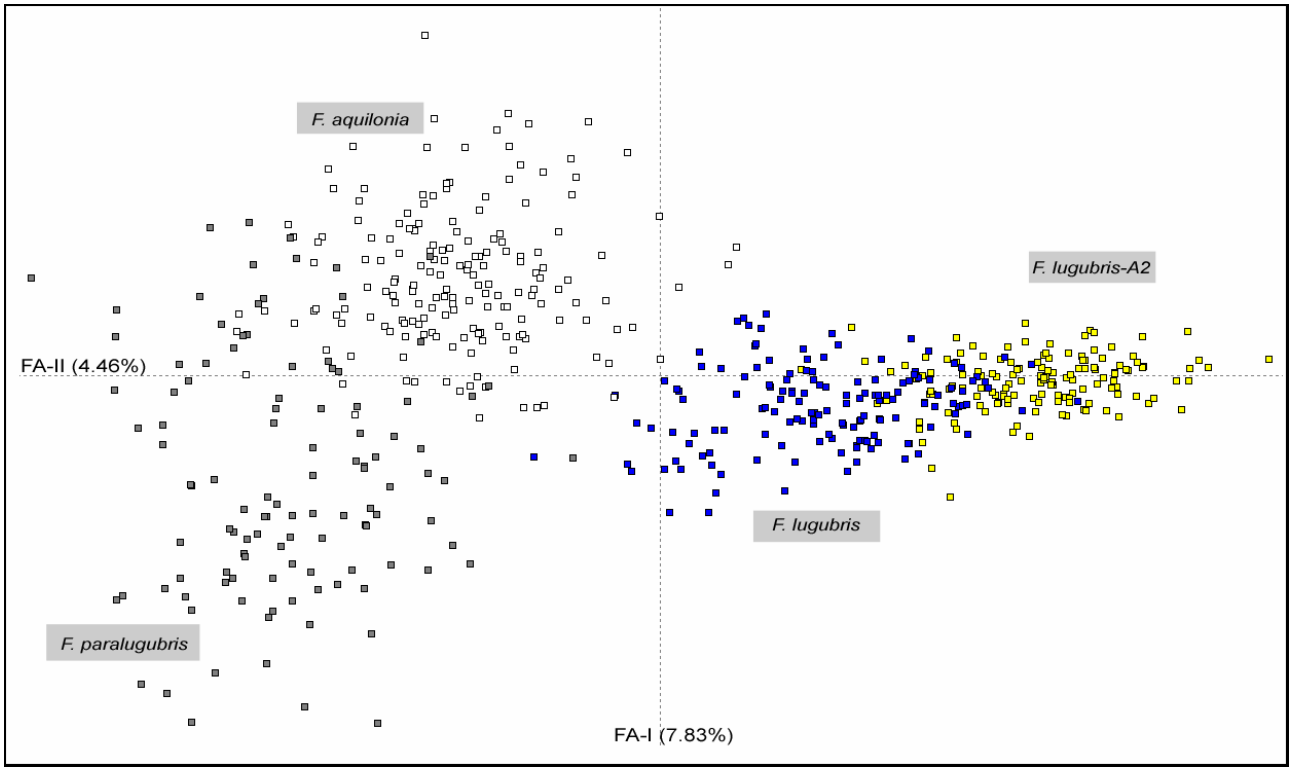

Fig. 5: Factorial Correspondence Analysis showing relationships among the multilocus genotypes of the four red wood ant species living at high altitude in the Alps: *F. aquilonia* (white), *F. paralugubris* (grey), *F. lugubris* (blue) and *F. lugubris*-A2 (yellow). Coloured points represent the individual genotype for each sample. FA-I and FA-II are the first and second principal factors of variability.

Tab. 4: Paired F_{ST} values between red wood ant species. All the values differ significantly from zero with $P < 0.001$.

	***F. lugubris*-A2**	***F. paralugubris***	***F. aquilonia***	***F. rufa***	***F. polyctena***	***F. pratensis***
F. lugubris	0.101	0.196	0.130	0.323	0.207	0.226
F. lugubris-A2	–	0.326	0.230	0.434	0.339	0.334
F. paralugubris		–	0.117	0.297	0.201	0.284
F. aquilonia			–	0.261	0.178	0.257
F. rufa				–	0.341	0.501
F. polyctena					–	0.360
F. pratensis						–

lugubris samples collected in the study area and in those analyzed previously (BERNASCONI & al. 2010). The results of the phylogenetic reconstructions showed that all *F. lugubris*-A2 samples share the same haplotype (EMBL Accession Number GU946453), which differs from the ones observed in *F. lugubris* (Fig. 6). These results are in accordance with those shown by the microsatellites and confirm the genetic distinction between *F. lugubris* and *F. lugubris*-A2.

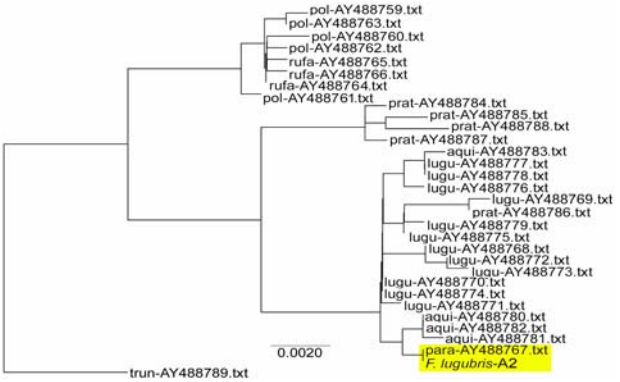

Fig. 6: Phylogenetic tree (ML) obtained with the mtDNA sequences of GOROPASHNAYA & al. (2004) (EMBL Accession numbers: AY488759 - AY488791). Several *F. lugubris*-A2 individuals have also been sequenced with the same primers used by GOROPASHNAYA & al. (2004) but only a single one has been recovered and added to the phylogenetic tree (EMBL Accession number GU946453). It clusters with *F. paralugubris* (in yellow).

Discussion

Number of genetic units: Microsatellite data indicate that the six morphologically identified red wood ant species represent seven genetic units in the Swiss National Park and the surrounding area. Samples belonging to *F. rufa*, *F. polyctena*, *F. aquilonia*, *F. paralugubris*, and *F. pratensis* form different genetic pools in accordance with the phylogenetic study conducted by GOROPASHNAYA & al. (2004). In addition, individuals with *F. lugubris* morphology surprisingly form two distinct genetic units, here called *F. lugubris* and *F. lugubris*-A2.

Formica pratensis is well separated from all the other species as shown by the FCA and by the F_{ST} values. The result is in accordance with GOROPASHNAYA & al. (2004), who did already show that *F. pratensis* forms a distinct clade within the *F. rufa* group. There have been some controversies on the species status of *F. pratensis* and its ecomorphs, in particular concerning *F. rufa pratensis* var. *nigricans* BONDROIT, 1912. Some authors considered *Formica nigricans* as separate from *F. pratensis* (e.g., KUTTER 1977, COLLINGWOOD 1979), while others never recognized it as a different species (e.g., DLUSSKII 1967, PARACHIVESCU 1972). The controversies were finally stopped by a detailed morphological and ecological investigation by SEIFERT (1992), who interpreted *Formica nigricans* as an ecomorph of *F. pratensis*. In the future, nuclear-DNA studies on these two ecomorphs could be useful to better understand patterns of genetic diversity within *F. pratensis*.

The species *F. rufa* and *F. polyctena* frequently hybridize at least in some areas in Central Europe (SEIFERT 1991, CZECHOWSKI 1996). The mtDNA haplotypes suggest incomplete lineage sorting and both species have very little sequence diversity (GOROPASHNAYA & al. 2004). Our present results confirm the previous finding from Sweden that sympatric populations of the two species form separate gene pools (GYLLENSTRAND & al. 2004). No hybrids were observed and the level of genetic differentiation ($F_{ST} = 0.341$) was higher than in some other species pairs. It is possible that hybridization in this species pair is localized in some areas.

Molecular data revealed that the species *F. aquilonia* and *F. paralugubris* are genetically close to each other. Some individuals with *F. paralugubris* morphology showed signs of admixture with *F. aquilonia* within the Park and all the hybrid individuals were sampled in the same valley.

As morphological identifications have been carried out carefully and some of them have also been blindly checked, our results are not influenced by identifications flaws. These two species have highly similar mtDNA haplotypes with very little geographical variation, suggesting a recent divergence (GOROPASHNAYA & al. 2004). It has been speculated that *F. paralugubris* probably originated as a result of a past hybridization between *F. aquilonia* and *F. lugubris* (GOROPASHNAYA & al. 2004). Our data agree with this hypothesis in that *F. paralugubris* workers are genetically close to *F. aquilonia* but morphologically similar to *F. lugubris*.

***Formica lugubris* and *F. lugubris*-A2:** The microsatellite data revealed that individuals morphologically iden-

tified as *F. lugubris* surprisingly form two distinct genetic units, named here *F. lugubris* and *F. lugubris*-A2. This distinction was indicated both by nuclear microsatellites and by the mtDNA haplotypes. The haplotype of *F. lugubris*-A2 is indeed clearly different from all other *F. lugubris* workers collected in the present study and from the European *F. lugubris* samples analyzed previously (BERNASCONI & al. 2010). In fact, the mtDNA haplotype clusters *F. lugubris*-A2 with *F. paralugubris* (Fig. 6), but the microsatellite data, particularly the FCA, suggests that *F. paralugubris* is further removed from *F. lugubris*-A2 than from other *F. lugubris* samples.

The F_{ST} value indicates that, even though it is significantly different from zero, the genetic distance between *F. lugubris* and *F. lugubris*-A2 (F_{ST} = 0.101) is lower than between two *F. lugubris* populations collected within the same area and analyzed in a previous work (F_{ST} = 0.156; BERNASCONI & al. 2005). This seems to argue that *F. lugubris*-A2 could be considered a different *F. lugubris* population rather than a different species. However, the distinct mtDNA haplotypes indicate that there is at least no influx of *F. lugubris* genes into the *F. lugubris*-A2 population via immigration of *F. lugubris* queens. It can also be noted that the distance between *F. lugubris* and *F. lugubris*-A2 is comparable to the genetic inter-specific distance observed between *F. aquilonia* and *F. paralugubris* (F_{ST} = 0.117) and between *F. lugubris* and *F. aquilonia* (F_{ST} = 0.130).

To date, *F. lugubris*-A2 has been found only in one valley, Val Mingèr, within the Swiss National Park. Could the genetic separation observed between the populations classified as *F. lugubris* and *F. lugubris*-A2 be due to geographical isolation? To our knowledge, there is no evident barrier in Mingèr Valley that could prevent ants from freely moving in and out of it. It therefore seems that the detected genetic difference cannot be associated with geographical isolation of this valley from other areas inhabited by *F. lugubris*. Moreover, *F. aquilonia*, which shares similar social structure and reproductive strategies with *F. lugubris*-A2 (MAEDER 2006; C. Bernasconi, unpubl.), is also present in the Mingèr Valley. These *F. aquilonia* nests do not genetically differ from the other *F. aquilonia* samples within the study area. There is also no clear indication that the population of *F. lugubris*-A2 would represent ongoing hybridization between *F. lugubris* and either *F. paralugubris* or *F. aquilonia*. The admixture analyses and the FCA showed that *F. lugubris*-A2 genotypes are not a mix between two other species – hybrid genotypes would have been located in the middle of their parental species in the FCA, as observed for hybrids between *F. aquilonia* and *F. paralugubris*.

We therefore suggest that *F. lugubris*-A2, which is morphologically similar to *F. lugubris*, but is genetically distinct from it, might represent an undescribed cryptic species of the *F. rufa* group. Combining the nuclear and mitochondrial data indicates that the population may have originated via hybridization as the mtDNA haplotype associates it with *F. paralugubris* and the microsatellite alleles and morphology link it with *F. lugubris*. The situation would be very similar to that of *F. paralugubris*, which has been described as a separate species (SEIFERT 1996a). Its discovery was prompted because alarm pheromones (CHERIX 1983) and behaviour (ROSENGREN & CHERIX 1981, ROSENGREN & al. 1994) showed variation within a population morphologically considered as *F. lugubris* in the Swiss Jura Mountains, and allozymes demonstrated the existence of two separate gene pools (PAMILO & al. 1992).

The divergence between *F. lugubris*-A2 and *F. lugubris* is similar to that observed between the mitochondrial lineages of *F. paralugubris* and *F. lugubris*, which diverged about 100 thousand years ago (GOROPASHNAYA & al. 2004). We can therefore suggest that *F. lugubris*-A2 probably originated at about the same period during the last glaciation in an Alpine valley, which was not covered by ice. As mentioned above, *F. paralugubris* also has genetic features suggesting its hybridogenous speciation. As for the origin of *F. paralugubris* (GOROPASHNAYA & al. 2004), hybridization is indeed known to have played a role in the evolution of the *Formica rufa* group (SEIFERT 1991, CZECHOWSKI 1996, SEIFERT & GOROPASHNAYA 2004) as well as in other ants (PEARSON 1983, SEIFERT 1999, 2006, SCHWANDER & al. 2008) and hybridization has also been suggested as a mechanism leading to speciation in social insects (NONACS 2006a, b) and other animals (SCHWARZ & al. 2005, GOMPERT & al. 2006, MAVÀREZ & al. 2006, MALLET 2007, MAVÀREZ & LINARES 2008). Furthermore, NONACS (2006b) pointed out that hybridization could be suitable as it may allow colonies to survive and prosper in microhabitats that are unfavourable to pure species or make colonies competitively superior to parental species. Capability to hybridize is therefore particularly important in times of environmental changes (NONACS 2006b). On the other hand, if population densities are low, it may be better to hybridize rather than have no reproductive success at all (NONACS 2006b).

Considering the particular situation of *F. lugubris*-A2, our data also suggest that hybrid speciation is probably more common than we thought in Alpine red wood ants. In order to corroborate this hypothesis, it would be interesting to add more samples, including *F. lugubris*-A2 individuals, to the phylogenetic tree obtained by GOROPASHNAYA & al. (2004).

The possible occurrence of a new cryptic species within the Swiss National Park would be of great interest for this nature reserve and for conservation planning in the area. It is, however, necessary to first verify the species status of *F. lugubris*-A2 and to clarify the general role of hybridization in speciation within the *F. rufa* group ants. Such a project should expand the geographical scale and it would benefit from integrative taxonomy, which gathers data by different techniques (DAYRAT 2005, WILL & al. 2005, VALDECASAS & al. 2008, SEIFERT 2009, SCHLICK-STEINER & al. 2010, but see CARDOSO & al. 2009) and has been successfully used in other ant genera (LUCAS & al. 2002, SCHLICK-STEINER & al. 2006a, b, SEIFERT 2009). Possible tools which have already been applied to study the *Formica rufa* group include behavioural tests based on recognition (ROSENGREN & CHERIX 1981, ROSENGREN & al. 1994, MAEDER & al. 2005) and chemical analyses of cuticular hydrocarbons (MARTIN & al. 2008). Further, the analysis of sex pheromones (WALTER & al. 1993) could be very suitable to highlight potential prezygotic barriers.

Acknowledgements

Authors are indebted to Riitta Jokela, Lumi Viljakainen, Jonna Kulmuni, and Kukka Haapaniemi of the University of Oulu (Finland) for their precious help in the laboratory

and to Birgit Schlick-Steiner, Florian Steiner, Herbert Zettel, Michele Pestalozzi, and two anonymous referees for fruitful revisions on earlier versions of the manuscript. We are also grateful to Monica Kaiser-Benz, Anne Freitag, Matthieu Fleury for their help during sampling. We thank Patrick Basset, Guillaume Evanno, Thomas Broquet and Gwenaëlle Le Lay for support in statistical analyses. We are also grateful to the Swiss National Park staff and to the Canton of Grisons for providing authorization for sample collections and the Academy of Finland (122210) for financial support.

Zusammenfassung

Wegen ihres günstigen Einflusses auf Waldökosysteme sind Waldameisen der *Formica rufa*-Gruppe in vielen europäischen Ländern gesetzlich geschützt. Sie werden als die verlässlichsten Bioindikatoren für die Stabilität dieser Ökosysteme angesehen. Ihre Taxonomie ist jedoch umstritten und wird wenig bearbeitet, was hauptsächlich in dem für die morphologische Artbestimmung erforderlichen hohen Zeitaufwand und großen individuellen Erfahrungshintergrund begründet ist. Wir nutzten 9 Mikrosatellitenloci sowie mitochondriale DNA (das COI Gen), um die Eignung genetischer Marker zur Artbestimmung und Darstellung kryptischer Biodiversität dieser Ameisen in den östlichen Schweizer Alpen zu zeigen. Wir analysierten 83 Nester aller im Schweizer Nationalpark vertretenen Waldameisenarten. Genetische Daten zeigten, dass diese Arten verschiedene Genpools repräsentieren. Weiterhin zeigten die Daten, dass *Formica aquilonia* YARROW, 1955 und *F. paralugubris* SEIFERT, 1996 im Park häufig hybridisieren, eng verwandt sind und sich wahrscheinlich erst vor evolutionsbiologisch kurzer Zeit aufgespalten haben. Die Mikrosatellitendaten zeigten ferner, dass eine große, morphologisch als *F. lugubris* ZETTERSTEDT, 1838, identifizierte Population aus dem Val Mingèr von allen anderen *F. lugubris*-Populationen und Waldameisenarten dieser Region verschieden ist. Diese durch Analysen mitochondrialer DNA unterstützten Daten sprechen für die Existenz einer bislang unbekannten kryptischen Waldameisenart in den östlichen Schweizer Alpen. Diese mutmaßliche kryptische Art wird hier provisorisch als *F. lugubris*-A2 benannt. Diese Resultate haben eine große Bedeutung für künftige Naturschutzvorhaben und Studien über Verbreitung und Evolution dieser geschützten Ameisen.

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