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CONTENTS

ABSTRACT

Mound building red wood ants (species of the *Formica rufa* group) belong to one of the most studied groups of ants in Europe and have fundamental roles and positive effects in forested habitats of the northern hemisphere. In addition, they are considered among the most promising bioindicators of forest ecosystems. Because of their importance, these ants are protected by law in many European countries, including Switzerland. However, despite this protection, they are included on the red list of threatened species edited by the International Union for Conservation of Nature (IUCN) and on the red list of some particular countries like Switzerland. Because of their similar morphology and a high intraspecific variability, the morphological identification of these species can be quite complicated. In addition, they are sometimes able to hybridize or to form mixed colonies. Consequently, the taxonomy of this group of ants has been much debated during the past decades. Based on a phylogenetic study, today the group is considered to count six species in Europe: *F. rufa*, *F. polyctena*, *F. lugubris*, *F. paralugubris*, *F. aquilonia* and *F. pratensis*. Nevertheless, the taxonomy of the group is often neglected mainly due to the lack of reliable and easy to use identification methods.

Considering the importance of correct species assessment in conservation biology, in this study we want to disentangle the taxonomical difficulties within the *Formica rufa* group and to clarify the diversity of these protected ants, by using an integrative approach.

We first analyzed the distribution of the sibling species *F. lugubris* and *F. paralugubris* in the Italian Alps by collecting new samples on the field and by examining one of the major red wood ant collections, which is deposited at the University of Pavia, Italy. After that, we developed a molecular tool based on mitochondrial DNA, which provides a reliable and easy-to-use technique for the identification of *F. lugubris* and *F. paralugubris*. Afterwards, we extended the use of molecular markers for species identification to the whole *F. rufa* group and made a microsatellite analysis. Results confirm that molecular markers are consistent tools for species identification and that the six known species represent six different genetic pools. In addition, genetic data highlighted the existence of a new cryptic species in the Swiss Alps, called *Formica lugubris-X*.

The presence of a new species can have a great influence on future conservation plans in favour of these protected ants and consequently for forested habitats. We therefore completed molecular data by behavioural (pupae recognition) and chemical analyses based on sex pheromones of the entire *F. rufa* group. Both approaches are in accordance to genetic results and confirm that *F. lugubris-X* really represents a new cryptic species of red wood ant within the Swiss National Park (Eastern Swiss Alps).

Results obtained in this study have a great importance in terms of biodiversity. Moreover, they provide important taxonomical information, reliable tools for species identifications and future perspectives for a consequent conservation of red wood ant species.

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INTRODUCTION

To date, about 12'500 species of ants have been described on Earth (Agosti & Johnson, www.antbase.org). Nevertheless, this represents only a part of their real diversity. New species are indeed discovered every year thanks to the investigation of less studied habitats and to technical advances. As a comparison, only 8'800 ant species were known in 1990 (Holldöbler & Wilson 1990). Ants are everywhere and play a major role in many terrestrial ecosystems (Holldöbler & Wilson 1990; Passera & Aron 2005). Considering their ubiquitous distribution, the facility of sampling, their sensitivity to environmental variables and their importance at many trophic levels, ants are considered among the most suitable species in monitoring (Underwood & Fisher 2006). For example, ants can be employed for detecting trends of endangered species or for monitoring ecosystems variation, like habitat fragmentation or climate change (Punttila 1996; Underwood & Fisher 2006). Because of their importance and because of their fascinating social life, these insects have been the topic of numerous studies all over the World (see Vander Meer *et al*. 1990; Holldöbler & Wilson 1990; Passera & Aron 2005 and references therein). In most of these studies as well as in monitoring projects, correct taxonomic assessment is a fundamental prerequisite.

In the face of the biodiversity crisis, there is indeed an increasing need for reliable taxonomic information in order to allow us to understand, manage and preserve the natural world. This is particularly true if we consider that species already considered endangered might be composed of a number of species that are even more rare than earlier supposed. Taxonomic works should therefore be prioritized. Unfortunately, taxonomy - the science of naming and classifying organisms - is nowadays facing a crisis (Wheeler 2004; Wilson 2004; Agnarsson & Kuntner 2007), in particular because it often receives less grants than other disciplines and because taxonomists are underrepresented within the biological community (Wilson 2002, 2004; Wheeler 2004). In addition, morphology-based methods for species recognition often require lots of experience and time and act as limiting factors in biological studies and biomonitoring.

Among ants, for example, despite considerable progress in the morphometrical analysis (e.g. Seifert 2002), groups with small interspecific differences and high intraspecific variation are often poorly resolved by morphological methods alone (Lucas *et al*. 2002; Knaden *et al*. 2005; Ross & Shoemaker 2005; Steiner *et al*. 2005; Schlick-Steiner *et al*. 2006a, b). Moreover, cryptic species, which are morphologically hardly distinguishable (Bickford *et al*. 2007), represent another

major problem to correct species classification and a large number of them have been recently discovered in ants (Lucas *et al*. 2002; Ross & Shoemaker 2005; Schlick-Steiner *et al*. 2006a, b; Seifert 2009).
Thanks to technical advances, new tools for species delimitation exist today and can be helpful in taxonomical studies. For example, DNA-based methods, like DNA-barcoding, have been proposed to help in taxonomic investigations and biodiversity surveys (e.g. Hebert *et al*. 2004; Lambert *et al*. 2005; Ward *et al*. 2005). Even if DNA-barcoding received considerable critiques (e.g. Wheeler 2004; Will & Rubinoff 2004; Will *et al*. 2005), several studies showed the utility of molecular markers for species identification and also for discovering hidden biodiversity in apparently well-studied groups of organisms (e.g. Hebert *et al*. 2004; Schwartz *et al*. 2006; Bickford *et al*. 2007), with some good examples also among ants (Macaranas *et al*. 2001; Gyllenstrand *et al*. 2004; Knaden *et al*. 2005; Ross & Shoemaker 2005; Smith *et al*. 2005; Steiner *et al*. 2005; Pusch *et al*. 2006; Schlick-Steiner *et al*. 2006a, b). Molecular data are therefore increasingly employed to solve problems in taxonomy and species delimitation, but they are frequently discordant with the traditional taxa boundaries based on morphological data. To solve taxonomic problems considering such discrepancy, recent works suggested to employ an integrative taxonomy, which gathers data from different techniques for delimiting species boundaries (Dayrat 2005; Will *et al.* 2005; Valdecasas *et al*. 2008). Such an integrative approach has also been successfully used in some ant genera (Lucas *et al*. 2002; Schlick & Steiner *et al*. 2006a, b; Steiner *et al*. 2006). Besides the great number of studies based on molecular data, several works employed chemical compounds (such as cuticular hydrocarbons or gland contents) or behaviour for taxonomical revisions in ants and these techniques have proven their usefulness during last years (Bagnères *et al*. 1991; Rosengren & Cherix 1981; Rosengren *et al*. 1994; Maeder *et al*. 2005; Maeder 2006; Dahbi *et al*. 2008; Martin *et al*. 2008a, b).

Mound building red wood ants (species of the *Formica rufa* group) belong to one of the most studied groups of ants in Europe (see Cotti 1963, 1995, 1996; Cherix *et al*. 2006). Red wood ant species - so called because of their reddish and brown coloration and because of their preference for forested habitats - have fundamental roles and positive effects in forest ecosystems of the northern hemisphere: they reduce the density of pest species and other invertebrates of the forest floor thanks to their super-predator behaviour (Pavan 1959, 1981); they are major seed disperser and improve soil aeration processes, favouring plant colonization and growth; they modify their habitat by hunting many other invertebrates and by structuring ant communities (Savolainen & Vepsäläinen 1988; Savolainen *et al.* 1989); they cultivate and

protect honeydew-producing homopterans, which benefit to other species like honeybees (Wellenstein 1960); they are key component of the diet of other animals like the European brown bear (Grosse *et al*. 2003); their nests provide an excellent habitat for numerous other species (Laakso & Setala 1997, 1998); they take part to nutrient cycles, like phosphorus and carbon mineralization, by stimulating the transformation of soil organic matter (Domisch *et al*. 2008); they increase soil heterogeneity and are crucial to the functioning of forest ecosystems (Jurgensen *et al*. 2008). Therefore, red wood ants are considered among the most promising species in forest ecosystems monitoring (Gösswald 1990).

Because of their importance, these insects are protected by law in many European countries (Gösswald 1989), including Switzerland (Loi fédérale du 1er juillet 1966 sur la protection de la nature et du paysage, modifiée le 19 juin 2000). However, despite this protection, these ants are included on the red list of threatened species edited by the International Union for Conservation of Nature (IUCN) (Wells *et al*. 1983; Agosti 1994; Hilton-Taylor 2000) and on the red list of some particular countries like Switzerland (Agosti & Cherix 1994).

During the past decade, many researches have focussed on red wood ant taxonomy (e.g. Vepsäläinen & Pisarski 1981; Collingwood 1987; Seifert 1991; Goropashnaya *et al*. 2004). All these species have indeed a very similar morphology and a high intraspecific variability. In addition, they are sometimes able to hybridize (Seifert 1999; Seifert & Goropashnaya 2004) or to form mixed colonies (Seifert 1991; Czechowski 1996; Czechowski & Radchenko 2006). Consequently, the morphological identification of these ants can be quite complicated (Seifert 2007) and their taxonomy has been much debated (Vepsäläinen & Pisarski 1981; Collingwood 1987; Seifert 1991). The recent phylogenetic study conducted by Goropashnaya *et al*. (2004) suggested that at present time the group is formed by six species in Europe: *F. rufa* LINNAEUS, 1758, *F. polyctena* FÖRSTER, 1850, *F. lugubris* ZETTERSTEDT, 1838, *F. paralugubris* SEIFERT, 1996, *F. aquilonia* YARROW, 1955 and *F. pratensis* RETZIUS, 1783. However, the actual taxonomy of the group is often neglected mainly due to the lack of reliable and easy to use identification methods (e.g. Bonera 2002, but see Groppali and Bonera 2004; Boudjema *et al*. 2006). *Formica lugubris* and *F. paralugubris* are a good example of difficult taxonomy within the *F. rufa* group. In fact, until 1996 the two species were identified as a single species, named *F. lugubris*. Nevertheless, the discovery of two morphotypes among *F. lugubris* queens by Kutter (1967, 1977) and research on alarm pheromones (Cherix 1983), allozymes (Pamilo *et al*. 1992) and behaviour (Rosengren & Cherix 1981; Rosengren *et al*. 1994) indicated the existence of two different *F. lugubris* types in the Swiss Jura Mountains. This finally led to

the description of *F. paralugubris* as a sibling species of *F. lugubris* (Seifert 1996).

Considering the importance of correct species assessment and the need of reliable tools for species identification in conservation biology, with the present study we want to disentangle the taxonomical difficulties within the *Formica rufa* group and to clarify the diversity of this protected ants, by using an integrative approach. We first decided to analyze more in detail the distribution of *F. lugubris* and *F. paralugubris* in the Italian Alps by collecting new samples on the field and by examining one of the major red wood ant collections, which is deposited at the University of Pavia, Italy (chapter 1 = Bernasconi *et al*. 2006). The collection was initiated by Prof. M. Pavan and Prof. G. Ronchetti and consists of about 2860 samples that were collected from about 500 stations within the Italian Alps (Pavan 1959, 1981, Ronchetti & Groppali 1995). Afterwards, we developed a molecular tool based on mitochondrial DNA in order to provide an easy and reliable method for the identification of *F. lugubris* and *F. paralugubris* (chapter 2). Considering the promising results obtained for *F. lugubris* and *F. paralugubris*, we extended the use of molecular markers for species identification to the whole *F. rufa* group and made a microsatellite analysis (chapter 3). Molecular data highlighted the existence of a potential new cryptic species in the Swiss Alps. We therefore wanted to verify our hypothesis by conducting a behavioural study based on the capacity of workers to discriminate between pupa of their species and those of another species (chapter 4). Finally, in order to have further confirmation, we employed chemotaxonomy, by comparing sex pheromones - produced by the Dufour gland of virgin queens - of all red wood ant species (chapter 5).

This work has been mainly conducted within the Swiss National Park area. Created in 1914, the Swiss National Park (SNP) is a strict nature reserve (Category IA - IUCN) located in the east of Switzerland in Engadin Valley, Canton of Grisons. It is the largest natural reserve in Switzerland and, until now, its unique national park. It covers a surface of 172,4 km^2, from which 100 km^2 are forests and alpine and subalpine meadows. It is crossed by 80 km of trails, which are the only accessible places. The SNP and surrounding area is the most suitable place for studying red wood ants in Switzerland and probably in the Alps. *F. lugubris*, *F. aquilonia* and *F. paralugubris* are very abundant within the SNP while *F. rufa*, *F. polyctena* and *F. pratensis* live in the adjacent region. Thus, the *F. rufa* group in its whole is represented in the Swiss National Park area (Cherix *et al*. 2007). In addition, the Park offers the unique opportunity to study the evolution of red wood ant populations in unmanaged forests.

REFERENCES

Agnarsson I. & Kuntner M. 2007. Taxonomy in a changing world: seeking solutions for a science in crisis. *Systematic Biology*, 56, 531-539.

Agosti D. 1994. The conservation of social insects: a joint initiative of IUSSI and IUCN. In: Lenoir A., Arnold G. & Lepage M. (eds), *Les insectes sociaux. 12th congress of the International Union for the Study of Social Insects, Paris, Sorbonne, 21-27 August 1994*, 369. Université Paris Nord.

Agosti D. & Cherix D. 1994. Liste rouge des fourmis menacées de Suisse. Office fédéral de l'environnement, des forêts et du paysage (OFEFP) (eds). Berne, Switzerland.

Bagnères A-G., Morgan D. & Clement J-L. 1991. Species-specific secretions of the Dufour glands of three species of Formicine ants (Hymenoptera: Formicidae). *Biochemical Systematics and Ecology*, 19, 25-33.

Bernasconi C., Maeder A., Freitag A., & Cherix D. 2006 *Formica paralugubris* (Hymenoptera, Formicidae) in the Italian Alps from new data and old data revisited. *Myrmecological News*, 8, 251-256.

Bickford D., Lohman D.J., Sohdi N.S., Ng P.K.L., Meier R., Winker K., Ingram K. and Das I. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution*, 22, 148-155.

Bonera G. 2002. Analisi del comportamento intraspecifico in colonie differenti di *Formica lugubris* Zett. (Hymenoptera: Formicidae). Tesi di Laurea, Università di Pavia.

Boudjema G., Lempérière G., Deschamps-Cottin M. & Molland D.G. 2006. Analysis and nonlinear modeling of the mound-building ant *Formica lugubris* spatial multi-scale dynamic in a larch-tree stand of the southern French Alps. *Ecological Modelling*, 190, 147-158.

Cherix D. 1983. Intraspecific variations of alarm pheromones between two populations of the red wood ant *Formica lugubris* Zett. (Hymenoptera, Formicidae). *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, 56, 57-65.

Cherix D., Freitag A. & Maeder A. 2006. Les fourmis des bois du Parc jurassien vaudois. Parc jurassien vaudois et Musée cantonal de Zoologie, St-George, Lausanne.

Cherix D., Devenoges D., Freitag A., Bernasconi C. & Maeder A. 2007. Premier recensement des fourmis des bois (groupe *Formica rufa*) au Parc National Suisse. *Nationalpark-Forschung in der Schweiz*, 94, 69-79.

Collingwood C. A. 1987. Taxonomy and zoogeography of the *Formica rufa* L. species group. In: *Chemistry and Biology of Social Insects* (J. Eder and H. Rembold, Eds), München: Verlag J. Peperny. pp. 65-67.

Cotti G. 1963. Bibliografia ragionata 1930-1961 del gruppo *Formica rufa* in Italiano, Deutch, English. Ministero dell'agricoltura e delle foreste. Roma. 413pp.

Cotti G. 1995. Bibliografia ragionata 1962-1981 del gruppo *Formica rufa* in Italiano, Français, Deutsch. Istituto di Entomologia dell'Università di Pavia, Centro Interdisciplinare di Bioacustica e di Ricerche Ambientali. Roma, 520 pp.

Cotti G. 1996. A bibliography of the *Formica rufa* group (Hymenoptera, Formicidae). *Insect Social Life*, 1, 133-136.

Czechowski W. 1996. Colonies of hybrids and mixed colonies, interspecific nest takeover in wood ants (Hymenoptera, Formicidae). *Memorabilia Zoo*logica*,* 50, 1-116.

Czechowski W. & Radchenko A., 2006. Do permanently mixed colonies of wood ants (Hymenoptera: Formicidae) really exist? *Annales Zoologici*, 56, 667-673.

Dahbi A., Hefetz A. and Lenoir A. 2008. Chemotaxonomy of some *Cataglyphis* ants from Morocco and Burkina Faso. *Biochemical Systematics and Ecology*, 36, 564-572.

Dayrat B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85, 407-415.

Domisch T., Ohashi M., Finer L., Risch A.C., Sundstrom L., Kilpelainen J. & Niemela P. 2008. Decomposition of organic matter and nutrient mineralisation in wood ant (*Formica rufa* group) mounds in boreal coniferous forests of different age. *Biology and Fertility of Soils*, 44, 539-545.

Goropashnaya A.V., Fedorov V.B., Pamilo P. 2004. Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. *Molecular Phylogenetics and Evolution*, 32, 198-206.

Gösswald K. 1989. Die Waldameise. Band 1: Biologische Grundlagen, Ökologie und Verhalten. AULA-Verlag Wiesbaden.

Gösswald K. 1990. Die Waldameise. Band 2: Die Waldameise in Ökosystem Wald, ihr Nutzen und ihre Hege. AULA-Verlag Wiesbaden.

Groppali R. & Bonera G. 2004. Supercolony hypothesis and relationship among *Formica paralugubris* nests in the protected area "Giovetto delle Palline". *Insect Social Life*, 5, 63-68.

Grosse C., Kaczensky P. & Knauer F. 2003. Ants: a food source sought by Slovenian brown bears (*Ursus arctos*)? *Canadian Journal of Zoology*, 81, 1996-2005.

Gyllenstrand N., Seppä P., Pamilo P. 2004. Genetic differentiation in sympatric wood ants, *Formica rufa* and *F. polyctena*. *Insectes Sociaux*, 51, 139-145.

Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H. & Hallwachs H. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerators*.

Proceedings of the National Academy of Sciences of the United States of America, 101, 14812-14817.

Hilton-Taylor C. (compiler). 2000. IUCN red list of threatened species. IUCN, Gland, Switzerland and Cambridge, UK.

Hölldobler B. &.Wilson E.O. 1990. The Ants. Springer-Verlag. 732pp.

Jurgensen M.F., Finer L., Domisch T., Kilpelainen J., Punttila P., Ohashi M., Niemela P., Sundstrom L., Neuvonen S. & Risch A.C. 2008. Organic mound-building ants: their impact on soil properties in temperate and boreal forests. *Journal of Applied Entomology*, 132, 266-275.

Knaden M., Tinaut A., Cerda X., Wehner S., Wehner R. 2005. Phylogeny of three parapatric species of desert ants, *Cataglyphis bicolor, C. viatica* and *C. savigny*: a comparison of mitochondrial DNA, nuclear DNA and morphological data. *Zoology*, 108, 169-177.

Kutter H. 1967. Variationsstatische Erhebungen an Weibchen von *Formica lugubris* Zett. *Bull Soc Entomol Suisse*, 40, 63-77.

Kutter H. 1977. Formicidae-Hymenoptera. Insecta Helvetica. Entomologisches Institut der ETH.

Laakso J. & Setala H. 1997. Nest mounds of red wood ants (*Formica aquilonia*): hot spots for litter-dwelling earthworms. *Oecologia*, 111, 565-569.

Laakso J. & Setala H. 1998. Composition and trophic structure of detrital food web in ant nest mounds of *Formica aquilonia* and in the surrounding forest soil. *Oikos*, 81, 266-278.

Lambert D.M., Baker A., Huynen L., Haddrath O., Hebert P.D.N. & Millar C.D. 2005. Is a large-scale DNA-based inventory of ancient life possible? *Journal of Heredity*, 96, 279-284.

Lucas C., Fresneau D., Kolmer K., Heinze J., Delabie J.H.C. & Pho D.B. 2002. A multidisciplinary approach to discriminating different taxa in the species complex *Pachycondyla villosa* (Formicidae). *Biological Journal of the Linnean Society*, 75, 249-259.

Macaranas J.M., Colgan D.J., Major R.E., Cassis G., Gray M.R. 2001. Species discrimination and population differentiation in ants using microsatellites. *Biochemical Systematics and Ecology*, 29, 125-136.

Maeder A. 2006. Contribution to the conservation biology of the wood ant *Formica lugubris* (Hymenoptera, Formicidae) in Switzerland. PhD Thesis, University of Lausanne.

Maeder A., Freitag A. & Cherix D. 2005. Species- and nestmate brood discrimination in the sibling wood ant species *Formica paralugubris* and *Formica lugubris*. *Annales Zoologici Fennici*, 42, 201-212.

Martin S.J., Helanterä H. and Drijfhout F.P. 2008. Colony-specific hydrocarbons identify nest mates in two species of *Formica* ants. *Journal of Chemical Ecology*, 34, 1072-1080.

Martin S.J., Helanterä H. and Drijfhout F.P. 2008. Evolution of species-specific cuticular hydrocarbon patterns in *Formica* ants. *Biological Journal of the Linnean Society*, 95, 131-140.

Pamilo P., Chautems D., Cherix D. 1992. Genetic differentiation of disjunct populations of the ants *Formica aquilonia* and *Formica lugubris* in Europe. *Insectes Sociaux*, 39, 15-29.

Passera L. & Aron S. 2005. Les fourmis: comportement, organisation sociale et évolution. Publication du programme de monographie du CNRC (Conseil National de recherches du Canada).

Pavan M. 1959. Attività italiana per la lotta biologica con formiche del gruppo *Formica rufa* contro gli insetti dannosi alle foreste. Ministero dell'agricoltura e delle foreste, Roma. Collana verde 4, 1-70.

Pavan M. 1981. Utilizzazione delle formiche del gruppo *Formica rufa* nella difesa biologica delle foreste. In : Studi sulle formiche utili alle foreste, Ministero dell'agricoltura e delle foreste, Roma. Collana verde 59.

Punttila P. 1996. Succession, forest fragmentation, and the distribution of wood ants. *Oikos*, 75, 291-298.

Pusch K., Seifert B., Foitzik S. and Heinze J. 2006. Distribution and genetic divergence of two paraptric sibling ant species in Central Europe. *Biological Journal of the Linnean Society*, 88, 223-234.

Ronchetti G. and Groppali R. 1995. Quarantacinque anni di protezione forestale con *Formica lugubris* Zett. (Hym. Formicidae). L'esperienza di monte d'Alpe (Appennino ligure in provincia di Pavia). MURST, Istituto di entomologia dell'università di Pavia.

Rosengren R., Cherix D. 1981. The pupa carrying test as a taxonomic tool in the *Formica rufa* group. In: Biosystematics of Social Insects. Systematics Association Special Volume No.19 (P.E Howse and J.L. Clément, Eds), London: Academic Press. pp 263-281.

Rosengren R., Chautems D., Cherix D., Fortelius., Keller L. 1994. Separation of two sibling species of *Formica* L. ants by a behavioural choice test based on brood discrimination. *Memorabilia Zoologica*, 48, 237-249.

Ross K.G. & Shoemaker D. 2005. Species delimitation in native South American fire ants. *Molecular Ecology*, 14, 3419-3438.

Savolainen R. & Vepsäläien K. 1988. A competition hierarchy among boreal ants: impact on resource partitioning and community structure. *Oikos*, 51, 135-155.

Savolainen R., Vepsäläien K. & Wuorenrinne H. 1989. Ant assemblages in the taiga biome: testing the role of trerritorial wood ants. *Oecologia*, 81, 481-486.

Schlick-Steiner B.C., Steiner F.M. Konrad H., Marko B., Csösz S., Heller G., Ferencz B., Sipos B., Christian E., Stauffer C. 2006a. More than one species of *Messor* harvester ants (Hymenoptera: Formicidae) in Central Europe. *European Journal of Entomology*, 103, 469-476.

Schlick-Steiner B.C., Steiner F.M., Moder K., Seifert B., Sanetra M., Dyreson E., Stauffer C., Christian E. 2006b. A multidisciplinary approach reveal cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). *Molecular Phylogenetics and Evolution*, 40, 259-273.

Schwartz M., Luikart G. and Waples R.S. 2006. Genetic monitoring as a promising tool for conservation and management. *Trends in Ecology and Evolution*, 22, 25-33.

Seifert B. 1991. The phenotypes of the *Formica rufa* complex in East Germany. *Abhandlungen und Berichte des Naturkundemuseum Görlitz,* 65, 1-27.

Seifert B. 1996. *Formica paralugubris* nov. spec. – a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). *Reichenbachia Museum Tierkunden*. Dresden 31, 193-201.

Seifert B. 1999. Interspecific hybridisations in natural populations of ants by example of a regional fauna (Hymenoptera, Formicidae). *Insectes Sociaux*, 46, 45-52.

Seifert B. 2002. How to distinguish most similar insect species – improving the stereomicroscopic and the mathematical evaluation of external characters by example of ants. *Journal of Applied Entomology*, 126, 1-9.

Seifert B. 2007. Die Ameisen Mittel- und Nordeuropas. Lutra Verlags- und Vertriebsgesellschaft, Görlitz.

Seifert B. 2009. Cryptic species in ants (Hymenoptera: Formicidae) revisited: we need a change in the alpha-taxonomic approach. *Myrmecological News* 12: 149-166.

Seifert B. & Goropashnaya A. 2004. Ideal phenotypes and mismatching haplotypes – errors of mtDNA treeing in ants (Hymenoptera, Formicidae) detected by standardized morphometry. *Organism, Diversity & Evolution*, 4, 295-305.

Smith A., Fisher B.L. and Hebert P.D.N. 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society of London B*, 360, 1825-1834.

Steiner F.M., Schlick-Steiner B.C., Sanetra M., Ljubomirov T., Antonova V., Christian E., Stauffer C. 2005. Towards DNA-aided biogeography: An example from *Tetramorium* ants (Hymenoptera, Formicidae). *Annales Zoologici Fennici*, 42, 23-35.

Steiner F.M., Schlick-Steiner B.C., Konrad H., Moder K., Christian E., Seifert B., Crozier R.H., Stauffer C. & Buschinger A. 2006. No sympatric speciation here: multiple data sources show that the ant *Myrmica microrubra* is not a separate species but an alternate reproductive morph of *Myrmica rubra*. *Journal of Evolutionary Biology*, 19, 777-787.

Underwood E.C. & Fisher B.L. 2006. The role of ants in conservation monitoring: If, when and how. *Biological Conservation*, 132, 166-182.

Valdecasas A.G., Williams D. and Wheeler Q.D. 2008. 'Integrative taxonomy' then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society*, 93, 211-216.

Vander Meer R.K., Jaffe K. & Cedeno A. 1990. Applied myrmecology: a world perspective, edited by Robert Vander Meer, Klaus Jaffe and Aragua Cedeno. Westview Press Boulder, San Francisco & Oxford.

Vespäläinen K., Pisarski B. 1981. The taxonomy of the *Formica rufa* group: chaos before order. In: Biosystematics of Social Insects. Systematics Association Special Volume No.19 (P.E Howse and J.L. Clément, Eds), London: Academic Press. pp 27-36.

Ward R.D., Zemlak T.S., Innes B.H., Last P.R. & Hebert P.D.N. 2005. DNA barcoding Australia's fish species. *Philosophical Transactions of The Royal Society London B*, 360, 1847-1857.

Wellenstein G. 1960. Ergebnisse vierjähriger Untersuchungen über di Steigerung der Waldbienentracht. *Z. Angew. Entomol.*, 47, 32-41.

Wells S.M., Pyle R.M. & Collins N.M. 1983. Wood ants of Europe, *The IUCN invertebrate red data book*, 499-502. IUCN, Gland, Switzerland.

Wheeler Q.D. 2004. Taxonomic triage and the poverty of phylogeny. *Philosophical Transactions of the Royal Society of London B*, 359, 571-583.

Will K.W. & Rubinoff D. 2004. Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistic*, 20, 47-55.

Will K.W., Mishler B.D, Wheeler QD. 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, 54, 844-851.

Wilson E.O. 2002. The encyclopedia of life. *Trends in Ecology and Evolution*, 18, 77-80.

Wilson E.O. 2004. Taxonomy as a fundamental discipline. *Philosophical transactions of the Royal Society of London B*, 359, 739.

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Formica paralugubris (Hymenoptera: Formicidae) in the Italian Alps from new data and old data revisited

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Abstract

We provide evidence that *Formica paralugubris* SEIFERT, 1996, a species of wood ant recently described from Switzerland, is present in the Italian Alps. Until 1996, this species was confounded with *F. lugubris* ZETTERSTEDT, 1838. We examine the wood ant collection deposited at the University of Pavia (Italy) and collect new samples within the Italian Alps. *Formica paralugubris* seems to be more abundant than *F. lugubris*. Moreover, both species are found in sympatry in some localities.

Key words: *Formica rufa* group, *Formica paralugubris*, *Formica lugubris*, sympatry, entomological collections.

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Introduction

Red wood ants (*Formica rufa* LINNAEUS, 1761 group) have been one of the most studied groups of ants in Europe during the last century (COTTI 1963, 1995, 1996). Under the direction of Prof. Mario Pavan (1918 - 2003), several studies examining the biological control, distribution and ecology of red wood ants were conducted in Italy (PAVAN 1959, 1981, RONCHETTI & GROPPALI 1995).

Wood ant species are morphologically very similar and consequently difficult to distinguish. Moreover, they are able to hybridize (SEIFERT 1991, CZECHOWSKI 1996, SEIFERT & GOROPASHNAYA 2004). As a result, the taxonomy of the *F. rufa* group has always been difficult and controversial (VEPSÄLÄINEN & PISARSKI 1981, COLLINGWOOD 1987, SEIFERT 1991). At the present time, this group is considered to number six species in Europe (SEIFERT 1996a, 1996b, GOROPASHNAYA & al. 2004): *F. rufa*, *F. polyctena* FÖRSTER, 1850, *F. lugubris* ZETTERSTEDT, 1838, *F. paralugubris* SEIFERT, 1996, *F. aquilonia* YARROW, 1955, and *F. pratensis* RETZIUS, 1783.

Since the discovery of a super-colony of *F. lugubris* (now identified as *F. paralugubris*) in the Swiss Jura (GRIS & CHERIX 1977) we have investigated several aspects of wood ant biology and ecology. In particular, we have focused on the reproductive strategies of the two species *F. lugubris* and *F. paralugubris* (see CHERIX & al. 2004 for a review, MAEDER 2006).

Formica paralugubris has been described recently as a sibling species of *F. lugubris* on the basis of morphological criteria (SEIFERT 1996b). A high level of experience is necessary for species identification and the method is time consuming even for specialists. Before 1996 the two species were considered as a single one under the name of *F. lugubris*, referred to as *F. lugubris* sensu lato in this paper. For that reason, the distribution of each species remains unclear. *Formica lugubris* sensu lato was considered as a boreo-alpine species ranging from 600 m up to 2200 m (GÖSSWALD & al. 1965, GÖSSWALD 1989) and widely distributed in Europe (PAVAN 1981, RONCHETTI 1981). Since its description, *F. paralugubris* has been found in the Pyrenees (A. MAEDER unpubl.), in Austria (SEIFERT 1996a, GLASER 2000, 2001, 2005, STEINER & al. 2002), in the Swiss Alps (MAEDER & CHERIX 2001, NEUMEYER & SEIFERT 2005), the French Alps (Isère, Hautes-Alpes, A. Maeder unpubl.), the Italian Alps (Vinschgaus / Val Venosta, GLASER 2003), and in the Swiss and French Jura Mountains (SEIFERT 1996a, MAEDER & CHERIX 2001, NEUMEYER & SEIFERT 2005). Unfortunately, data on its distribution in the Southern Alps and in other European regions are rare (CHERIX & al. 2004, C. Bernasconi unpubl.). However, *F. lugubris* sensu lato has been reported almost everywhere in the Italian Alps (RONCHETTI & GROPPALI 1995). Our objectives are first to confirm the presence of *F. paralugubris* in the Italian Alps and, second, to make an initial survey of its distribution on a wide range. Therefore we decided to investigate the collection of red wood ants made by Prof. M. Pavan and colleagues that are archived at the University of Pavia (Italy) and to re-sample some areas of the Italian Alps.

Methods

In March 2003, we examined the red wood ant collection (University of Pavia, Italy) that was initiated in 1955 under the supervision of Prof. M. Pavan and Prof. G. Ronchetti. This collection consists of about 2860 wood ant samples (mounted specimens) that were collected from about 500 stations within the Italian Alps (PAVAN 1959). We selected and reanalyzed 36 samples previously identified as *F. lugubris* coming from 14 different stations throughout the Italian Alps. In order to have rapid and relatively reliable species identification, only samples with queens were se-

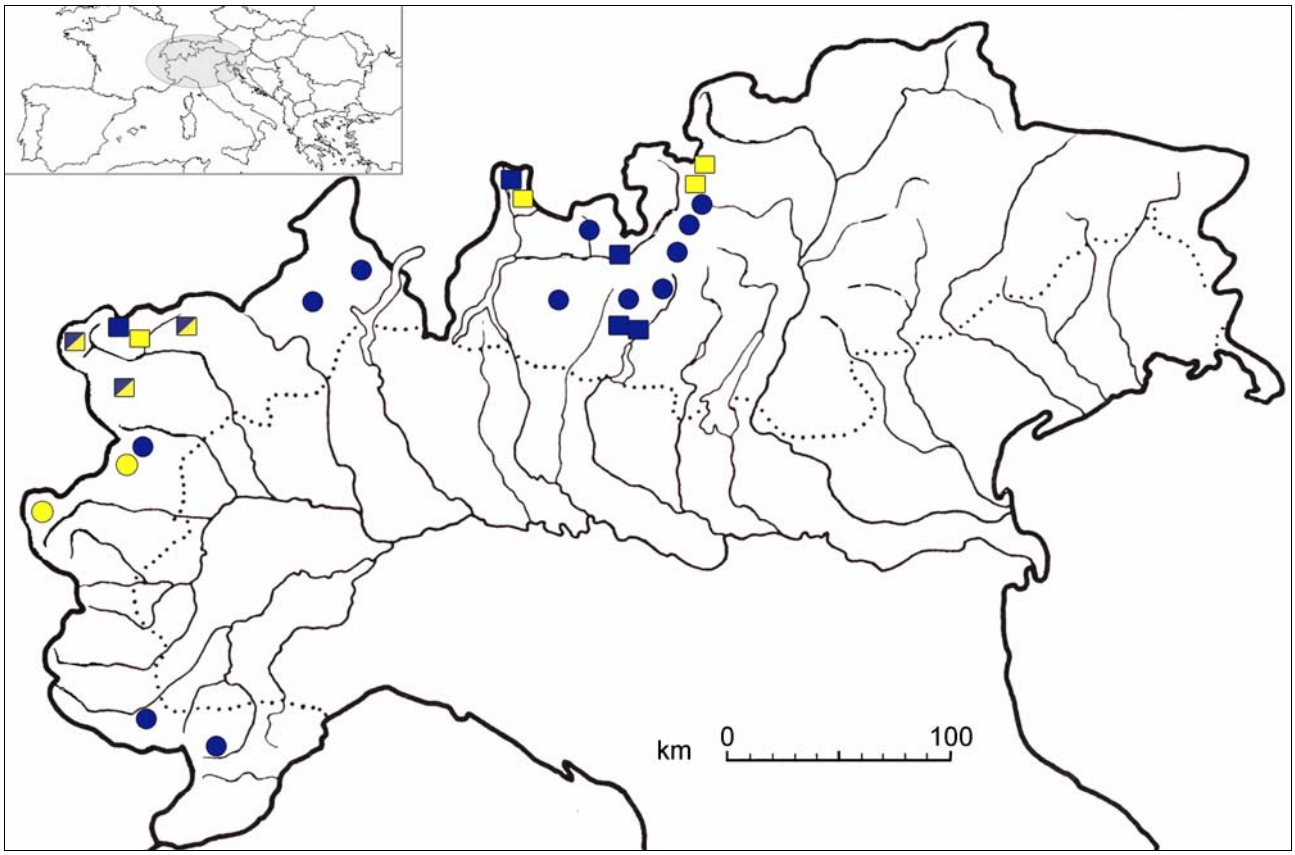

Fig. 1: Location of *Formica paralugubris* (blue) and *F. lugubris* (yellow) in the Italian Alps. Circles: samples collected in the 1950's. Squares: new field samples collected in 2005. Bicolor squares: stations in which the two species were found in sympatry. Dotted line: southern limit of the Italian Alps.

lected. Species identification was carried out according to SEIFERT (1996b) by measuring morphological traits in queens and also by comparing queens with reference material coming from the Swiss Jura and Swiss Alps. This reference material (workers and queens) was identified based on the morphological traits of workers (SEIFERT 1996b, B. Seifert pers. com.). We encountered some ambiguous queen specimens that were thus discarded from our study, only very clear specimens were considered. Some of these problematic specimens are stored at the museum of zoology in Lausanne and are available for future careful analysis.

In addition, in 2005 we collected 50 new samples from 12 different stations within the northwestern Italian Alps. The sampling regions were selected on the basis of previous work (PAVAN & al. 1971). Only areas where wood ants were previously confirmed were selected. Within each selected region we collected workers from nests along the pathways in order to sample along an altitudinal transect from about 1200 m to the upper limit of the forest. From each nest we collected about 20 workers. Ten ants were prepared for collection and ten were stored in ethanol 95 % for future genetic analysis. The new samples are stored at the Museum of Zoology (Lausanne, Switzerland) as voucher specimens.

Results

According to species re-identification we found that 33 / 36 (92 %) samples of *F. lugubris* sensu lato (Pavia's collection) belong to *F. paralugubris*, while only 3 / 36 (8 %) were identified as *F. lugubris* (Tab. 1, Fig. 1). Within the new samples 34 / 50 (68 %) have been recognized as *F. paralugubris* and 16 / 50 (32 %) as *F. lugubris* (Tab. 2, Fig. 1). The two species were found in sympatry in three stations from the new field samples, which were all located in the Aosta Valley (Fig. 1).

Discussion

Our results show that the wood ant *F. paralugubris* is present and widely distributed in the Italian Alps. Both deposited samples and fieldwork seem to indicate that *F. paralugubris* is more abundant than *F. lugubris* in term of occupied localities. However, this apparent dominance is relative because of potential biases in the sampling methods and differences in the social structure of the two species. *Formica paralugubris* forms obligately large colonies of numerous interconnected nests (polydomy) containing a huge number of laying queens (polygyny) (CHERIX 1980). On the other hand, *F. lugubris* is socially polymorphic with both monogynous and polygynous colonies (discovered at present only in the Swiss Alps, BERNASCONI & al. 2005). Re-analyzed samples of the Pavia collection were selected according to the presence of queens. These queens were collected at the nest surface during the sunny period in spring for 44 % of the samples. Consequently, as it was probably very difficult to find the *F. lugubris* queen from the monogynous nests during the sunny period, and thus, there was probably a bias toward *F. paralugubris* samples. Visited localities during the fieldwork were not select-

Tab. 1: Samples of *Formica lugubris* sensu lato collected by Professors M. Pavan and G. Ronchetti (PAVAN 1959) and deposited at the University of Pavia (Italy) that have been re-identified as *F. lugubris* or *F. paralugubris*. Province, station, locality, altitude of the station and sampling date are noted. Geographic coordinates were not available.

Province	Station	Locality	Date	Altitude (m)	Species
Cuneo	Ormea	Navette	9.IV.1955	1400	*F. paralugubris*
Cuneo	Ormea	Navette	9.IV.1955	1600	*F. paralugubris*
Cuneo	Valdieri	Casermetta	29.VII.1956	1600	*F. paralugubris*
Novara	Ceppo Morelli	Pizzo Camino	7.VI.1955	1110	*F. paralugubris*
Novara	Ceppo Morelli	Pizzo Camino	7.VI.1955	1160	*F. paralugubris*
Novara	Ceppo Morelli	Pizzo Camino	7.VI.1955	1260	*F. paralugubris*
Novara	Malesco	Capretto	23.IV.1955	1100	*F. paralugubris*
Novara	Malesco	Faedo	23.IV.1955	1300	*F. paralugubris*
Novara	Malesco	Orsera	11.IV.1955	1300 / 1600	*F. paralugubris*
Novara	Malesco	Riolata	11.IV.1955	1300 / 1600	*F. paralugubris*
Torino	Ala di Stura	Regione Rio Chiesa	11.IV.1955	1700	*F. lugubris*
Torino	Chialamberto	Pessé-Comba Creus	11.IV.1955	1600	*F. paralugubris*
Torino	Chialamberto	Pessé-Comba Creus	21. VI.1956	1650	*F. paralugubris*
Torino	Chialamberto	Leisan-Inv. Leisan	26. VI.1956	1500	*F. paralugubris*
Torino	Bardonecchia	Bacini	29.V.1957	1900	*F. lugubris*
Torino	Bardonecchia	Prà Reimond	28.V.1957	1800	*F. lugubris*
Bergamo	Vilminore	Clusorina	26.V.1955	1400	*F. paralugubris*
Bergamo	Vilminore	Paghera di Polzone	9.V.1956	1200	*F. paralugubris*
Bergamo	Vilminore	Giovetto	11.VI.1954	1300 / 1400	*F. paralugubris*
Bergamo	Vilminore	Giovetto	4.VI.1958	1200 / 1450	*F. paralugubris*
Bergamo	Piazza Brembana	Zucco Stremareggia	22.V.1953	1400	*F. paralugubris*
Bergamo	Piazza Brembana	Paris	21.V.1953	1600	*F. paralugubris*
Bergamo	Piazza Brembana	Foppabona	25.VI.1955	1400	*F. paralugubris*
Bergamo	Piazza Brembana	Foppabona	25.VI.1955	1400	*F. paralugubris*
Bergamo	Piazza Brembana	Foppabona	25.VI.1955	1400	*F. paralugubris*
Brescia	Cedegolo	Fontana Suta	19. VII.1956	1600	*F. paralugubris*
Brescia	Edolo	Paghera Lezza	04.VI.1955	1650	*F. paralugubris*
Brescia	Edolo	Paghera Lezza	04.VI.1955	1600	*F. paralugubris*
Brescia	Ponte di legno	Gasso	03.V.1954	1300	*F. paralugubris*
Brescia	Ponte di legno	Gasso	03.V.1954	1500	*F. paralugubris*
Brescia	Ponte di legno	Gasso	03.V.1954	1400	*F. paralugubris*
Brescia	Vezza d'Oglio	Fondo val Paghera	11.VI.1959	1300	*F. paralugubris*
Brescia	Vezza d'Oglio	Fondo val Paghera	12.VI.1959	1301	*F. paralugubris*
Brescia	Vezza d'Oglio	Fondo val Paghera	13.VI.1959	1302	*F. paralugubris*
Sondrio	Valmolenco	Gaspoggio	28.V.1953	1450	*F. paralugubris*
Sondrio	Valmolenco	Gaspoggio	28.V.1953	1450	*F. paralugubris*

Tab. 2: Field samples collected in 2005 and deposited at the Museum of Zoology (Lausanne, Switzerland). Province, station, locality, date, geographic coordinates and altitude of the station are given.

Province	Station	Locality	Date	Latitude	Longitude	Altitude	Species
Brescia	Borno	Giovetto	2.V.2005	N 45°57'28"	E 10°07'19"	1294 m	*F. paralugubris*
Brescia	Borno	Giovetto	2.V.2005	N 45°57'30"	E 10°07'26"	1332 m	*F. paralugubris*
Brescia	Borno	Giovetto	2.V.2005	N 45°57'28"	E 10°07'28"	1354 m	*F. paralugubris*
Brescia	Borno	Giovetto	2.V.2005	N 45°57'28"	E 10°07'30"	1405 m	*F. paralugubris*
Brescia	Borno	Giovetto	2.V.2005	N 45°57'28"	E 10°07'31"	1360 m	*F. paralugubris*
Brescia	Borno	Giovetto	2.V.2005	N 45°57'28"	E 10°07'32"	1374 m	*F. paralugubris*
Brescia	Borno	Giovetto	2.V.2005	N 45°57'28"	E 10°07'46"	1433 m	*F. paralugubris*
Brescia	Borno	Giovetto	2.V.2005	N 45°57'29"	E 10°07'46"	1432 m	*F. paralugubris*
Bergamo	Azzone	Giovetto-Giuadel	3.V.2005	N 45°57'36"	E 10°07'10"	1181 m	*F. paralugubris*
Bergamo	Azzone	Giovetto-Giuadel	3.V.2005	–	–	–	*F. paralugubris*
Bergamo	Azzone	Giovetto-Giuadel	3.V.2005	–	–	–	*F. paralugubris*
Bergamo	Azzone	Giovetto-Giuadel	3.V.2005	–	–	–	*F. paralugubris*
Bergamo	Azzone	Giovetto-Giuadel	3.V.2005	N 45°57'51"	E 10°07'17"	1183 m	*F. paralugubris*
Sondrio	Campodolcino	Gualdera	3.V.2005	N 46°23'29"	E 09°21'44"	1430 m	*F. lugubris*
Sondrio	Madesimo	Pian del Lanzo	4.V.2005	N 46°25'13"	E 09°20'52"	1577 m	*F. paralugubris*
Sondrio	Madesimo	Pian del Lanzo	4.V.2005	N 46°25'09"	E 09°20'56"	1574 m	*F. paralugubris*
Sondrio	Madesimo	Pian del Lanzo	4.V.2005	N 46°25'09"	E 09°20'56"	1622 m	*F. paralugubris*
Sondrio	Madesimo	Pian del Lanzo	4.V.2005	N 46°25'03"	E 09°20'56"	1565 m	*F. paralugubris*
Sondrio	Madesimo	Pian del Lanzo	4.V.2005	N 46°25'05"	E 09°20'56"	1567 m	*F. paralugubris*
Sondrio	Aprica	Magnolta	1.VIII.2005	–	–	–	*F. paralugubris*
Sondrio	Aprica	Magnolta	1.VIII.2005	N 46°08'39"	E 10°08'24"	1396 m	*F. paralugubris*
Sondrio	Aprica	Magnolta	1.VIII.2005	N 46°08'20"	E 10°08'05"	1561 m	*F. paralugubris*
Sondrio	Aprica	Magnolta	1.VIII.2005	N 46°08'23"	E 10°08'23"	1643 m	*F. paralugubris*
Sondrio	Aprica	Magnolta	1.VIII.2005	N 46°08'07"	E 10°08'21"	1804 m	*F. paralugubris*
Sondrio	Aprica	Magnolta	1.VIII.2005	N 46°08'11"	E 10°08'36"	1716 m	*F. paralugubris*
Sondrio	St.Caterina	Passo di Gavia	2.VIII.2005	N 46°23'57"	E 10°29'43"	2123 m	*F. lugubris*
Sondrio	St.Caterina	Passo di Gavia	2.VIII.2005	N 46°24'01"	E 10°29'54"	2056 m	*F. lugubris*
Sondrio	St.Caterina	Passo di Gavia	2.VIII.2005	N 46°27'16"	E 10°29'55"	1531 m	*F. lugubris*
Aosta	Etroubles	Pozon	27.VI.2005	N 45°48'36"	E 07°13'40"	1567 m	*F. lugubris*
Aosta	Etroubles	Pozon	27.VI.2005	N 45°48'36"	E 07°13'39"	1584 m	*F. lugubris*
Aosta	Etroubles	Pozon	27.VI.2005	N 45°48'35"	E 07°13'36"	1616 m	*F. lugubris*
Aosta	Courmayeur	Val Vény	27.VI.2005	N 45°48'03"	E 06°55'27"	1516 m	*F. lugubris*
Aosta	Courmayeur	Visailles	28.VI.2005	N 45°46'58"	E 06°53'43"	1730 m	*F. paralugubris*
Aosta	Courmayeur	Visailles	28.VI.2005	N 45°46'47"	E 06°53'22"	1831 m	*F. paralugubris*
Aosta	Courmayeur	Visailles	28.VI.2005	N 45°46'39"	E 06°53'01"	2008 m	*F. paralugubris*
Aosta	Courmayeur	Visailles	28.VI.2005	N 45°46'41"	E 06°53'01"	2007 m	*F. paralugubris*
Aosta	Courmayeur	Visailles	28.VI.2005	N 45°46'43"	E 06°53'10"	2026 m	*F. paralugubris*
Aosta	Courmayeur	Visailles	28.VI.2005	N 45°47'28"	E 06°54'35"	1561 m	*F. lugubris*
Aosta	Valsavarenche	Crottes	28.VI.2005	N 45°35'35"	E 07°11'56"	1816 m	*F. lugubris*
Aosta	Valsavarenche	Crottes	28.VI.2005	N 45°35'34"	E 07°11'55"	1817 m	*F. lugubris*
Aosta	Valsavarenche	Crottes	28.VI.2005	N 45°35'29"	E 07°11'53"	1879 m	*F. paralugubris*
Aosta	Valsavarenche	Le Pont	29.VI.2005	N 45°33'25"	E 07°12'41"	1740 m	*F. lugubris*
Aosta	Valtournenche	La Magdeleine	29.VI.2005	N 45°47'53"	E 07°36'40"	1540 m	*F. paralugubris*
Aosta	Valtournenche	La Magdeleine	29.VI.2005	N 45°49'10"	E 07°36'27"	1864 m	*F. paralugubris*
Aosta	Valtournenche	La Magdeleine	29.VI.2005	N 45°49'21"	E 07°36'45"	1980 m	*F. paralugubris*
Aosta	Valtournenche	La Magdeleine	29.VI.2005	N 45°49'20"	E 07°36'49"	1981 m	*F. lugubris*
Aosta	Valtournenche	La Magdeleine	29.VI.2005	N 45°49'21"	E 07°37'01"	1984 m	*F. lugubris*
Aosta	Valtournenche	La Magdeleine	29.VI.2005	N 45°48'07"	E 07°36'48"	1575 m	*F. lugubris*
Aosta	Bourg-St.Rhémy	–	29.VI.2005	N 45°50'35"	E 07°10'33"	1806 m	*F. paralugubris*
Bolzano	Stelvio	–	3.VIII.2005	N 46°36'30"	E 10°32'33"	1632 m	*F. lugubris*

ed following a randomized and stratified sampling protocol which may also introduce a potential species-specific bias.

In addition, this study reveals that in Italy both species also live in local sympatry, in accordance with previous observations made in Switzerland (MAEDER & CHERIX 2001, CHERIX & al. 2004).

Considering our results, we strongly recommend caution in further studies on wood ants. For example, it is surprising that a very recent work (BOUDJEMA & al. 2006) completely ignored current wood ant taxonomy and related literature. Fortunately, since our visit to Pavia, some studied wood ant colonies located in the Giovetto natural reserve (see Tab. 2) were appropriately reattributed to *F. paralugubris* (GROPPALI & BONERA 2004).

Besides morphological identification, sometimes difficult even for specialists, it is possible to ensure species identification by using complementary tools. For instance, we demonstrated that the two species can be discriminated by their cuticular hydrocarbons profiles (Chemotaxonomy, MAEDER 2006) and by their worker behaviour (Pupa carrying test, MAEDER & al. 2005). Moreover, a genetic tool is in development (C. Bernasconi unpubl.).

This study is a first survey of the distribution of the two wood ant species *F. lugubris* and *F. paralugubris* confounded as *F. lugubris* sensu lato before 1996. With respect to conservation biology, their respective distributions are obviously more fragmented than what was previously thought. Correct species identification should ensure appropriate conservation measures and is paramount in any scientific study.

Finally, our work once more demonstrates the importance of voucher specimens and collections deposited in museums of natural history or other institutions (FRANCOEUR 1976, ALBERCH 1993, SCHLICK-STEINER & al. 2003). Thanks to the huge work carried out by Professors M. Pavan, G. Ronchetti and colleagues (PAVAN 1959) we had the opportunity to report that *F. paralugubris* was already sampled in Italy about 50 years ago.

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Zusammenfassung

Wir berichten über Vorkommen der Waldameise *Formica paralugubris* SEIFERT, 1996 in den italienischen Alpen. Die Art wurde erst in jüngerer Zeit aus der Schweiz beschrieben. Bis 1996 wurde sie als *F. lugubris* ZETTERSTEDT, 1838 aufgefasst. Wir untersuchten die Waldameisensammlungen, die an der Universität Pavia (Italien) deponiert sind und machten neue Aufsammlungen in den italienischen Alpen. *Formica paralugubris* ist offenbar häufiger als *F. lugubris*. Beide Arten kommen an einigen Stellen syntop vor.

References

ALBERCH, P. 1993: Museums, collections and biodiversity inventories. – Trends in Ecology and Evolution 8: 372-375.

BERNASCONI, C., MAEDER, A., CHERIX, D. & PAMILO, P. 2005: Diversity and genetic structure of the wood ant *Formica lugubris* in unmanaged forests. – Annales Zoologici Fennici 42: 189-199.

BOUDJEMA, G., LEMPERIERE, G., DESCHAMPS-COTTIN, M. & MOLLAND, D.G. 2006: Analysis and nonlinear modeling of the mound-building ant *Formica lugubris* spatial multi-scale dynamic in a larch-tree stand of the southern French Alps. – Ecological Modelling 190: 147-158.

CHERIX, D. 1980: A preliminary note about structure, phenology and diet of a super-colony of *Formica lugubris* ZETT. – Insectes Sociaux 27: 226-236.

CHERIX, D., MAEDER, A., BERNASCONI, C., CASTELLA, G. & FREITAG, A. 2004: Red wood ants: new species and new questions! – Insect Social Life 5: 47-54.

COLLINGWOOD, C.A. 1987: Taxonomy and zoogeography of the *Formica rufa* L. species group. In: EDER, J. & REMBOLD, H. (Eds.): Chemistry and biology of social insects. – Verlag J. Peperny, München, pp. 65-67.

COTTI, G. 1963: Bibliografia ragionata 1930-1961 del gruppo *Formica rufa* in Italiano, Deutsch, English. – Ministero dell' agricoltura e delle foreste, Roma, 413 pp.

COTTI, G. 1995: Bibliografia ragionata 1962-1981 del gruppo *Formica rufa* in Italiano, Français, Deutsch. – Istituto di Entomologia dell'Università di Pavia, Centro Interdisciplinare di Bioacustica e di Ricerche Ambientali, Roma, 520 pp.

COTTI, G. 1996: A bibliography of the *Formica rufa* group (Hymenoptera, Formicidae). – Insect Social Life 1: 133-136.

CZECHOWSKI, W. 1996: Colonies of hybrids and mixed colonies, interspecific nest takeover in wood ants (Hymenoptera, Formicidae). – Memorabilia Zoologica 50: 1-116.

FRANCOEUR, A. 1976: The need for voucher specimens in behavioural and ecological studies. – Bulletin of Entomological Society of Canada 8: 23.

GLASER, F. 2000: Checkliste der Ameisen (Hymenoptera, Formicidae) Vorarlbergs – eine Zwischenbilanz. – Vorarlberger Naturschau Dornbirn 8: 97-111.

GLASER, F. 2001: Die Ameisenfauna Nordtirols – eine vorläufige Checkliste (Hymenoptera: Formicidae). – Berichte des Naturwissenschaftlich-Medizinischen Vereins Innsbruck 88: 237-280.

GLASER, F. 2003: Die Ameisenfauna (Hymenoptera, Formicidae) des Vinschgaus (Südtirol, Italien) – eine vorläufige Artenliste. – Gredleriana 3: 209-230.

GLASER, F. 2005: Rote Liste gefährdeter Ameisen Vorarlbergs. – Rote Listen 3, Vorarlberger Naturschau, Dornbirn, 127 pp.

GOROPASHNAYA, A., FEDOROV, V.B. & PAMILO, P. 2004: Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. – Molecular Phylogenetics and Evolution 32: 198-206.

GÖSSWALD, K. 1989: Die Waldameise. Band 1: Biologische Grundlagen, Ökologie und Verhalten. – AULA-Verlag, Wiesbaden, 660 pp.

GÖSSWALD, K., KNEITZ, G. & SCHIRMER, G. 1965: Die geographische Verbreitung der hügelbauenden *Formica*-Arten (Hymenoptera, Formicidae) in Europa. – Zoologische Jahrbücher, Abteilung für Systematik, Ökologie, Geographie und Biologie der Tiere 92: 369-404.

GRIS, G. & CHERIX, D. 1977: Les grandes colonies de fourmis des bois du Jura (groupe *Formica rufa*). – Mitteilungen der Schweizerischen Entomologischen Gesellschaft 50: 249-250.

GROPPALI, R. & BONERA, G. 2004: Supercolony hypothesis and relationship among *Formica paralugubris* nests in the protected area "Giovetto delle Palline". – Insect Social Life 5: 63-68.

MAEDER, A. 2006: Contribution to the conservation biology of the wood ant *Formica lugubris* (Hymenoptera, Formicidae) in Switzerland. – Unpublished PhD Thesis, University of Lausanne.

MAEDER, A. & CHERIX, D. 2001: Problèmes posés par *Formica paralugubris*, une nouvelle espèce de fourmis des bois. – Actes des Colloques Insectes Sociaux 14: 21-25.

MAEDER, A., FREITAG, A. & CHERIX, D. 2005: Species and nestmate brood discrimination in the sibling wood ant species *Formica paralugubris* and *Formica lugubris*. – Annales Zoologici Fennici 42: 201-212.

NEUMEYER, R. & SEIFERT, B. 2005: Kommentierte Liste der frei lebenden Ameisen (Hymenoptera: Formicidae) in der Schweiz. – Mitteilungen der Schweizerischen Entomologischen Gesellschaft 78: 1-17.

PAVAN, M. 1959: Attività italiana per la lotta biologica con formiche del gruppo *Formica rufa* contro gli insetti dannosi alle foreste. – Ministero dell'agricoltura e delle foreste, Roma, Collana verde 4, 78 pp.

PAVAN, M. 1981: Utilizzazione delle formiche del gruppo *Formica rufa* nella difesa biologica delle foreste. In: Studi sulle formiche utili alle foreste. – Ministero dell'agricoltura e delle foreste, Roma. Collana verde 59, 338 pp.

PAVAN, M., RONCHETTI, G. & VENDEGNA, V. 1971: Corologia del gruppo *Formica rufa* in Italia (Hymenoptera Formicidae). – Ministero dell'agricoltura e delle foreste, Roma, Collana verde 30, 93 pp.

RONCHETTI, G. 1981: Distribuzione delle formiche del gruppo *Formica rufa* in Europa. In: Studi sulle formiche utili alle foreste. – Ministero dell'agricoltura e delle foreste, Roma, Collana verde 59, pp. 227-236.

RONCHETTI, G. & GROPPALI, R. 1995: Quarantacinque anni di protezione forestale con *Formica lugubris* ZETT. (Hym. Formicidae). L'esperienza di monte d'Alpe (Appennino ligure in provincia di Pavia). – Ministero dell'Università e della ricerca scientifica e tecnologica, Roma, 256 pp.

SCHLICK-STEINER, B.C., STEINER, F.M. & SCHÖDL, S. 2003: A case study to quantify the value of voucher specimens for invertebrate conservation: Ant records in Lower Austria. – Biodiversity and Conservation 12: 2321-2328.

SEIFERT, B. 1991: The phenotypes of the *Formica rufa* complex in East Germany. – Abhandlungen und Berichte des Naturkundemuseum Görlitz 65: 1-27.

SEIFERT, B. 1996a: Ameisen beobachten, bestimmen. – Naturbuch Verlag, Augsburg, 351 pp.

SEIFERT, B. 1996b: *Formica paralugubris* nov. spec. – a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). – Reichenbachia Museum Tierkunden, Dresden 31: 193-201.

SEIFERT, B & GOROPASHNAYA, A. 2004: Ideal phenotypes and mismatching haplotypes - errors of mtDNA treeing in ants (Hymenoptera: Formicidae) detected by standardized morphometry. – Organisms, Diversity & Evolution 4: 295-305.

STEINER, F.M., SCHÖDL, S. & SCHLICK-STEINER, B.C. 2002: Liste der Ameisen Österreichs (Hymenoptera: Formicidae), Stand Oktober 2002. – Beiträge zur Entomofaunistik 3: 17-25.

VESPÄLÄINEN, K. & PISARSKI, B. 1981: The taxonomy of the *Formica rufa* group: chaos before order. In: HOWSE, P.E. & CLÉMENT, J.L. (Eds.): Biosystematics of social insects. – Systematics Association Special Volume No.19, Academic Press, London, pp. 27-36.

CHAPTER 2

Molecular markers allow sibling species identification in red wood ants (*Formica rufa* group).

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ABSTRACT

Protected in many European countries, red wood ants (*Formica rufa* group) are a group of species, which are considered to be among the most promising bioindicators in forest ecosystems. Nevertheless, because of their morphological similarity and intraspecific variability, morphological species identification can be very tough. For example a high level of experience is necessary for discriminating between the sibling species *F. lugubris* and *F. paralugubris*, two species that often live in sympatry in the same Alpine forests. New taxonomic tools providing rapid and reliable species identification are therefore needed.

In this study we present a simple and reliable molecular technique based on mtDNA (COI gene) and restriction enzyme for discriminating between *F. lugubris* and *F. paralugubris*. We also confirm the validity of this method with a Bayesian analysis based on microsatellites. This new molecular tool represents a clear breakthrough in discriminating *F. lugubris* and *F. paralugubris* and will be really helpful in large-scale biomonitoring.

KEYWORDS: Cryptic species, red wood ants, mt-DNA, microsatellites, species identification

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INTRODUCTION

The *Formica rufa* group (red wood ants) has been one of the most studied groups of ants in Europe during the last century and many researches have been devoted to their biology and ecology (see Cotti, 1963, 1995, 1996; Gösswald, 1989, 1990). Because of their beneficial impact on forest ecosystems, these ants are protected by law in many European countries. Furthermore, all red wood ant species have a very similar morphology and, in some cases, are able to hybridize (Seifert & Goropashnaya, 2004) or to form mixed colonies (Seifert, 1991; Czechowski, 1996). As a consequence, the taxonomy of the group has been much debated (Vepsäläinen & Pisarski, 1981; Collingwood, 1987; Seifert, 1991). However, a recent phylogenetic study suggested that the group consists of six species in Europe (Goropashnaya *et al*., 2004) which are *Formica rufa* Linnaeus, 1761, *F. polyctena* Förster, 1850, *F. lugubris* Zetterstedt, 1838, *F. paralugubris* Seifert, 1996, *F. aquilonia* Yarrow, 1955 and *F. pratensis* Retzius, 1783.

Formica lugubris and *F. paralugubris* are a good example of difficult taxonomy within the *F. rufa* group. Until 1996 the two species were pooled together as a single species, named *F. lugubris*. Nevertheless, studies on alarm pheromones (Cherix, 1983), allozymes (Pamilo *et al*., 1992) and behaviour (Rosengren & Cherix, 1981; Rosengren *et al*., 1994) have shown the existence of two different *F. lugubris* types in the Swiss Jura Mountains. *Formica paralugubris* was finally described in 1996 as a sibling species of *F. lugubris* on the basis of morphological criteria (Seifert, 1996a). Other approaches based on cuticular hydrocarbons and behaviour (Maeder, 2006) as well as recent research based on microsatellites and sexual pheromones (Bernasconi *et al*., a,b, in prep.) have confirmed the earlier genetic results (Pamilo *et al*., 1992; Goropashnaya *et al*., 2004): the two species are clearly separated and should be treated as two distinct taxa. However, their morphological identification remains difficult.

Formica lugubris and *F. paralugubris* often live in sympatry in the Alps (Bernasconi *et al*., 2006) and, since its formal description, *F. paralugubris* has been found in Austria (Seifert, 1996a; Glaser, 2000, 2001, 2005; Steiner *et al*., 2002), in the Swiss Alps (Maeder & Cherix, 2001; Neumeyer & Seifert, 2005), the French Alps (Isère, Hautes-Alpes, Maeder unpubl.), the Italian Alps (Vinschgaus / Val Venosta, Glaser 2003; Bernasconi *et al*., 2006), and in the Swiss and French Jura Mountains (Seifert, 1996a; Maeder & Cherix, 2001; Neumeyer & Seifert, 2005). Nevertheless, even after its description and confirmation of its presence in many Alpine regions, the existence of *F. paralugubris* is still often neglected due to the lack of reliable identification

means (e.g. Bonera, 2002, but see Groppali and Bonera, 2004; Boudjema *et al*., 2006). New taxonomic tools are thus needed to fill this gap.
To date, the best way to discriminate between these two sibling species is to use morphological criteria. However, the method is rather complex, necessitating lots of experience and time (Seifert, 1996a, b); the procedure is based on the comparison of the external morphology of queens and workers. The best results are obtained with multiple discriminant functions based on nest samples using characters such as body part measures, numbers of setae and hair length. Statistical analyses give satisfactory results for queens of the two species but a lower discrimination for the workers (i.e. samples from northern Europe and British isles should be taken with care). In addition this method is not applicable to males. Consequently, since correct species identification is fundamental for studies in conservation and evolutionary biology, the aim of the present work is to provide a simple and reliable molecular tool based on mitochondrial DNA to distinguish *F. lugubris* from *F. paralugubris*.

METHODS

Sampling

Individuals of the two species were collected between 2005 and 2007. In total 244 *F. lugubris* nests were sampled. Most of them were sampled within the Swiss, Italian and Slovenian Alps. A couple of nests were also sampled in Ireland, Scotland, Denmark, Finland, Bulgaria and Pyrenees. According to the *F. paralugubris* distribution, 230 nest of this species were sampled within the Swiss, Italian and Slovenian Alps trying to select the same locations in which *F. lugubris* was also present. For both species, 30 workers per nest were collected on nest surface and stored in absolute ethanol until DNA extraction. For each nest, at least 10 ants were deposited at the Museum of Zoology of Lausanne as voucher specimens.

Morphological identification

Species identification was first carried out on the basis of the morphological criteria described by Seifert (1996a, b, 2007): traits were measured in workers (Seifert, 1996b, 2007; B. Seifert pers. com.) and, when possible, compared with traits measured in queens from the reference material already deposited at the Museum of Zoology of Lausanne (Switzerland).

Mt-DNA identification

One worker per nest (Table 1; $N_{lugubris}$ = 244, $N_{paralugubris}$ = 230) was used for genetic analysis. Genomic DNA, taking the whole individual, was isolated using QIAamp DNA Mini Kit (Qiagen). Ten individuals of each species had been first sequenced at the mitochondrial COI fragment to identify potential restriction sites. The restriction enzyme BamHI was selected as its restriction site 5'…GGATCC…3' is present in the COI sequences of *F. lugubris* (haplotype I) but not in *F. paralugbris* (haplotype II). All individuals were thus analyzed by enzyme digestion to check whether haplotypes I and II are species-specific.
All individuals were amplified at the mitochondrial COI region by polymerase chain reaction (PCR). All PCR were carried out in 25 µl solutions comprising: 2.5 µl PCR Qiagen Buffer (containing 45 pmol $MgCl_2$), 1.5 µl dNTPs (2.5 mM each), 0.2 µl of $MgCl_2$ (Qiagen), 0.3 µl Taq Polymerase (Qiagen), 17.5 µl distilled-deionized water and 1 µl of each forward primer (5'- ttg att ttt tgg tca tcc aga agt -3'), reverse primer (5'- tag gtg aat ttg aat ttt gta atg -3') and template DNA. The PCR cycles were as follows: 94°C for 1 min followed by 35 cycles at 92°C for 1 min, 45°C for 1 min and 72°C for 3 min; with a final 10 min extension period at 72°C. PCR products were digested by BamHI restriction enzyme following manufacturer instructions (Promega Corporation) and at 37°C for 2h. After digestion, products were analyzed by electrophoresis on a 1.5% agarose gel. Fragment sizes were compared to a 100bp ladder (Promega Corporation).

Microsatellites amplifications

In order to validate the mtDNA identifications and to check whether hybridization events could influence the results obtained with the restriction enzyme approach, we selected 20 *F. lugubris* nests and 21 *F. paralugubris* nests from our sampling (Table 1). These nests occurred in sympatry in four different alpine forests of the Southern Swiss Alps (Canton of Tessin) and allowed us to perform a local scale comparison.
For this second analysis, eight workers from each nest (Table 1; $N_{lugubris}$ = 160, $N_{paralugubris}$ = 168) were genotyped using nine microsatellite loci: FL12, FL20, FL21, FL29 (Chapuisat, 1996), and FE13, FE19, FE37, FE38, FE51 (Gyllenstrand *et al*., 2002). PCR conditions were mainly as described by Chapuisat (1996) and Gyllenstrand *et al*. (2002), with slight modifications of the amplification conditions following optimisation by Mäki-Petäys *et al.* (2005). The 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 and genotyping

was carried out using the computer program GeneMapper (Applied Biosystems).

Microsatellites analyses

All microsatellite genotypes were then assigned to a group using a Bayesian admixture procedure implemented in STRUCTURE 2.2 (Pritchard *et al*., 2000; http://pritch.bsd.uchicago.edu). This model has been designed to identify the number (K) of genetic clusters present within the individuals. At the same time, it evaluates the relative probability of each individual to belong to one or more clusters (i.e. if they are genetically admixed as a result of hybridization). In our case, we expect individuals to pool within two distinct groups, one for each species.
STRUCTURE was run using the admixture model, and 10 repetitions of 100000 iterations following by a burn-in period of 20000 iterations. Other parameters were set to default values. To decide for the most probable number of K genetic clusters, posterior probability values for K ('Log probability of data'; L(K)) were estimated assigning a prior from 1 to 5. Using the posterior probability as described by Pritchard *et al*. (2000) it was not clear which number of clusters K best fits our data set. Therefore, we calculated the ΔK statistic, proposed by Evanno *et al*. (2005). We chose the value of K=2 which showed the highest ΔK and then evaluated the individual membership coefficient (q_{ind}) to the two inferred clusters. Individuals with a proportion of membership to each cluster $q_{ind} < 0.90$ (admixed individual) were assigned to more than one cluster whereas individuals with $q_{ind} > 0.90$ were assigned to only 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 *et al*., 2002; Cegelski *et al*., 2003; Basset *et al.*, 2006). Then we assessed the average membership coefficient (q_{group}) of each morphologically defined species to each cluster. Similarly, each species was assigned to one cluster if its q_{group} was > 0.90, or jointly more to one cluster if its q_{group} to each cluster was < 0.90.

RESULTS

Mt-DNA identification

A 950bp COI fragment was amplified for one worker from each of 244 *F. lugubris* nests and 230 *F. paralugubris* nests (Table 1). After digestion with BamHI, two bands were present in the digested COI sequences (haplotype I) of 234 *F. lugubris*, while no digestion (haplotype II) occurred in 222 *F. paralugubris*

samples (Table 1). Morphology and mtDNA data were therefore concordant in 96% of the *F. lugubris* workers and in 96.5% of the *F. paralugubris* workers.

Microsatellite identification

We used Bayesian analyses implemented in STRUCTURE to detect admixture between the two species within our data set ($N_{lugubris}$ = 160; $N_{paralugubris}$ = 168) on the basis of microsatellite genotypes. Using the method of Evanno *et al*. (2005), it was clear that our samples included two distinct groups. The average proportions of membership (q_{group}) of the two sampled species indicated that these two groups corresponded to the two species: all *F. lugubris* workers, except one, grouped in cluster I ($q_{group\text{-}lugubris}$ = 0.99), and all *F. paralugubris* workers, except one, grouped in cluster II ($q_{group\text{-}paralugubris}$ = 0.99) (Table 1). Only two individuals (0.6%) showed signs of admixture with $q_{ind} < 0.90$: one *F. lugubris* had a q_{ind}=0.86 and one *F. paralugubris* had a q_{ind}=0.82. However, their q_{ind} are high indicating that most of their genome is assigned to the correct putative species.
Afterwards (not shown in Table 1), all these individuals were also analysed with the mtDNA approach, and the results are concordant with microsatellites indicating that all *F. lugubris* workers share the haplotype I, while all *F. paralugubris* workers share the haplotype II.

DISCUSSION

Our data show that the method based on mtDNA and restriction enzyme is a powerful technique for species identification. Mitochondrial DNA data was concordant with morphology in more than 96% of the samples. Furthermore, microsatellite data were always concordant with mtDNA results, indicating that this latter method is highly reliable and that discordances between morphology and mtDNA are likely explained by errors in morphological identification. Measuring morphological traits in wood ants, especially when comparing *F. lugubris* and *F. paralugubris*, can indeed lead to subjective mistakes because of wrong measurements or intraspecific variation (B. Seifert, pers. com.).
Discordance between morphology and mtDNA could also be explained by past hybridization events between these two species. Nevertheless, according to microsatellites, hybridization is very rare within the four alpine forest populations that were genotyped, although nests of *F. lugubris* and *F. paralugubris* are found in close vicinity. In addition, similar results were also found in other sympatric *F. lugubris* and *F. paralugubris* populations within the Swiss National Park (Eastern Swiss Alps) (Bernasconi *et al*., a, in prep), in the Swiss Jura

Mountains and the Italian Alps (C. Bernasconi, unpublished data). Since hybridization does not seem to influence our results, the COI gene appears as a very reliable character for discriminating between these two wood ant species.

During the past decade, species identification based on the COI region (DNA barcoding) came up as a new technique to provide rapid and accurate species identification (Hebert *et al*., 2003; Hebert & Gregory, 2005). Even if this method has been severely criticized (e.g. Wheeler, 2004; Will & Rubinoff, 2004; Will *et al*., 2005), the utility of DNA barcodes for species identification has been successfully demonstrated in a number of taxonomic groups (Hebert *et al*., 2004; Lambert *et al*., 2005; Ward *et al*., 2005; Ellis *et al*., 2006) and in other ant genera such as *Tetramorium* (Steiner *et al*., 2005; Schlick-Steiner *et al*., 2006), *Temnothorax* (Pusch *et al*., 2006) and *Solenopsis* (Ross & Shoemaker, 2005). The mtDNA identification method is consistent and faster than morphology. When 5 to 10 workers per nest are necessary for morphological species identification, only one is enough when using mtDNA, assuming that no mixed nests exist. Thus, depending on the availability of laboratory facilities, this genetic method allows analysing a large number of ants in a short time. However, even if this technique saves time, it is not intended to completely replace morphology or to identify all wood ant species. For example, preliminary results indicate that it is not possible to discriminate between *F. pratensis* and *F. lugubris*, nor *F. aquilonia* and *F. paralugubris* by only looking at the restriction site used here (C. Bernasconi, unpublished data). Morphological investigation or mtDNA sequencing remains necessary in these cases and the restriction fragment analysis should be applied only for separating samples of *F. lugubris* and *F. paralugubris*. Moreover, to avoid incomplete digestion or enzyme inhibition leading to a misidentification, we advise to include known control individuals of both species at each PCR and digestion.

In addition, our results indicate that microsatellites markers analysed in a Bayesian framework are also an efficient tool for species identification, as each individual were correctly assigned to its putative species. Similarly, Gyllenstrand *et al*. (2004) showed by using microsatellites that the species *F. rufa* and *F. polyctena*, which frequently hybridize at least in some areas (Seifert, 1991; Czechowski, 1996), formed locally two distinct gene pools. The utility of microsatellites has been demonstrated in other species (Macaranas *et al*., 2001; Schiffer *et al*., 2004; Basset *et al*., 2006) and further studies on red wood ants indicate that this molecular technique could be successfully applied on the whole *F. rufa* group (Bernasconi *et al*., a, in prep).

In conclusion, the present study provides two reliable molecular techniques to identify *F. lugubris* and *F. paralugubris* and also confirms that these two species do represent two sibling species. Therefore, these ants should be treated as

separate units and the proposed methods represent a helpful breakthrough in demonstrating that and in showing how to identify the samples reliably.
The fast and reliable restriction enzyme-based method presented in this study, as well as the microsatellite approach will be of great interest to researchers working on the conservation biology of the *F. rufa* group species, in particular in alpine forests in which *F. lugubris* and *F. paralugubris* live in sympatry. Wood ants are protected by law in many European countries and considered to be among the most reliable bioindicators in forest ecosystems (Gösswald, 1990). The mtDNA identification tool is very useful as it helps to perform rapid and consistent species identification in large-scale biomonitoring. Microsatellites are also helpful for species identification and, compared to mtDNA, can give additional information on hybridization events. However, microsatellites are more expensive and laborious than the restriction fragment method. Moreover they should be preferentially used in local scale studies to avoid geographical influences on Bayesian analyses.

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REFERENCES

Basset P., Yannic G. & Hausser J. 2006. Genetic and karyotypic structure in the shrews of the *Sorex araneus* group: are they independent? *Molecular Ecology*, 15, 1577-1587.

Bernasconi C., Maeder A., Freitag A., & Cherix D. 2006. *Formica paralugubris* (Hymenoptera, Formicidae) in the Italian Alps from new data and old data revisited. *Myrmecological News*, 8, 251-256.

Bonera G. 2002. Analisi del comportamento intraspecifico in colonie differenti di *Formica lugubris* Zett. (Hymenoptera: Formicidae). Tesi di Laurea, Università di Pavia.

Boudjema G., Lempérière G., Deschamps-Cottin M. & Molland D.G. 2006. Analysis and nonlinear modelling of the mound-building ant *Formica lugubris* spatial multi-scale dynamic in a larch-tree stand of the southern French Alps. *Ecological Modelling*, 190, 147-158.

Cegelski C.C., Waits L.P., Anderson N.J. 2003. Assessing population structure and gene flow in Montana wolverines (*Gulo gulo*) using assignement-based approaches. *Molecular Ecology*, 12, 2907-2918.

Chapuisat M. 1996. Characterization of microsatellite loci in *Formica lugubris B* and their variability in other ant species. *Molecular Ecology,* 5, 599-601.

Cherix D. 1983. Intraspecific variations of alarm pheromones between two populations of the red wood ant *Formica lugubris* Zett. (Hymenoptera, Formicidae). *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, 56, 57-65.

Collingwood C. A. 1987. Taxonomy and zoogeography of the *Formica rufa* L. species group. *Chemistry and biology of social insects* (ed. by J. Eder and H. Rembold). Verlag J. Peperny, München, 65-67.

Cotti G. 1963. Bibliografia ragionata 1930-1961 del gruppo *Formica rufa* in Italiano, Deutsch, English. Ministero dell'agricoltura e delle foreste, Roma.

Cotti G. 1995. Bibliografia ragionata 1962-1981 del gruppo *Formica rufa* in Italiano, Français, Deutsch. Istituto di Entomologia dell'Università di Pavia, Centro Interdisciplinare di Bioacustica e di Ricerche Ambientali, Roma.

Cotti G. 1996. A bibliography of the *Formica rufa* group (Hymenoptera, Formicidae). *Insect Social Life*, 1, 133-136.

Czechowski W. 1996. Colonies of hybrids and mixed colonies, interspecific nest takeover in wood ants (Hymenoptera, Formicidae). *Memorabilia Zoologica,* 50, 1-116.

Ellis J.S., Knight M.E., Carvell C. & Goulson D. 2006. Cryptic species identification: a simple diagnostic tool for discriminating between two problematic bumblebee species. *Molecular Ecology Notes*, 6, 540-542.

Evanno G., Regnaut S., Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, 14, 2611-2620.

Glaser F. 2000. Checkliste der Ameisen (Hymenoptera, Formicidae) Voralbergs - eine Zwischenbilanz. *Voralberger Naturschau* 8*, Dornbirn*, 97 - 111.

Glaser F. 2001. Die Ameisenfauna Nordtirols – eine vorläufige Checkliste (Hymenoptera: Formicidae). *Berichte des Naturwissenschaftlich-Medizinischer Verein, Innsbruck*, 88, 237-280.

Glaser F. 2003. Die Ameisenfauna (Hymenoptera, Formicidae) des Vinschgaus (Südtirol, Italien) - eine vorläufige Artenliste. *Gredleriana*, 3, 209 - 230.

Glaser F. 2005. *Rote Liste gefährdeter Ameisen Voralbergs*. Rote Listen 3, Voralberger Naturschau, Dornbirn.

Goropashnaya A., Fedorov V.B. & Pamilo P. 2004. Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. *Molecular Phylogenetics and Evolution*, 32, 198-206.

Gösswald K. 1989. Die Waldameise. Band 1: Biologische Grundlagen, Ökologie und Verhalten. AULA-Verlag, Wiesbaden.

Gösswald K. 1990. Die Waldameise. Band 2: Die Waldameise in Ökosystem Wald, ihr Nutzen und ihre Hege. AULA-Verlag, Wiesbaden.

Groppali R. & Bonera G. 2004. Supercolony hypothesis and relationship among *Formica paralugubris* nests in the protected area “Giovetto delle Palline”. *Insect Social Life*, 5, 63-68.

Gyllenstrand N., Gertsch P. J. & Pamilo P. 2002. Polymorphic microsatellites DNA markers in the ant *Formica exsecta*. *Molecular Ecology Notes*, 2, 67-69.

Gyllenstrand N., Seppä P., Pamilo P. 2004. Genetic differentiation in sympatric wood ants, *Formica rufa* and *F. polyctena*. *Insectes Sociaux*, 51 : 139-145.

Hebert P.D.N, Cywinska A., Ball S.L. & deWaard J.R. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B*, 270, 313-322.

Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H. & Hallwachs H. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerators*. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 14812-14817.

Hebert P.D.N. & Gregory T.R. 2005. The promise of DNA barcoding for taxonomy. *Systematic Biology*, 54, 852-859.

Lambert D.M., Baker A., Huynen L., Haddrath O., Hebert P.D.N. & Millar C.D. 2005. Is a large-scale DNA-based inventory of ancient life possible? *Journal of Heredity*, 96, 279-284.

Macaranas J.M., Colgan D.J., Major R.E., Cassis G., Gray M.R. 2001. Species discrimination and population differentiation in ants using microsatellites. *Biochemical Systematics and Ecology*, 29, 125-136.

Maeder A. 2006. Contribution to the conservation biology of the wood ant *Formica lugubris* (Hymenoptera, Formicidae) in Switzerland. PhD Thesis, University of Lausanne.

Maeder A. & Cherix D. 2001. Problèmes posés par *Formica paralugubris*, une nouvelle espèce de fourmis des bois. *Actes des Colloques Insectes Sociaux*, 14, 21-25.

Mäki-Petäys H., Zakharov A., Viljakainen L., Corander J. & Pamilo P. 2005. Genetic changes associated to declining populations of *Formica* ants in fragmented forest landscape. *Molecular Ecology*, 14, 733-742.

Manel S., Berthier P., Luikart G. 2002. Detecting wildlife poaching: identifying the origin of individuals with Bayesian assignment tests and multilocus genotypes. *Conservation Biology*, 16, 650-659.

Neumeyer R. & Seifert B. 2005. Kommentierte Liste der frei lebenden Ameisen (Hymenoptera: Formicidae) in der Schweiz. *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, 78, 1-17.

Pamilo P., Chautems D., Cherix D. 1992. Genetic differentiation of disjunct populations of the ants *Formica aquilonia* and *Formica lugubris* in Europe. *Insectes Sociaux*, 39, 15-29.

Pritchard J.K., Stephens M., Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics*, 155, 945-959.

Pusch K., Seifert B., Foitzik S., Heinze J. 2006. Distribution and genetic divergence of two parapatric sibling ant species in Central Europe. *Biological Journal of the Linnean Society*, 88, 223-234.

Rosengren R. & Cherix D. 1981. The pupa-carrying test as a taxonomic tool in the *Formica rufa* group. *Biosystematics of social insects*: *Systematics Association Special Volume No.19* (ed. by P.E. Howse and J.L. Clément). Academic Press, London, 263-281.

Rosengren R., Chautems D., Cherix D., Fortelius W., Keller L. 1994. Separation of two sympatric sibling species of *Formica* L. ants by a behavioural choice test based on brood discrimination. *Memorabilia Zoologica*, 48, 237-249.

Ross, K.G. & Shoemaker, D. 2005. Species delimitation in native South American fire ants. *Molecular Ecology*, 14, 3419-3438.

Schiffer M., Carew M.E., Hoffmann E.E. 2004. Molecular, morphological and behavioural data reveal the presence of a cryptic species in the widely studied *Drosophila serrata* species complex. *Journal of Evolutionary Biology*, 17, 430-442.

Schlick-Steiner B.C., Steiner F.M., Moder K., Seifert B., Sanetra M., Dyreson E., Stauffer C., Christian E. 2006. A multidisciplinary approach reveal cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). *Molecular Phylogenetics and Evolution*, 40, 259-273.

Seifert B. 1991. The phenotypes of the *Formica rufa* complex in East Germany. *Abhandlungen und Berichte des Naturkundemuseum Görlitz,* 65, 1-27.

Seifert B. 1996a. *Formica paralugubris* nov. spec. – a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). *Reichenbachia Museum Tierkunden*, *Dresden*, 31, 193-201.

Seifert B. 1996b. Ameisen beobachten, bestimmen. Naturbuch Verlag, Augsburg.

Seifert B. 2007. *Die Ameisen Mittel- und Nordeuropas*. Lutra - Verlags- und Vertriebsgesellschaft, Görlitz.

Seifert B. & Goropashnaya, A. 2004. Ideal phenotypes and mismatching haplotypes – errors of mtDNA treeing in ants (Hymenoptera: Formicidae) detected by standardized morphometry. *Organisms, Diversity & Evolution*, 4, 295-305.

Steiner F.M., Schödl S. & Schlick-Steiner B.C. 2002. Liste der Ameisen Österreichs (Hymenoptera: Formicidae). Stand Oktober 2002. *Beiträge zur Entomofaunistik*, 3, 17-25.

Steiner F.M., Schlick-Steiner B.C., Sanetra M., Ljubomirov T., Antonova V., Christian E., Stauffer C. 2005. Towards DNA-aided biogeography: An example from *Tetramorium* ants (Hymenoptera, Formicidae). *Annales Zoologici Fennici*, 42, 23-35.

Vepsäläinen K., Pisarski B. 1981. The taxonomy of the *Formica rufa* group: chaos before order. *Biosystematics of social insects*: *Systematics Association Special Volume No.19* (ed. by P.E. Howse and J.L. Clément). Academic Press, London, 27-36.

Ward R.D., Zemlak T.S., Innes B.H., Last P.R. & Hebert P.D.N. 2005. DNA barcoding Australia's fish species. *Proceedings of the Royal Society of London B*, 360, 1847-1857.

Wheeler Q.D. 2004. Taxonomic triage and the poverty of phylogeny. *Philosophical transactions of the Royal Society of London B*, 359, 571-583.

Will K.W. & Rubinoff D. 2004. Myth of the molecule: DNA barcodes for species cannot replace morphology for identification and classification. *Cladistics*, 20, 47-55.

Will K.W., Mishler B.D, Wheeler QD. 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, 54, 844-851.

Table 1. Genetic assignment of morphologically identified individuals. The number of sampled nests and the number of analyzed individuals of the two species *F. lugubris* and *F. paralugubris* are indicated for each molecular marker: A) mtDNA and B) microsatellites. For each analysis the number of individuals characterized by the haplotype I or II (mtDNA), or the average proportion of membership of each morphologically defined species (q_{group}) to group I or II (microsatellites) are indicated.

Molecular marker		*F. lugubris*	*F. paralugubris*
A) mtDNA (COI)	Number of nests	244	230
	Number of individuals	244	230
	Haplotype I	234	8
	Haplotype II	10	222
B) Microsatellites	Number of nests	20	21
	Number of individuals	160	168
	Group I ($q_{group\_lugubris}$)	0.99	0.01
	Group II ($q_{group\_paralugubris}$)	0.01	0.99

CHAPTER 3

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

Christian Bernasconi, Daniel Cherix & Pekka Pamilo

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 bio-indicators 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 identification requests lots of time and experience. We therefore employed 9 microsatelltites loci and mitochondrial DNA (COI gene) to verify the power of genetic markers for red wood ants species identification 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 within the Swiss National Park area. Genetic data indicated that these species represent different genetic pools. Moreover, results showed that *F. aquilonia* and *F. paralugubris* 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*, 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 mtDNA analyses, suggest the existence of a new cryptic species within the Eastern Swiss Alps. This putative cryptic species has been named *F. lugubris-X*. These results have a great importance for future conservation plans, monitoring and evolutionary studies on these protected ants.

Keywords: Microsatellites, *Formica rufa* group, cryptic species, species identification, biodiversity

INTRODUCTION

Ants are everywhere and have a major role in many terrestrial ecosystems (Holldöbler & 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, closely related ant species and populations within a species are often used to assess special questions in evolutionary biology like shift from single-queen colonies to multi-queen colonies (e.g. Zhu *et al*., 2003; Gyllenstrand *et al*. 2005) and from ordinary life style to a social parasite (e.g. Mori *et al*. 2001; Savolainen & Vepsäläinen 2003). In monitoring studies, as well as in ecology, evolutionary biology and conservation biology in general, correct species identification is a fundamental prerequisite (Sites & Marshall 2003; Mace 2004).

Cryptic species, which are morphologically hardly distinguishable (Bickford *et al*. 2007), represent a major problem to correct species classification and biodiversity studies. A large number of cryptic species have already been discovered in ants (Lucas *et al*. 2002; Ross & Shoemaker 2005; Schlick-Steiner *et al*. 2006a, 2006b; Seifert 2009) in which species identification is often based on morphologically variable worker ants. Despite considerable progress in the morphometrical analysis (e.g. Seifert 2002), ant species with small interspecific differences and high intraspecific variation are often poorly resolved by morphological methods alone (Lucas *et al*. 2002; Knaden *et al*. 2005; Ross & Shoemaker 2005; Steiner *et al*. 2005; Schlick-Steiner *et al*. 2006a, 2006b).

Molecular approaches have confirmed to be extremely useful in the delimitation of species and in monitoring the cryptic diversity in well-studied groups of organisms (e.g. Hebert *et al*. 2004; Lambert *et al*. 2005; Ward *et al*. 2005; Schwartz *et al*. 2006; Bickford *et al*. 2007). Some good examples also exist among ants, in which morphologically similar species may differ markedly in their mitochondrial DNA (Knaden *et al*. 2005; Ross & Shoemaker 2005; Smith *et al*. 2005; Steiner *et al*. 2005; Pusch *et al*. 2006; Schlick-Steiner *et al*. 2006a, 2006b; Steiner *et al*. 2006; Bernasconi *et al*. submitted) or microsatellites (Macaranas *et al*. 2001; Gyllenstrand *et al*. 2004; Bernasconi *et al*. submitted).

The European red wood ants (*Formica rufa* group) belong to one of the most studied groups of ants in Europe as regards 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 bio-indicators 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). Consequently, the morphological identification of these species can be quite complicated and the taxonomy of the group has been much debated (Vepsäläinen & Pisarski 1981; Collingwood 1987; Seifert 1991). The recent phylogenetic study conducted by Goropashnaya *et al*. (2004) suggested that at present time the group is formed by six species in Europe: *F. rufa* LINNAEUS, 1761, *F. polyctena* FÖRSTER, 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 identification methods (e.g. Bonera 2002, but see Groppali and Bonera 2004; Boudjema *et al*. 2006). The species pair *Formica lugubris* and *F. paralugubris* are a good example of difficult taxonomy and until 1996 they were identified as a single species (*F. lugubris*). However, alarm pheromones (Cherix 1983), allozymes (Pamilo *et al*. 1992) and behaviour (Rosengren & Cherix 1981; Rosengren *et al*. 1994) indicated the existence of two different *F. lugubris* types in the Swiss Jura Mountains. This finally led to the description of *F. paralugubris* as a sibling species of *F. lugubris* (Seifert 1996a). Our genetic studies (Bernasconi *et al*. submitted) demonstrate that the two species can be reliably distinguished from each other on the basis of mtDNA-based markers and nuclear microsatellites.

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 (Gyllenstrand *et 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 sympatry or at least close to each other. The alpine region is a suitable place for such a study. First, all red wood ant species are present in this area. Second, 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; Stehlik 2003; Schönswetter *et al*. 2005; Parisod & Besnard 2007; Haubrich & Schmitt 2007; Parisod 2008). Our main focus is in *F. lugubris* because this species has a high level of mtDNA haplotype diversity in Eurasia, whereas the species *F. aquilonia*, *F. rufa*, *F. polyctena* and *F. paralugubris* have almost no intraspecific mtDNA variation within Eurasia (Goropashnaya *et al*. 2004).

In this paper we present our results obtained by using microsatellites (i) to investigate on the cryptic diversity of this well studied group of ants and (ii) to

test the utility of genetic markers for the identification of these protected species.

METHODS

Sample collection

Sampling was conducted between 2005 and 2008 within the Swiss National Park (Canton of Grisons) and surrounding area (Figure 1). Created in 1914, this strict natural reserve offers the unique opportunity to study the evolution of wood ant populations in unmanaged forests. Moreover, all red wood ant species are present in this region (Devenoges 1999; Cherix *et al*. 2007; C. Bernasconi, unpublished data). 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. According to 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*, 3 to *F. polyctena*, 5 to *F. rufa* and 4 to *F. pratensis*) and a total of 683 worker individuals were analyzed for this study (Appendix 1).

Morphological identification

Species identification was assessed on the basis of morphological criteria according to Seifert (1996a, 1996b, 2007) by measuring morphological traits in workers (Seifert 1996a, 1996b, 2007; B. Seifert pers. com.) 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 microsatellites 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 *et al*. 2002). In total, 683 individuals were genotyped. PCR conditions were mainly as described by Chapuisat (1996) and Gyllenstrand *et al*. (2002) with slight modifications of the amplification conditions following optimisation by Mäki-Petäys *et 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 and genotyping was carried out 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 *et al*. 2000; http://pritch.bsd.uchicago.edu). This model was designed to identify the unknown number of K genetic clusters of origin 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 results of hybridization. STRUCTURE was run with the admixture model, and 10 repetitions of 100000 iterations following by 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 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 sympatry 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 have also been found in close vicinity in the study area. We analyzed the two groups independently by evaluating the number of K clusters, which best fits our datasets. We assumed that ants of the two groups belong to an unknown number of K genetically distinct clusters (K). Posterior probability values for K ('Log likelihood; ln L) were estimated assigning a prior from 1 to 10. Using this parameter as described by Pritchard *et al*. (2000) it was not clear which number of clusters K best fits our data set. Therefore, we calculated the ΔK statistic, proposed by Evanno *et 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 individual) 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 *et al*. 2002; Cegelski *et al*. 2003).

Factorial Correspondence Analysis

A Factorial Correspondence Analysis (FCA) of individual multilocus scores was used to describe patterns of differentiation, and it was computed using

GENETIX 4.02. Individuals were considered part of distinct groups, according to assignment analysis performed with STRUCTURE.

Analyses of genetic variation and population structure

The software package GENETIX 4.02 (Belkhir *et al*. 2001) was also used to calculate the allele frequencies, allele number, observed (Ho) and expected (He) heterozygosities for each species or genetic groups. Deviations from Hardy-Weinberg equilibrium and the genetic structure of the K populations defined with STRUCTURE was 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 jack-knifing over nests and confidence intervals were obtained by permutation tests over loci (5000 permutations) (Goudet 1995).

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 cluster of *F. polyctena*, *F. rufa* and *F. pratensis*, and four groups in the other cluster including *F. aquilonia*, *F. lugubris* and *F. paralugubris*. Next we assessed the average membership coefficient (q_{group}) of each species to each genetic group. Each species was assigned to one group if its 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 3 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 (Table 2, Figure 2).
As mentioned above, *F. aquilonia*, *F. paralugubris* and *F. lugubris*, were split into four different groups. All *F. aquilonia* and *F. paralugubris* workers, respectively, formed separate groups of their own. The individual and average membership coefficients indicated that admixture occurs between *F. paralugubris* and *F. aquilonia* in such a way that some samples identified morphologically as *F. paralugubris* showed genetic affinity with *F. aquilonia* (Table 3, Figure 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-X* throughout the rest of this paper. A few admixed individuals were observed between *F. lugubris* and *F. lugubris-X* (Table 3, Figure 3).
All the pairwise F_{ST} values between the seven genetic groups were significantly greater than zero (Table 4). The smallest F_{ST} value (0.101) was between *F. lugubris* and *F. lugubris-X*. 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 analyses of the individual genotypes (Figure 4a,b) 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 sympatry, the species form clearly distinct gene pools.
On the contrary, *F. aquilonia* and *F. paralugubris* are genetically close and the genotype distributions overlap quite a lot (Figure 4b), with overlapping genotypes corresponding to putatively hybrid individuals already detected by STRUCTURE. The distribution of the individual data points in the FCA analysis also showed that the genotypes of *F. lugubris-X* are located marginally and outside the group formed by *F. lugubris*, *F. aquilonia* and *F. paralugubris*. Furthermore, there was only little overlap between the distributions of the *F. lugubris-X* 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, the H_{obs} values being lower than expected and the average F_{IS} values being positive (from 0.083 to 0.246). This suggests deviations from the expected Hardy-Weinberg genotype frequencies (Table 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 *et al*. 1995). This should, however, not much affect the above cluster analyses.

F_{ST} values calculated between nests within each group are significantly different from zero and vary from 0.067 of *F. lugubris-X* to 0.240 of *F. lugubris* (Table 1).

Mitochondrial DNA investigations

As the microsatellite results suggested genetic separation between *F. lugubris* and *F. lugubris-X*, we checked whether they also show differences in the mtDNA haplotypes. For this we used the restriction method developed earlier to distinguish between *F. lugubris* and *F. paralugubris* (Bernasconi *et al*., submitted). It has indeed been shown that European *F. lugubris* share some specific mutations on the COI gene that are not present in other red wood ant species (Bernasconi *et al*., submitted). We thus analyzed 1 to 5 individuals from each *F. lugubris-X* nest. The results clearly indicate that *F. lugubris*-X samples have a different haplotype when compared to other *F. lugubris* samples collected in the study area and to those analyzed in our previous work (Bernasconi *et al*., submitted).
Moreover, further analyses indicate that *F. lugubris-X* haplotype also differ from *F. lugubris* samples when comparing the same mtDNA fragment used by Gorospashnaya *et 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 (Figure 5). Both results are in accordance with microsatellites and confirm the genetic distinction between *F. lugubris* and *F. lugubris-X*.

DISCUSSION

Number of genetic units

Microsatellites data indicate that the six red wood ant species represent seven genetic units within the Swiss National Park and 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 *et al*. (2004). In addition, individuals morphologically identified as *F. lugubris* surprisingly pooled within two distinct genetic units, *F. lugubris* and *F. lugubris-X*.
Formica pratensis is well separated from all the other species of the group as shown by the FCA and by the F_{ST} values. The result is in accordance with Goropashnaya *et al*. (2004), which already showed that *F. pratensis* form a separate phylogenetic cluster within the *F. rufa* group. In the past there have been some controversies on the species status of *F. pratensis* and its

ecomorphs, in particular due to the description of *F. rufa pratensis* var. *nigricans* by Emery (1909). Some authors considered *Formica nigricans* as separated from *F. pratensis* (Kutter 1977; Collingwood 1979), while others never recognized it as a different species (Dlusskii 1967; Parachivescu 1972). The controversies were finally stopped by a detailed morphological and ecological investigation conducted by Seifert (1992), which described *Formica nigricans* as an ecomorph of *F. pratensis*. In the future, microsatellite 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 that the lineage sorting between the species is not complete and both species have very little sequence diversity (Goropashnaya *et al*. 2004). Yet, our present results confirm the previous finding from northern Europe (Sweden) that sympatric populations of the two species form separate gene pools Gyllenstrand *et al*. (2004). No hybrids were observed and the level of genetic differentiation (F_{ST} = 0.341) was higher than between some other species pairs. It is possible that hybridization between this species pair is localized in some areas, even though we cannot completely rule out occasional hybridization on the basis of the small number of nests within our study area.

Molecular data revealed that the species *F. aquilonia* and *F. paralugubris* are genetically close to each other. Some individuals morphologically identified as *F. paralugubris*, showed signs of admixture with *F. aquilonia* within the Park and hybrid individuals were all sampled in the same valley. Interestingly, hybridization was mainly observed in samples morphologically identified as *F. paralugubris*, rather than in *F. aquilonia* workers. This could be a consequence of the morphological species identification: there is indeed a higher risk to erroneously identify *F. aquilonia* samples as *F. paralugubris* than the opposite.

These two species have highly similar mtDNA haplotypes with very little geographical variation, suggesting a recent divergence (Goropashnaya *et 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 *et 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*. Hybridization is 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 1991; Schwander *et al*. 2009), and hybridization has also been suggested as a mechanism leading to speciation in these social insects (Nonacs 2006a,b) and other animals (Mallet 2007). Considering the following lines and the particular

position of *F. lugubris-X*, our data suggest that hybrid speciation is probably more common as we thought in alpine red wood ants.

F. lugubris and F. lugubris-X

The microsatellite data revealed that individuals morphologically identified as *F. lugubris* surprisingly form two distinct genetic units, named here *F. lugubris* and *F. lugubris-X*. This distinction was indicated both by nuclear microsatellites and by the mtDNA haplotypes. The haplotype of *F. lugubris-X* is indeed clearly different from all other *F. lugubris* workers collected in the present study and from the European *F. lugubris* samples analyzed in our previous work (Bernasconi *et al*. submitted). In fact, the mtDNA haplotype clusters *F. lugubris-X* with *F. paralugubris* (Figure 5), but the microsatellite data, particularly the FCA analysis, suggests that *F. paralugubris* is nuclearly further removed from *F. lugubris-X* than from other *F. lugubris* samples.
The F_{ST} value indicates that, even if it is significantly different from zero, the genetic distance between *F. lugubris* and *F. lugubris-X* (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 *et al.* 2005). This seems to argue that *F. lugubris-X* could be considered a different *F. lugubris* population rather than a different species. Nevertheless, the distance between *F. lugubris* and *F. lugubris-X* 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-X* population has been found only in one valley, Val Mingèr, within the Swiss National Park. Could the genetic separation between *F. lugubris* and *F. lugubris-X* be due to geographical isolation? Some *F. aquilonia* nests are also present in the same valley and these nests do not genetically differ from the other *F. aquilonia* samples within the study area. Moreover, field observations indicate that there is no evident barrier in Mingèr valley that could prevent ants to freely move in and out from it. It therefore seems that the population is not geographically isolated from other areas inhabited by *F. lugubris*. There is also no clear indication that the population of *F. lugubris-X* would represent ongoing hybridization between *F. lugubris* and either *F. paralugubris* or *F. aquilonia*. The admixture analyses and the FCA showed that *F. lugubris-X* genotypes are not a mix between two other species. In this case hybrid genotypes would have been located in the middle of their parental species on the FCA, as observed for hybrids between *F. aquilonia* and *F. paralugubris*.

We therefore suggest that *F. lugubris-X*, which is morphologically similar to *F. lugubris*, but is genetically distinct from it, might represent an undescribed cryptic species of red wood ant. 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 the morphology link it with *F. lugubris*. The situation is very similar to that of *F. paralugubris*, which was described recently as a new species (Seifert 1996a). Alarm pheromones (Cherix 1983) and behaviour (Rosengren & Cherix 1981; Rosengren *et 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 *et al*. 1992). As discussed above, also *F. paralugubris* has genetic features which point to a role of hybridization in its development.

Cryptic species and integrative taxonomy

The possibility 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-X* and to clarify the general role of hybridization in speciation within the *F. rufa* group ants. Molecular data are increasingly employed to solve problems in taxonomy and species delimitation (e.g. Ross & Shoemaker 2005; Roy *et al*. 2006; Vogler & Monaghan 2007; Rowe & Beebee 2007; Boissin *et al*. 2008; Gattolliat *et al*. 2008; Valentini *et al*. 2008; Bernasconi *et al*. submitted), but they are frequently discordant with the traditional taxa boundaries based on morphological data (e.g. Cardoso & Vogler 2005; Heckman *et al*. 2006; Schlick-Steiner *et al*. 2006a, b; Steiner *et al*. 2006). Recent works have suggested an integrative taxonomy, which gathers data by different techniques for delimiting species boundaries (Dayrat 2005; Will *et al.* 2005; Valdecasas *et al*. 2008, but see Cardoso *et al*. 2009; Seifert 2009). The integrative approach has already been successfully used in other ant genera (Lucas *et al*. 2002; Schlick-Steiner *et al*. 2006a, b; Seifert 2009) and we strongly believe that it could help in solving red wood ant taxonomy. For example, behavioural tests based on recognition (aggression or pupa carrying) have proven useful (Rosengren & Cherix 1981; Rosengren *et al*. 1994; Maeder *et al*. 2005) and could be helpful in the present situation. Moreover, chemical analyses could also be practical to verify the existence of a new cryptic species. Cuticular hydrocarbons were recently used by Martin *et al*. (2008). Even though the study did not consider all the *F. rufa* species, the results indicated that chemical cues could offer another powerful tool for species discrimination. An investigation on chemical compounds such

as sex pheromones could be very suitable to highlight eventual prezygotic barriers between the different genetic groups. Moreover, further samplings efforts will be necessary to check whether nests sharing the same genetic characteristics than *F. lugubris-X* are present in other geographical areas.

CONCLUSION

In this paper we presented results based on microsatellites and Bayesian analyses to identify red wood ant species. Our data show that molecular markers are powerful tools for species identification and at the same time revealed the existence of a new cryptic species within the Swiss National Park area. We note that this technique is more objective than morphology in identifying red wood ant species and give simultaneously important information on hybridization events. Similar results have also been found in other organisms (Pierpaoli *et al*. 2003; Basset *et al*. 2006; Randi 2008). Therefore, genetic monitoring can give fundamental information for conservation planning. In fact, as suggested by Schwartz *et al*. (2006) “species already considered endangered might be composed of multiple species that are even more rare than previously supposed”. This seems to be true for red wood ants.
More generally, molecular markers can also help in improving our knowledge on the real diversity on earth (Bickford *et al*. 2006). About two millions of species have been described to date (Stork 1997), but, in spite of the massive work accomplished so far, this represents only a part of the real diversity on Earth (Wilson 2003). Consequently, given that most species remain undescribed, efforts to catalogue and explain biodiversity need to be prioritized (Gotelli 2004; Bickford *et al*. 2006). We believe that molecular markers, combined with other types of data are useful tools also for alpha taxonomists and could help taxonomy to come out of its actual crisis (Wilson 2004; Wheeler 2004).

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REFERENCES

Basset P., Yannic G. & Hausser J. 2006. Genetic and karyotipic structure in the shrews of the *Sorex araneus* group : are they independent ? *Molecular Ecology*, 15, 1577-1587.

Belkhir K., Borsa P., Chikhi L., Raufaste N. & Bonhomme F. 1996-2004 GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).

Bernasconi C., Maeder A., Cherix D. & Pamilo P. 2005. Diversity and genetic structure of the wood ant *Formica lugubris* in unmanaged forests. *Annales Zoologici Fennici*, 42, 189-199.

Bickford D., Lohman D.J., Sohdi N.S., Ng P.K.L., Meier R., Winker K., Ingram K. and Das I. 2007. Cryptic species as a window on diversity and conservation. *Trends in Ecology and Evolution* 22, 148-155.

Boissin E., Féral J.P. & Chenuil A. 2008. Defining reproductively isolated units in a cryptic and syntopic species complex using mitochondrial and nuclear markers: the brooding brittle star, *Amphipholis squamata* (Ophiuroidea). *Molecular Ecology*, 17, 1732-1744.

Bonera G. 2002. Analisi del comportamento intraspecifico in colonie differenti di *Formica lugubris* Zett. (Hymenoptera: Formicidae). Tesi di Laurea, Università di Pavia.

Boudjema G., Lempérière G., Deschamps-Cottin M. & Molland D.G. 2006. Analysis and nonlinear modeling of the mound-building ant *Formica lugubris* spatial multi-scale dynamic in a larch-tree stand of the southern French Alps. *Ecological Modelling*, 190, 147-158.

Cardoso A, Vogler A.P. 2005. DNA taxonomy, phylogeny and Pleistocene diversification of the *Cicindela hybrida* species group (Coleoptera: Cicindelidae). *Molecular Ecology*, 14, 3531-3546.

Cardoso A., Serrano A. and Vogler A.P. 2009. Morphological and molecular variation in tiger beetles of the *Cicindela hybrida* complex: is an “integrative taxonomy” possible? *Molecular Ecology*, 18, 648-664.

Cegelski C.C., Waits L.P., Anderson N.J. 2003. Assessing population structure and gene flow in Montana wolverines (*Gulo gulo*) using assignement-based approaches. *Molecular Ecology*, 12, 2907-2918.

Chapuisat M. 1996. Characterization of microsatellite loci in *Formica lugubris B* and their variability in other ant species. *Molecular Ecology,* 5, 599-601.

Cherix D. 1983. Intraspecific variations of alarm pheromones between two populations of the red wood ant *Formica lugubris* Zett. (Hymenoptera, Formicidae). *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, 56, 57-65.

Cherix D., Devenoges D., Freitag A., Bernasconi C. & Maeder A. 2007. Premier recensement des fourmis des bois (groupe *Formica rufa*) au Parc National Suisse. *Nationalpark-Forschung in der Schweiz* 94, 69-79.

Collingwood C. A. 1979. The Formicidae of Fennoscandia and Denmark. Fauna Entomologica Scandinavica 8. Klampenborg.

Cotti G. 1963. Bibliografia ragionata 1930-1961 del gruppo *Formica rufa* in Italiano, Deutsch, English. Ministero dell'agricoltura e delle foreste, Roma.

Cotti G. 1995. Bibliografia ragionata 1962-1981 del gruppo *Formica rufa* in Italiano, Français, Deutsch. Istituto di Entomologia dell'Università di Pavia, Centro Interdisciplinare di Bioacustica e di Ricerche Ambientali, Roma.

Cotti G. 1996. A bibliography of the *Formica rufa* group (Hymenoptera, Formicidae). *Insect Social Life*, 1, 133-136.

Czechowski W. 1996. Colonies of hybrids and mixed colonies, interspecific nest takeover in wood ants (Hymenoptera, Formicidae). *Memorabilia Zoologica,* 50, 1-116.

Dayrat B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85, 407-415.

Devenoges A. 1999. Distribution des fourmis des bois (groupe *rufa*) au Parc National Suisse, PNS. Applications à l'aide d'un système d'information géographique, SIG. Diplôme de Biologie, Univ. De Neuchâtel.

Dlusskii G. 1967. Ants of the genus Formica. (In Russian). Izd. Nauka, Mosow. 236 pp.

Evanno G., Regnaut S., Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, 14, 2611-2620.

Gattolliat JL., Hughes S.J., Monaghan M.T. & Sartori M. 2008. Revision of Madeiran mayflies (Insecta, Ephemeroptera). *Zootaxa,* 1957, 52-68.

Goropashnaya A., Fedorov V.B. & Pamilo P. 2004. Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. *Molecular Phylogenetics and Evolution*, 32, 198-206.

Gösswald K. 1990. Die Waldameise. Band 2: Die Waldameise in Ökosystem Wald, ihr Nutzen und ihre Hege. AULA-Verlag, Wiesbaden.

Gotelli N.J. 2004. A taxonomic wish-list for community ecology. *Philosophical Transactions of the Royal Society of London B*, 359, 585-597.

Goudet J. 1995. FSTAT (Version 1.2): a computer program to calculate F-statistics. *The Journal of Heredity*, 86, 485-486.

Groppali R. & Bonera G. 2004. Supercolony hypothesis and relationship among *Formica paralugubris* nests in the protected area "Giovetto delle Palline". *Insect Social Life*, 5, 63-68.

Gyllenstrand N., Gertsch P. J. & Pamilo P. 2002: Polymorphic microsatellites DNA markers in the ant *Formica exsecta*. *Molecular Ecology Notes*, 2, 67-69.

Gyllenstrand N., Seppä P., Pamilo P. 2004. Genetic differentiation in sympatric wood ants, *Formica rufa* and *F. polyctena*. *Insectes Sociaux*, 51, 139-145.

Gyllenstrand N., Seppä P., Pamilo P. 2005. Restricted gene flow between two social forms in the ant *Formica truncorum*. *Journal of Evolutionary Biology*, 18, 978-984.

Haubrich K. & Schmitt T. 2007. Cryptic differentiation in alpine-endemic, high altitude butterflies reveals down-slope glacial refugia. *Molecular Ecology*, 16, 3643-3658.

Hebert P.D.N., Penton E.H., Burns J.M., Janzen D.H. & Hallwachs H. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerators*. *Proceedings of the National Academy of Sciences of the United States of America*, 101, 14812-14817.

Heckman K.L., Rasoazanabary E., Machlin E., Godfrey L.R. and Yoder A.D. 2006. Incongruence between genetic and morphological diversity in *Microcebus griseorufus* of Beza Mahafaly. *BMC Evolutionary Biology*, 6, 98-108.

Hölldobler B &.Wilson E.O. 1990. The Ants. Springer-Verlag. 732pp.

Knaden M., Tinaut A., Cerda X., Wehner S., Wehner R. 2005. Phylogeny of three parapatric species of desert ants, *Cataglyphis bicolor, C. viatica* and *C. savigny*: a comparison of mitochondrial DNA, nuclear DNA and morphological data. *Zoology*, 108, 169-177.

Kutter H. 1977. Hymenoptera – Formicidae. Fauna Insecta Helvetica 6. Zürich. 298 pp.

Lambert D.M., Baker A., Huynen L., Haddrath O., Hebert P.D.N. & Millar C.D. 2005. Is a large-scale DNA-based inventory of ancient life possible? *Journal of Heredity*, 96, 279-284.

Lucas C., Fresneau D., Kolmer K., Heinze J., Delabie J.H.C. & Pho D.B. 2002. A multidisciplinary approach to discriminating different taxa in the species complex *Pachycondyla villosa* (Formicidae). *Biological Journal of the Linnean Society,* 75, 249-259.

Macaranas J.M., Colgan D.J., Major R.E., Cassis G., Gray M.R. 2001. Species discrimination and population differentiation in ants using microsatellites. *Biochemical Systematics and Ecology*, 29, 125-136.

Mace G.M. 2004. The role of taxonomy in species conservation. *Philosophical Transactions of the Royal Society of London B*, 359, 711-719.

Maeder A., Freitag A. & Cherix D. 2005. Species- and nestmate brood discrimination in the sibling wood ant species *Formica paralugubris* and *Formica lugubris*. *Annales Zoologici Fennici*, 42, 201-212.

Mäki-Petäys H., Zakharov A., Viljakainen L., Corander J. & Pamilo P. 2005. Genetic changes associated to declining populations of *Formica* ants in fragmented forest landscape. *Molecular Ecology*, 14, 733-742.

Mallet J. 2007. Hybrid speciation. *Nature*, 446, 279-283.

Manel S., Berthier P., Luikart G. 2002. Detecting wildlife poaching: identifying the origin of individuals with Bayesian assignment tests and multilocus genotypes. *Conservation Biology*, 16, 650-659.

Martin S.J., Helanterä H. and Drijfhout F.P. 2008. Evolution of species-specific cuticular hydrocarbon patterns in *Formica* ants. *Biological Journal of the Linnean Society*, 95, 131-140.

Mori A., Grasso D.A., Visicchio R., Le Moli F. 2001. Comparison of reproductive strategies and raiding behaviour in facultative and obligatory slave-making ants: the case of *Formica sanguinea* and *Polyergus rufescens*. *Insectes Sociaux*, 48, 302-314.

Nonacs P. 2006. The ecology and evolution of hybridization in ants. *Ecology*, 87, 2141-2142.

Nonacs P. 2006. Interspecific hybridization in ants: at the intersection of ecology, evolution and behavior. *Ecology*, 87, 2143-2147.

Pamilo P., Chautems D., Cherix D. 1992. Genetic differentiation of disjunct populations of the ants *Formica aquilonia* and *Formica lugubris* in Europe. *Insectes Sociaux*, 39, 15-29.

Paraschivescu D. 1972. Pozitia sistematica a speciilor *Formica pratensis* Retz. Si *F. nigricans* Emery. Stud. Cercet. Biol. *Seria Zoologie*, 24(6), 527-535.

Parisod C. 2008. Postglacial recolonization of plants in the western Alps of Switzerland. *Botanica Helvetica*, 118, 1-12.

Parisod C. & Besnard G. 2007. Glacial *in situ* survival in the Western Alps and polytopic autopolyploidy in *Biscutella levigata* L. (Brassicaceae). *Molecular Ecology*, 16, 2755-2767.

Passera L. & Aron S. 2005. Les fourmis: comportement, organisation sociale et évolution. Publication du programme de monographie du CNRC (Conseil National de recherches du Canada).

Pearson B. 1983. Hybridization between the ant species *Lasius niger* and *Lasius alienus*, the genetic evidence. *Insectes Sociaux*, 30, 402-411.

Pemberton J.M., Slate J., Bancroft R. and Barret A. 1995. Nonamplifying alleles at microsatellite loci: a caution for parentage and population studies. *Molecular Ecology*, 4, 249-252.

Pierpaoli M., Birò Z.S., Herrmann M., Hupe K., Fernandes M., Ragni B., Szemethy L. & Randi E. 2003. Genetic distinction of wildcat (*Felis silvestris*) populations in Europe, and hybridization with domestic cats in Hungary. *Molecular Ecology*, 12, 2585-2598.

Pritchard J.K., Stephens M., Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics*, 155, 945-959.

Pusch K., Seifert B., Foitzik S., Heinze J. 2006. Distribution and genetic divergence of two parapatric sibling ant species in Central Europe. *Biological Journal of the Linnean Society*, 88, 223-234.

Randi E. 2008. Detecting hybridization between wild species and their domesticated relatives. *Molecular Ecology*, 17, 285-293.

Rosengren R. & Cherix D. 1981. The pupa-carrying test as a taxonomic tool in the *Formica rufa* group. *Biosystematics of social insects*: *Systematics Association Special Volume No.19* (ed. by P.E. Howse and J.L. Clément). Academic Press, London, 263-281.

Rosengren R., Chautems D., Cherix D., Fortelius W., Keller L. 1994. Separation of two sympatric sibling species of *Formica* L. ants by a behavioural choice test based on brood discrimination. *Memorabilia Zoologica*, 48, 237-249.

Ross K.G. & Shoemaker D. 2005. Species delimitation in native South American fire ants. *Molecular Ecology*, 14, 3419-3438.

Rowe G. & Beebee J.C. 2007. Defining population boundaries: use of three Bayesian approaches with microsatellites data from British natterjack toads (*Bufo calamita*). *Molecular Ecology*, 16, 785-796.

Roy V., Demanche C., Livet A. and Harry M. 2006. Genetic differentiation in the soil-feeding termite *Cubitermes* sp. *Affinis subarquatus*: occurrence of cryptic species revealed by nuclear and mitochondrial markers. *BMC Evolutionary Biology*, 6, 102-113.

Savolainen R. & Vepsäläinen K. 2003. Sympatric speciation through intraspecific parasitism. *Proceedings of the National Academy of Sciences USA*, 100, 7169-7174.

Schlick-Steiner B.C., Steiner F.M. Konrad H., Marko B., Csösz S., Heller G., Ferencz B., Sipos B., Christian E., Stauffer C. 2006a. More than one species of *Messor* harvester ants (Hymenoptera: Formicidae) in Central Europe. *European Journal of Entomology*, 103, 469-476.

Schlick-Steiner B.C., Steiner F.M., Moder K., Seifert B., Sanetra M., Dyreson E., Stauffer C., Christian E. 2006b. A multidisciplinary approach reveal cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). *Molecular Phylogenetics and Evolution*, 40, 259-273.

Schönswetter P., Stehlik I., Holderegger R., Tribsch A. 2005. Molecular evidence for glacial refugia of mountain plants in the European Alps. *Molecular Ecology*, 14, 3547-3555.

Schwander T., Suni S.S., Cahan S.H and Keller L. 2008. Mechanisms of reproductive isolation between an ant species of hybrid origin and one of its parents. *Evolution*, 62, 1635-1643.

Schwartz M., Luikart G. and Waples R.S. 2006. Genetic monitoring as a promising tool for conservation and management. *Trends in Ecology and Evolution*, 22, 25-33.

Seifert B. 1991. The phenotypes of the *Formica rufa* complex in East Germany. *Abhandlungen und Berichte des Naturkundemuseum Görlitz,* 65, 1-27.

Seifert B. 1992. *Formica nigricans* Emery, 1909 – an ecomorph of *Formica pratensis* Retzius, 1783 (Hymenoptera, Formicidae). *Entomologica Fennica*, 2, 217-226

Seifert B. 1996a. *Formica paralugubris* nov. spec. – a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). *Reichenbachia Museum Tierkunden*, *Dresden*, 31, 193-201.

Seifert B. 1996b. Ameisen beobachten, bestimmen. Naturbuch Verlag, Augsburg.

Seifert B. 2002. How to distinguish most similar insect species – improving the stereomicroscopic and the mathematical evaluation of external characters by example of ants. *Journal of Applied Entomology*, 126, 1-9.

Seifert B. 2007. *Die Ameisen Mittel- und Nordeuropas*. Lutra - Verlags- und Vertriebsgesellschaft, Görlitz.

Seifert B. 2009. Cryptic species in ants (Hymenoptera: Formicidae) revisited: we need a change in the alpha-taxonomic approach. *Myrmecological News* 12: 149-166.

Seifert B. & Goropashnaya A. 2004. Ideal phenotypes and mismatching haplotypes – errors of mtDNA treeing in ants (Hymenoptera: Formicidae) detected by standardized morphometry. *Organisms, Diversity & Evolution*, 4, 295-305.

Sites J.W. & Marshall J.C. 2003. Delimiting species: a Renaissance issue in systematic biology. *Trends in Ecology and Evolution*, 18, 462-470.

Smith A., Fisher B.L. and Hebert P.D.N. 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society B London*, 360, 1825-1834.

Stehlik I. 2000. Nunataks and peripheral refugia for alpine plants during quaternary glaciation in the middle part of the Alps. *Botanica Helvetica*, 110, 25-30.

Stehlik I. 2003. Resistance or emigration? Response of alpine plants to ice ages. *Taxon*, 52, 499-510.

Steiner F.M., Schlick-Steiner B.C., Sanetra M., Ljubomirov T., Antonova V., Christian E., Stauffer C. 2005. Towards DNA-aided biogeography: An example from *Tetramorium* ants (Hymenoptera, Formicidae). *Annales Zoologici Fennici*, 42, 23-35.

Steiner F.M., Schlick-Steiner B.C., Konrad H., Moder K., Christian E., Seifert B., Crozier R.H., Stauffer C. & Buschinger A. 2006. No sympatric speciation here: multiple data sources show

that the ant *Myrmica microrubra* is not a separate species but an alternate reproductive morph of *Myrmica rubra*. *Journal of Evolutionary Biology*, 19, 777-787.

Stork N.E. 1997. Measuring global biodiversity and its decline. Pages 41-68 in M.L. Reaka-Kudla, D.E. Wilson, and E.O. Wilson (eds.), *Biodiversity II*. Joseph Henry Press: Washington, D.C.

Underwood E.C. & Fisher B.L. 2006. The role of ants in conservation monitoring: If, when and how. *Biological Conservation*, 132, 166-182.

Valdecasas A.G., Williams D. and Wheeler Q.D. 2008. 'Integrative taxonomy' then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society*, 93, 211-216.

Valentini A., Pompanon F. and Taberlet P. 2008. DNA barcoding for ecologists. *Trends in Ecology and Evolution*, 42, 110-117.

Vepsäläinen K., Pisarski B. 1981. The taxonomy of the *Formica rufa* group: chaos before order. *Biosystematics of social insects*: *Systematics Association Special Volume No.19* (ed. by P.E. Howse and J.L. Clément). Academic Press, London, 27-36.

Vogler A.P. & Monaghan M.T. 2007. Recent advances in DNA taxonomy. *Journal of Zoological Systematics and Evolutionary Research*, 45, 1-10.

Ward R.D., Zemlak T.S., Innes B.H., Last P.R. & Hebert P.D.N 2005. DNA barcoding Australia's fish species. *Proceedings of the Royal Society of London B*, 360, 1847-1857.

Weir B.S. & Cockerham C.C. 1984. Estimating F-statistics for the analysis of population structure. *Evolution*, 38, 1358-1370.

Wheeler Q.D. 2004. Taxonomic triage and the poverty of phylogeny. *Philosophical Transactions of the Royal Society of London B*, 359, 571-583.

Will K.W., Mishler B.D, Wheeler Q.D. 2005 The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, 54, 844-851.

Wilson E.O. 2003. The encyclopedia of life. *Trends in Ecology and Evolution*, 18, 77-80.

Wilson E.O. 2004. Taxonomy as a fundamental discipline. *Philosophical Transactions of the Royal Society of London B*, 359, 739.

Wright S. 1943. Isolation by distance. *Genetics*, 28, 114-138.

Zhu D.Q., Chapuisat M. & Pamilo P. 2003. Highly variable social organisation of colonies in the ant *Formica cinerea*. *Hereditas*, 139, 7-12.

Table 1 Genetic diversity in red wood ant species over the 9 microsatellite loci. He, expected heterozygosity without bias (Nei 1978) ; Ho, observed heterozygosity ; standard deviation in parentheses. F_{IS}, Deviation from Hardy-Weinberg equilibrium following Weir & Cockerham (1984), ***P<0.002.

Species	No. of alleles	He	Ho	F_{IS}	F_{ST}
F. rufa	28	0.383 (0.228)	0.331 (0.202)	0150***	0.274***
F. polyctena	35	0.570 (0.092)	0.441 (0.143)	0.246***	0.204***
F. pratensis	34	0.444 (0.283)	0.381 (0.270)	0.159***	0.195***
F. lugubris	62	0.604 (0.248)	0.555 (0.230)	0.083***	0.240***
F. lugubris-X	46	0.486 (0.226)	0.440 (0.210)	0.098***	0.067***
F. paralugubris	49	0.652 (0.145)	0.498 (0.108)	0.241***	0.204***
F. aquilonia	62	0.665 (0.152)	0.508 (0.130)	0.239***	0.214***
Overall	95	0.549	0.450	0.180***	0.200***

Table 2. Average membership coefficient (q_{group}) of the lowland species. Each species is 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

Table 3. Average membership coefficient (q_{group}) of the four species living at high altitudes and in coniferous forests in the Alps. Each species is 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	Group IV
F. lugubris	0.88	0.09	0.02	0.02
F. lugubris-X	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

Table 4. Paired F_{ST} values between redwood ant species. ***P<0.002.

	F. lugubris	*F. lugubris-X*	*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-X		-	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							-

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

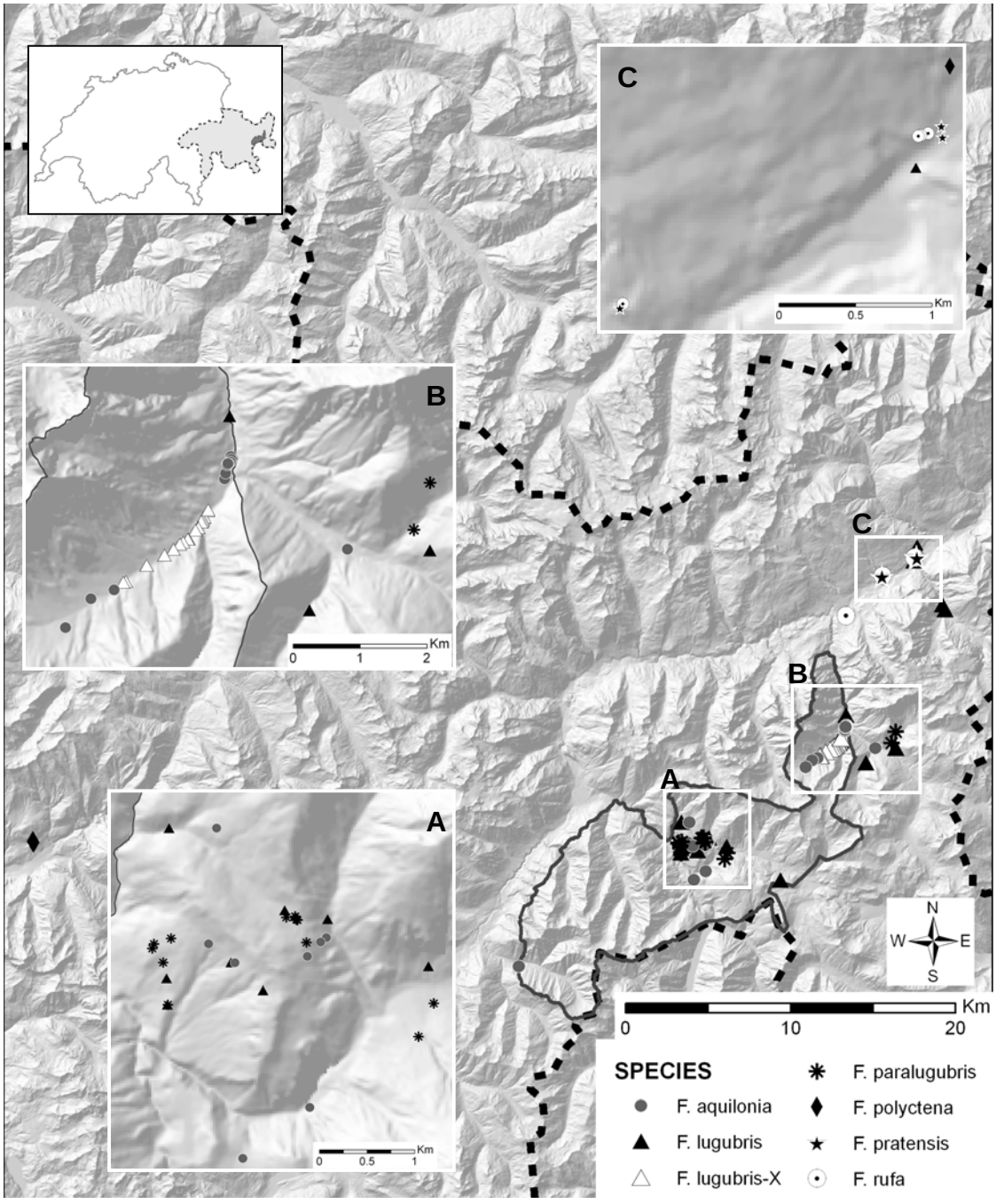


Figure 2: Individual membership coefficients (q_{ind}) of the lowland species *F. rufa* (blue), *F. polyctena* (red) and *F. pratensis* (green) analysed with the computer program STRUCTURE. Each individual is represented by a vertical line, which is partitioned into *k* coloured segments that represent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

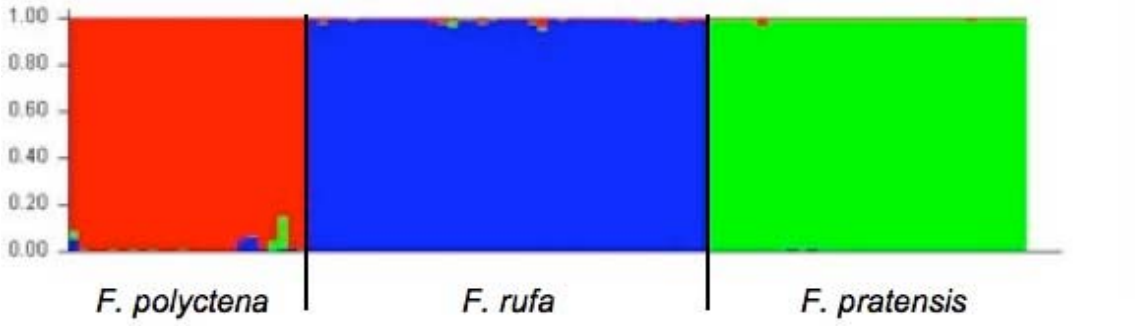


Figure 3: Individual membership coefficients (q_{ind}) of the 4 species living at high altitudes and in coniferous forest in the Alps *F. lugubris-X* (blue), *F. lugubris* (green), *F. aquilonia* (yellow), and *F. paralugubris* (red) analysed with the computer program STRUCTURE. Each individual is represented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

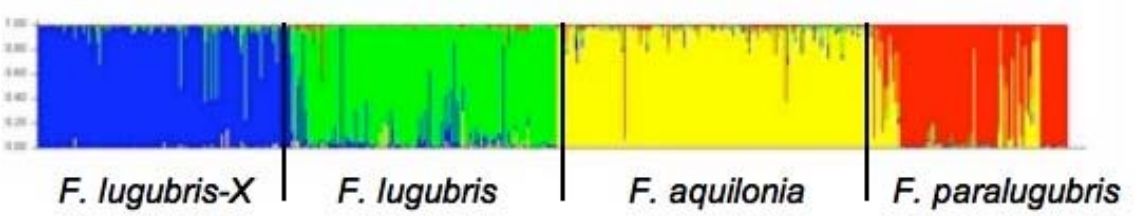


Figure 4a: Factorial Correspondance Analysis of the three red wood ant species living at lowland: *F. rufa* (blue), *F. polyctena* (yellow) and *F. pratensis* (grey). Coloured points represent the individual genotype for each sample.

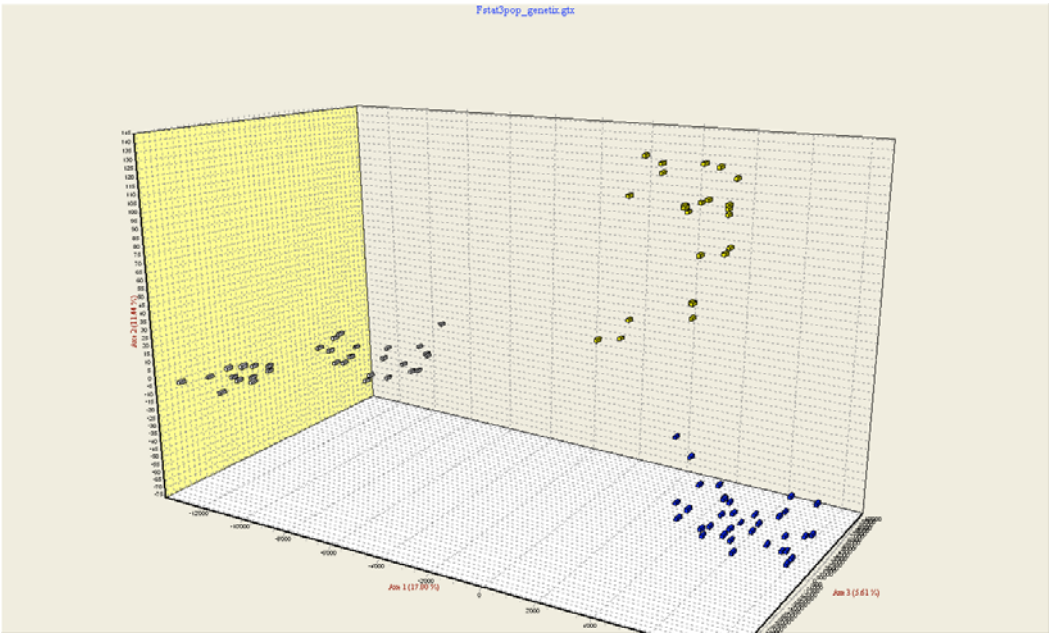


Figure 4b: Factorial Correspondance Analysis of the four red wood ant species living at thigh altitude in the Alps: *F. aquilonia* (grey), *F. paralugubris* (black), *F. lugubris* (blue) and *F. lugubris-X* (yellow). Coloured points represent the individual genotype for each sample.

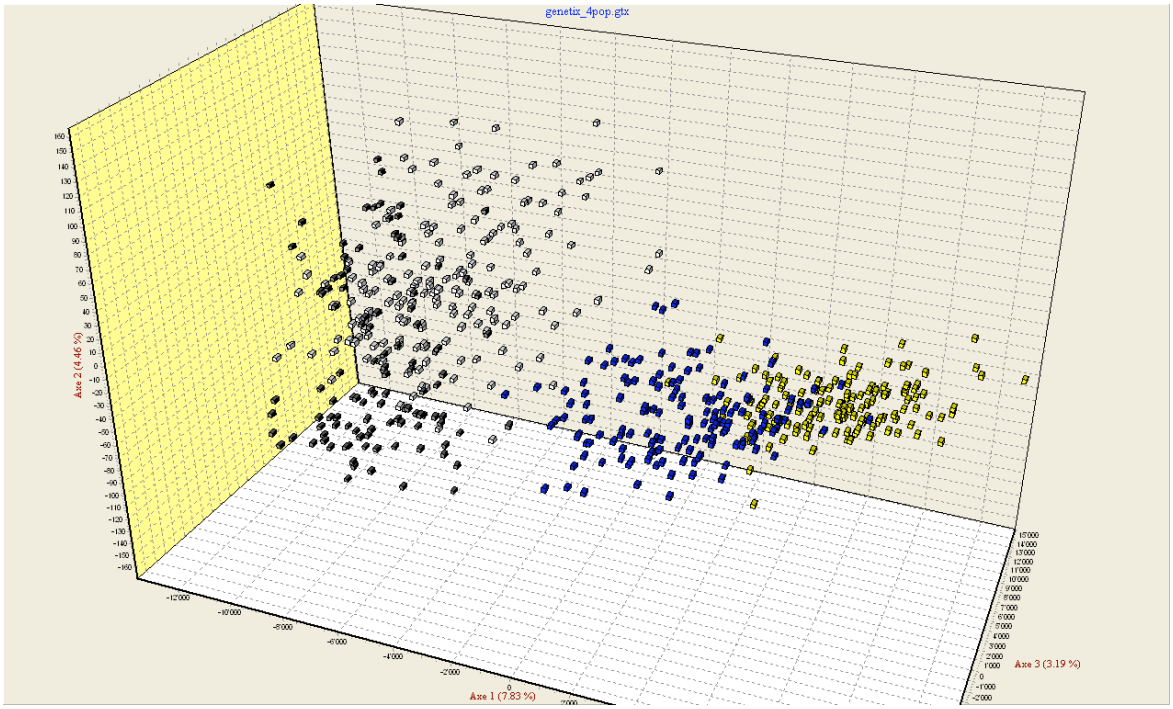
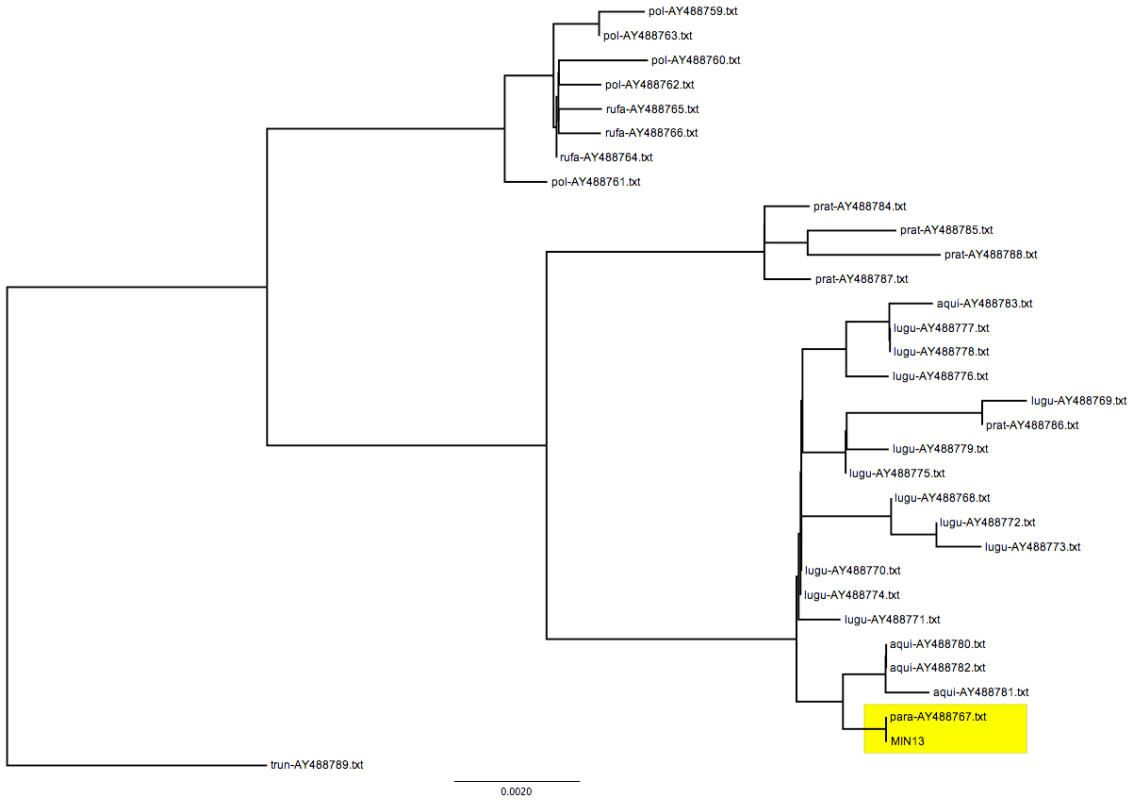


Figure 5. Phylogenetic tree (ML) obtained with the sequences of Goropashnaya *et al*. (2004) (EMBL Accession numbers: AY488759-AY488791). One *F. lugubris-X* individual (MIN13) has also been sequenced with the same primers used by Goropashnaya *et al*. 2004 and added to the phylogenetic tree. It clusters with *F. paralugubris* (in yellow).



Appendix 1. Species, Identity, Location , Swiss Coordinates and altitude of the analyzed nests.

Species	Sample	Locality	Region	Coordinate X	Coordinate Y	Altitude (m)
F. lugubris-X	MIN 6	Scuol	Val Minger	178588	818673	1746
F. lugubris-X	MIN 7	Scuol	Val Minger	178497	818617	1767
F. lugubris-X	MIN 8	Scuol	Val Minger	178499	818621	1788
F. lugubris-X	MIN 9	Scuol	Val Minger	178466	818603	1769
F. lugubris-X	MIN 10	Scuol	Val Minger	178440	818591	1794
F. lugubris-X	MIN 11	Scuol	Val Minger	178428	818575	1774
F. lugubris-X	MIN 12	Scuol	Val Minger	178344	818531	1795
F. lugubris-X	MIN 13	Scuol	Val Minger	178307	818491	1797
F. lugubris-X	MIN 14	Scuol	Val Minger	178175	818383	1824
F. lugubris-X	MIN 15	Scuol	Val Minger	178175	818374	1818
F. lugubris-X	MIN 16	Scuol	Val Minger	178128	818306	1812
F. lugubris-X	MIN 17	Scuol	Val Minger	178098	818253	1805
F. lugubris-X	MIN 18	Scuol	Val Minger	178019	818161	1850
F. lugubris-X	MIN 19	Scuol	Val Minger	177934	818024	1862
F. lugubris-X	MIN 20	Scuol	Val Minger	177780	817760	1911
F. lugubris-X	MIN 21	Scuol	Val Minger	177538	817448	1965
F. lugubris-X	MIN 22	Scuol	Val Minger	177526	817407	1980
F. lugubris-X	MIN 30	Scuol	Val Minger	178603	818680	1738
F. lugubris	P1-7	Zernez	Champlönch	173500	809000	2000
F. lugubris	P9K2	Zernez	Buffalora	170000	815000	1950
F. lugubris	SCU1	Scuol	Scharl	180000	819000	1600
F. lugubris	TAV 3	S-Charl	Tavru	177107	820196	1889
F. lugubris	CHP7	Zernez	Champlonch	172570	810330	2040
F. lugubris	SCH1	S-Charl	Plan d'Immetz	178000	822000	1900
F. lugubris	SCH2	S-Charl	Plan d'Immetz	178001	822001	1950
F. lugubris	SEN3	Sur-En	Val d'Uina	189319	823118	1148
F. lugubris	SEN7	Sur-En	Val d'Uina	186675	824835	1461
F. lugubris	SEN12	Sur-En	Val d'Uina	186350	825000	1550
F. lugubris	FUO1	Zernez	Il Fuorn	172040	811733	1847
F. lugubris	FUO7	Zernez	Il Fuorn	172538	810671	1914
F. lugubris	CHP9	Zernez	Champlönch	172631	810218	2045
F. lugubris	CRA1	Zernez	Crastatschas	171640	808982	1799
F. lugubris	CRA7	Zernez	Crastatschas	171913	808974	1818
F. lugubris	CRA17	Zernez	Crastatschas	172080	809653	1974
F. lugubris	CRA19	Zernez	Crastatschas	171791	809991	2085
F. aquilonia	MIN 1	Scuol	Val Minger	179398	819018	1680
F. aquilonia	MIN 23	Scuol	Val Minger	177411	817269	1987
F. aquilonia	MIN 24	Scuol	Val Minger	177280	816923	2064
F. aquilonia	MIN 25	Scuol	Val Minger	176848	816542	2145
F. aquilonia	MIN 26	Scuol	Val Minger	179284	818964	1688
F. aquilonia	MIN 29	Scuol	Val Minger	179063	818924	1718
F. aquilonia	MIN 2	Scuol	Val Minger	179354	819023	1650
F. aquilonia	MIN 3	Scuol	Val Minger	179314	819008	1663
F. aquilonia	MIN 4	Scuol	Val Minger	179290	818986	1689
F. aquilonia	MIN 5	Scuol	Val Minger	179146	818939	1707
F. aquilonia	MIN 27	Scuol	Val Minger	179269	818963	1719
F. aquilonia	MIN 28	Scuol	Val Minger	179292	818974	1659
F. aquilonia	C7	Zernez	Champlönch	173500	809500	2050
F. aquilonia	TAV 1	S-Charl	Tavru	178018	820763	1776
F. aquilonia	TRP2	S-chanf	Trupchun	164787	799145	1901
F. aquilonia	SCR1	Zernez	A.la Schera	170554	810482	1713
F. aquilonia	SCR5	Zernez	A.la Schera	170015	809776	1833
F. aquilonia	FUO8	Zernez	Il Fuorn	172342	810657	1923
F. aquilonia	FUO12	Zernez	Il Fuorn	172295	810597	1962
F. aquilonia	CHP12	Zernez	Champlönch	172143	810457	2070
F. aquilonia	CRA16	Zernez	Crastatschas	172276	809416	1993

F. aquilonia	CRA18	Zernez	Crastatschas	172076	809694	2027
F. paralugubris	P3B	Zernez	P3	171639	808988	1801
F. paralugubris	P3E	Zernez	P3	172226	808823	1879
F. paralugubris	P5B	Zernez	P5	171653	811789	1818
F. paralugubris	P5N3	Zernez	P5	171300	811629	1878
F. paralugubris	CHP4	Zernez	Champlonch	172527	810354	2036
F. paralugubris	CHP5	Zernez	Champlonch	172532	810341	2034
F. paralugubris	CHP6	Zernez	Champlonch	172566	810329	2041
F. paralugubris	CHP11	Zernez	Champlönch	172556	810236	2054
F. paralugubris	CHP16	Zernez	God la Drossa	172291	810447	2050
F. paralugubris	CRA8	Zernez	Crastatschas	172078	808939	1871
F. paralugubris	CRA15	Zernez	Crastatschas	172332	809021	1918
F. paralugubris	CRA12	Zernez	Crastatschas	172274	808841	1874
F. paralugubris	SES1	S-Charl	Sesvenna	178310	821762	1854
F. paralugubris	SES4	S-Charl	Sesvenna	179003	822003	1860
F. polyctena	ALV3	Alvaneu		172339	769646	1246
F. polyctena	ALV4	Alvaneu		172275	769702	1241
F. polyctena	SEN16	Sur-En	Val d'Uina	189983	823340	1199
F. rufa	SEN18	Sur-En		189544	823199	1156
F. rufa	SEN19	Sur-En		189530	823150	1160
F. rufa	SEN20	Sur-En		189526	823135	1171
F. rufa	SEN22	Sur-En		188430	821201	1250
F. rufa	SCU4	Scuol	Camping TCS	186000	819000	1750
F. pratensis	PRA1	Scuol		.	.	.
F. pratensis	SEN23	Sur-En		188402	821181	1249
F. pratensis	SEN1	Sur-En	Val d'Uina	189590	823285	1134
F. pratensis	SEN2	Sur-En	Val d'Uina	189518	823293	1112

CHAPTER 4

Behavioural species discrimination in red wood ants (*Formica rufa* group) in the Swiss National Park

Matthieu Fleury, Christian Bernasconi, Anne Freitag, Pekka Pamilo & Daniel Cherix

ABSTRACT

The taxonomy of European red wood ants (*Formica rufa* group) has always been controversial because the morphological method for species identification is rather complex. At present time, this group counts six species. However, during a previous work based on molecular markers, we showed the existence of one population morphologically identified as *F. lugubris*, but genetically different from all other analysed populations of this species. This population could represent a cryptic species within the Swiss National Park and has been named *F. lugubris-X*.

To verify our hypothesis we therefore conducted a behavioural test ("pupa-carrying test") based on the ability of ants to recognize pupae of their own species when compared to those of another species. The three red wood ant species present in the Swiss National Park (*F. lugubris*, *F. paralugubris* and *F. aquilonia*) and the *F. lugubris-X* population were used for our study. Results indicate that *F. lugubris-X* population differs from other *F. lugubris* and from all other species in the behaviour of its workers and in the way its pupae are discriminated by other workers. Additionally, we notice great similarity in behaviour patterns of *F. paralugubris* and *F. aquilonia* workers. Present results are thus in accordance with the genetic data.

This confirms the validity of the pupa-carrying test as a complementary taxonomic tool to identify red wood ants species. Moreover, these results strengthened our hypothesis on the existence of a new cryptic species within the Swiss Alps.

KEYWORDS: red wood ants, *Formica*, taxonomy, behaviour, cryptic species.

INTRODUCTION

The ability to discriminate between kin and nonkin plays an important role in social insects as a fundamental component of kin selection (Hamilton 1964; Agrawal 2001). It permits to focus altruistic behaviour toward conspecifics by rejecting or attacking heterospecific individuals (Beye *et al.* 1998). This discrimination behaviour can be directed against workers as well as against brood (Lenoir 1984; Panek & Gamboa 2000; Maeder *et al.* 2005) and allows the colony to avoid interspecific parasitism (Buschinger 1986; Lenoir *et al.* 2001).
Chemical compounds play an important role in recognition mechanisms of social insects (Fielde 1904; Bonavita-Cougourdan *et al.* 1987). These olfactory cues have endogenous and exogenous origins (Stuart 1987; Vander Meer & Morel 1998). Endogenous compounds represent genetically determined substances synthesized by the individuals (Beye *et al.* 1998; Giraud *et al.* 2002) which are either spread from one individual to another (e.g. queen pheromones; Carlin & Hölldobler 1986, 1987; Vander Meer & Alonso 1998) or produced by the individual itself (e.g. cuticular hydrocarbons; Singer 1998; Lahav *et al.* 1999; Howard 2005). On the contrary, exogenous cues consist in compounds acquired environmentally for example from food (Obin & Vander Meer 1988; Le Moli & Mori 1989; Silverman & Liang 2001) or nest material (Richard *et al.* 2004). All these compounds make up a common colony odour, described by Crozier & Dix (1979) as the "*Gestalt*". The relative importance of exogenous and endogenous components in recognition depends also on environmental factors (Downs & Ratnieks 1999) and, under homogenous environmental conditions, genetically based cues are expected to be more important.
The *Formica rufa* group (red wood ants) has been one of the most studied groups of ants in Europe during the last century and many researches have been devoted to their basic biology and ecology (see Cotti 1963, 1995, 1996; and Gösswald 1989, 1990). The recent phylogenetic study conducted by Goropashnaya *et al*. (2004) suggested that at present time the group consist of six species in Europe: *F. rufa* LINNAEUS, 1761, *F. polyctena* FÖRSTER, 1850, *F. lugubris* ZETTERSTEDT, 1838, *F. paralugubris* SEIFERT, 1996, *F. aquilonia* YARROW, 1955 and *F. pratensis* RETZIUS, 1783. Nevertheless, all these species have a very similar morphology and, in some cases, are able to hybridise (Czechowski 1993a; Seifert & Goropashnaya 2004) or to form mixed colonies (Seifert 1991; Czechowski 1993b, 1996; Czechowski & Radchenko 2006). As a consequence, the taxonomy of the group has always been debated and controversial (Vepsäläinen & Pisarski 1981; Collingwood 1987; Seifert 1991) and many investigations were conducted in order to clarify it (i.e. Mori & Le Moli 1993, Maeder & Cherix 2001).

A recent outcome of the numerous investigations on the *F. rufa* group was the description of *F. paralugubris* (Seifert 1996b). After the discovery of two distinct morphotypes among *F. lugubris* queens (Kutter 1967, 1977), a large diversity of taxonomic tools was used to examine the possibility of existence of diverse *F. lugubris* species. For example, the ability of ants to recognize homocolonial pupae by means of chemical cues was used in a behavioural experiment called "pupa-carrying test" (Rosengren *et al.* 1994) first developed by Rosengren & Cherix (1981). This taxonomic tool is based on natural reactions showed by workers when offered a choice between conspecific and heterospecific pupae. The results of this test, in association with other studies on alarm pheromones (Cherix 1983) and allozymes (Pamilo *et al.* 1992), provided clear evidence that *F. lugubris* was in fact composed of two distinct species. This led to a morphological comparative study and the description of *F. paralugubris* (Seifert 1996b). Afterwards, the "pupa-carrying test" conducted by Maeder *et al.* (2005) added further support to the species description.

Despite the large amount and diversity of studies on the *F. rufa* group, no complete comparative study was done exploring this group in its whole and on a local scale. In order to fill this gap, we recently made a microsatellites analysis on the six species of the *F. rufa* group within the Swiss National Park area (Eastern Swiss Alps) (Bernasconi *et al.*, in prep.). Besides the genetic differentiation of the six species, results also showed close genetic proximity between *F. aquilonia* and *F. paralugubris*. In addition, genetic data revealed the existence of a population morphologically described as *F. lugubris*, but genetically different to all other *F. lugubris* colonies and to all other wood ant species. As in Bernasconi *et al.* (in prep.), this population will be referred as *F. lugubris-X* in this paper.

In this study, our objectives are (1) to verify the behavioural status of the population *F. lugubris-X* compared to the species *F. lugubris*, *F. paralugubris*, *F. aquilonia*, and (2) to examine the genetic proximity between *F. aquilonia* and *F. paralugubris* by using a behavioural approach. We therefore used the pupa-carrying test to observe the behaviour displayed by the population *F. lugubris-X* and the species *F. lugubris*, *F. paralugubris*, and *F. aquilonia* when faced with pupae of their colony and of another population or species. We expected discrimination patterns in our experiment to correspond to genetic patterns observed in the microsatellites analyses (Bernasconi *et al.*, *in prep*.).

METHODS

Study species and study area

The population *F. lugubris-X* is located in the Mingèr Valley, situated between an altitude of 1700m and 2100m in the Eastern Swiss Alps, within the Swiss National Park (Bernasconi *et al.* in prep.). This population lives sympatrically with the three species *F. lugubris*, *F. paralugubris* and *F. aquilonia* in the unmanaged forests of this reserve (Cherix *et al*. 2007).
The Swiss National Park offers the unique possibility to observe red wood ants in a natural environment. At the same time, the relative small size of this strict nature reserve enables to study the *Formica rufa* group under reduced heterogeneity of environmental factors. As a result, genetic cues are expected to play a larger role than exogenous components in recognition processes in our discrimination tests.
In July 2008, we collected workers, worker pupae and nest material of the *F. lugubris-X* population and of the three species *F. lugubris*, *F. paralugubris*, and *F. aquilonia*. The collection was made in two sites 15km apart within the Swiss National Park. In each site, 2 to 3 nests per species were sampled (Table 1). The coordinates of each nest were taken by using a Geographical Positioning System (GPS). Sampling was made in July, when workers production and nest activity are at their peaks. Nests are therefore able to rapidly recover from perturbations due to our sampling. We collected only the necessary amount of pupae for our tests and to limit nest damages during collection. Controls were made to check if the nests well recovered after sampling. Sampled nests were already known from earlier studies (Devenoges 1999; Maeder 2006) and species identification has been conducted on a morphological base according to Seifert (1996a, 2007). These identifications were also confirmed by microsatellites (Bernasconi *et al.*, *in prep*). During the experiment, three nests have been sampled a second time because too few pupae were collected on the first sampling (Table 1). One of them has been substituted by a close one (connected with a trail) because no more pupae were found in the original sampled nest.
The collected material was kept in ventilated plastic boxes for few days before experiments (mean time ± S.D. between collection and tests was 4.7 ± 3.0 days). Workers were fed every day with water and a sugar mixture (honey in water) provided *ad libitum*.

Experimental procedures

We used the pupa-carrying test based on the “sequence-method” experiment described by Rosengren and Cherix (1981) and Rosengren *et al.* (1994). The design was slightly adapted from Maeder *et al.* (2005). Two different kinds of tests were conducted to assess the discrimination ability of workers. First, we made intraspecific tests, in which workers were offered homocolonial (from the same nest) and heterocolonial (from another nest of the same species) pupae (Table 2; combinations 1-2, 5-6, 11, 16). Second, we made interspecific tests, in which workers were offered conspecific and heterospecific pupae (Table 2; combinations 3-4, 7-10, 12-15). Nests used for each experiment were chosen randomly among all collected nests.

Fifteen workers were chosen according to their behaviour of pupae carriers. They were placed in an artificial nest consisting in a small plastic box filled with material of their own nest and containing 5 homocolonial pupae (Figure 1a). A round arena with a central entry hole was placed on this artificial nest. The entry hole was covered with a small piece of cardboard to prevent ants from entering the arena before the beginning of the test. The wall of the arena was covered with fluon to prevent ants from escaping. The casting plaster surface of the arena was divided in 20 numbered sectors (from 1 to 20) of equal area. In the aim to prevent ants from using external visual cues for orientation, the whole arena was surrounded by a 50cm high cardboard and was lighted centrally by a light bulb.

We placed 10 pupae of one kind in the odd-numbered sectors and 10 pupae of another kind in the even-numbered ones (Figure 1b). The experiment began when the arena entry hole was opened by removing the small piece of cardboard. We noted the order in which pupae were retrieved to the nest. The number of pupae of a kind remaining on the arena was reported when all pupae of the other kind had been retrieved to the nest. When no pupa was collected within 15 minutes or when the worker’s activity did not permit us to record the order in which pupae were retrieved to the nest, we discarded the replicate and repeated with new workers and pupae of the same nests.

After each experiment, workers were replaced in their ventilated plastic boxes and pupae were discarded. The arena surface was cleaned with water and forceps with alcohol, to prevent deposition of chemical cues. We fixed the number of tests to avoid a second sampling as much as possible. Fifteen tests were made when (1) *F. lugubris-X* pupae and/or workers were implicated (Table 2; combinations 1-5, 9, 13) and (2) both *F. paralugubris* and *F. aquilonia* were involved (Table 2; combinations 12 and 15). For all other combinations, 10 tests were conducted (Table 2; combinations 6-8, 10-11, 14, 16).

Statistical analyses

1 - Pupae discrimination

Pupae discrimination by workers of different species or population was analysed using the “worker choice test” developed by Rosengren *et al.* (1994) and adapted by Maeder *et al.* (2005). A matrix was constructed to report the order in which workers retrieved the pupae to the nest (Figure 2). If the workers discriminate, most observations should deviate from the diagonal of the matrix into an area of statistical significance, as described in Maeder *et al.* (2005) (Figure 2). A binomial test was used to compare the number of experiments in which workers significantly differentially retrieved both kinds of pupae with those in which workers showed no preference. The observed frequencies were tested against the expected frequencies under a binomial distribution with a probability parameter of 0.5. A significant difference ($p \leq 0.05$) indicates that workers of a species showed either a clear preference for homocolonial or conspecific pupae or an absence of discrimination of the heterocolonial or heterospecific pupae. If there is no significant difference ($p > 0.05$), none of these two conclusions can be emitted.

2 - Workers behaviour

A “discrimination index” (DI) was attributed to each test, in order to conduct a more powerful statistical analysis concerning the workers carrying behaviour. This index corresponded to the number of heterocolonial or heterospecific pupae remaining in the arena (1 to 10) when all pupae of the other kind were retrieved to the nest. If the ten heterocolonial or heterospecific pupae were retrieved first, the DI was the opposite of the number of pupae of the other kind remaining in the arena (-1 to -10) (Figure 2). A mean “discrimination index” was calculated for each workers-pupae combination. For each workers species, we made a one-way ANOVA and then tested the differences of treatment between intraspecific and interspecific tests in pairwise comparisons with a Tukey HSD test.

RESULTS

Out of a total of 231 replicates, 205 were conducted successfully. In the 26 other cases, 3 types of problems were observed: workers did not carry any pupae, too few pupae were retrieved, or the activity of workers was too important to record the order in which the pupae were retrieved to the nest.

1 - Pupae discrimination

F. lugubris-X pupae were always significantly discriminated when offered in interspecific tests to workers of *F. paralugubris* and *F. aquilonia* (Table 2; combinations 3-4). Similarly, they were also significantly discriminated when offered to *F. lugubris* workers (Table 2; combination 2). In intraspecific tests, homocolonial and heterocolonial *F. lugubris-X* pupae were significantly similarly retrieved to the nest (Table 2; combination 1).

F. lugubris pupae were significantly avoided by *F. aquilonia*, which preferred its own pupae (Table 2; combination 7). They also showed a tendency to be discriminated by *F. paralugubris* workers, (8 tests showing a discrimination against 2 presenting no preference), but the result of the binomial test is not significant (Table 2; combination 8). In intraspecific tests with *F. lugubris* workers, the number of tests with discrimination of heterocolonial pupae was equal to the number of tests with no preference, leading to a *p*-value of 1 with the binomial test (Table 2; combination 6). Concerning intraspecific tests with *F. lugubris-X* workers, *F. lugubris* pupae were significantly not discriminated (Table 2; combination 5).

F. aquilonia pupae were not significantly discriminated by *F. lugubris-X* and *F. paralugubris* workers in interspecific tests (Table 2; combinations 9, 12). When offered to workers of *F. lugubris*, these pupae had a tendency to be discriminated, although the *p*-value was not significant (Table 2; combination 10).

F. paralugubris pupae showed a tendency to be retrieved in the same way as heterospecific pupae when offered to *F. lugubris-X* and to *F. aquilonia* workers, but the results of the binomial tests are not significant (Table 2; combinations 13, 15). On the other hand, they had a tendency to be avoided by *F. lugubris* workers, although the *p*-value is > 0.05 (Table 2; combination 14).

Concerning intraspecific tests for both species *F. aquilonia* and *F. paralugubris*, the tendency of heterocolonial pupae to be similarly treated to homocolonial ones is significant for *F. aquilonia* (Table 2; combination 11) but not for *F. paralugubris* (Table 2; combination 16).

2 - Workers behaviour

The discrimination indexes (DI) for each interspecific and intraspecific test are presented in Table 2.

The number of pupae remaining on the arena at the end of the tests with *F. lugubris-X* workers was significantly lower in intraspecific tests with other *F. lugubris-X* pupae (mean DI ± S.E. = 0.3 ± 0.6; Figure 3a) than in interspecific tests with *F. aquilonia* (Tukey HSD: $p < 0.001$; Figure 3a) and *F. paralugubris* ($p = 0.003$; Figure 3a). A marginally significant difference in the same direction was obtained comparing the results of intraspecific tests with heterocolonial pupae of *F. lugubris-X* with those of interspecific tests with heterocolonial pupae of *F. lugubris* ($p = 0.060$; Figure 3a).

In tests with *F. lugubris* workers, the number of heterocolonial pupae of *F. lugubris* remaining in the arena in intraspecific tests (mean DI = 6.2 ± 1.2; Figure 3b) did not significantly differ from the remaining number of heterocolonial *F. lugubris-X* pupae ($p = 0.816$; Figure 3b) and heterospecific *F. aquilonia* ($p = 0.519$; Figure 3b) and *F. paralugubris* pupae ($p = 0.540$; Figure 3b).

Considering tests with *F. aquilonia* workers, the number of heterocolonial pupae remaining on the arena at the end of intraspecific tests (mean DI = 2.4 ± 1.0; Figure 3c) did not significantly differ from the number of *F. paralugubris* pupae ($p = 0.356$; Figure 3c) remaining at the end of interspecific tests. On the contrary, it differed significantly from the number of *F. lugubris-X* pupae ($p < 0.001$; Figure 3c) and *F. lugubris* pupae ($p < 0.001$; Figure 3c) left over on the arena in interspecific tests.

In tests with *F. paralugubris* workers, compared to the number of heterocolonial pupae remaining after intraspecific tests (mean DI = 2.0 ± 1.0; Figure 3d), the number of *F. aquilonia* pupae left over after interspecific tests did not significantly differ ($p = 0.227$; Figure 3d). On the contrary it significantly differed from the number of *F. lugubris-X* ($p < 0.001$; Figure 3d) and *F. lugubris* pupae ($p = 0.002$; Figure 3d) remaining after the other interspecific tests.

DISCUSSION

When offered the choice between conspecific pupae and *F. lugubris-X* pupae, workers of *F. lugubris*, *F. paralugubris* and *F. aquilonia* discriminated the latter, preferring to retrieve their own pupae to the nest. Moreover, by retrieving an important number of heterocolonial or heterospecific pupae, *F. lugubris-X* workers displayed a behaviour that differed greatly from the workers of *F. lugubris*, *F. paralugubris* and *F. aquilonia*. Despite they are morphologically identified as a single species, *F. lugubris* and *F. lugubris-X* showed the most conspicuous difference in their discriminative behaviours in our tests. *F. lugubris* displayed among the biggest "discrimination indexes" (Figure 3b) and *F. lugubris-X* showed among the smallest (Figure 3a). These results are consistent with aggression tests carried out previously (C. Bernasconi, unpublished data). High aggressiveness was observed when we put *F. lugubris* and *F. lugubris-X* workers together in a small plastic box, contrarily to what was noticed when we put workers of two different nests of either *F. lugubris* or *F. lugubris-X*. Furthermore, the particular behaviour displayed by *F. lugubris-X* workers and the way in which pupae of this population were discriminated are coherent with the results obtained in the recent genetic study (Bernasconi *et al.*, in prep.). The population of *F. lugubris-X* was found to genetically differ from *F. lugubris*, *F. aquilonia*, *F. paralugubris*, and from all other wood ant species of the *F. rufa* group.

Workers of *F. lugubris-X* displayed a particular behaviour that differentiated this population from the three species *F. lugubris*, *F. aquilonia* and *F. paralugubris*. These workers showed among the smallest discrimination indexes throughout the tests, meaning that they retrieved much more heterocolonial and heterospecific pupae to their nest than did the workers of *F. lugubris*, *F. aquilonia* and *F. paralugubris*. This behaviour might suggest that the population *F. lugubris-X* is a hybrid between two of these three genetically close species. Because of too similar genetic cues, workers of this population would consequently have difficulties in distinguishing between conspecific and heterospecific pupae. Nevertheless, this hypothesis can be clearly rejected by considering the strong discrimination displayed by the workers of the other species towards pupae of the population *F. lugubris-X*. Moreover, molecular data do not suggest that the population *F. lugubris-X* is a result of an ongoing hybridisation (Bernasconi *et al.*, *in prep.*).

The small propensity to discriminate presented by *F. lugubris-X* workers does not necessarily mean that they are unable to distinguish between different pupae. They might be able to make the distinction between two kinds of pupae but both of them are retrieved to the nest. The habit to retrieve heterocolonial or

heterospecific pupae to the nest is already known in *Solenopsis invicta* (Tschinkel 1992a, b). Workers of this species often steal the brood of small incipient conspecific nests in order to accelerate their own colony maturity. Although never observed in species of the *F. rufa* group, a similar slave-making behaviour could eventually explain the behaviour of *F. lugubris-X* in the pupa-carrying test (Kutter 1957, 1969). The ability to retrieve heterospecific pupae to their nest could also suggest a more extreme behaviour, displayed by slave-making ants (e.g. *Formica sanguinea*), which steal brood of different species in order to obtain slave workers (see Mori *et al.* 2000). To compare the behaviour of *F. lugubris-X* and *F. sanguinea*, we conducted complementary pupa-carrying tests in which workers of both species were offered a choice between their own pupae and pupae of other species (from the subgenera *Raptiformica*, *Coptoformica*, *Serviformica* and *Formica s. str.*). In these preliminary experiments, workers of *F. sanguinea* equally retrieved both kinds of pupae to the nest, independently of the subgenera of the tested heterospecific pupae. For its part, *F. lugubris-X* showed no discrimination when offered the choice with pupae of different species of the *Formica rufa* group but never retrieved pupae belonging to other subgenera (M. Fleury, unpublished data). Since these tests are only preliminary and the hypothesis of *F. lugubris-X* raiding brood was never observed in field or laboratory experiment, the ability of *F. lugubris-X* to steal brood needs to be verified. The particular pattern of discrimination displayed by the workers of this population is a matter of great interest for upcoming studies.

Besides the low discrimination level showed by *F. lugubris-X* workers, another particular behaviour was observed in our tests. A small tendency to discriminate was also noticed between *F. aquilonia* and *F. paralugubris*, both species retrieving to their nest many pupae of the other species. This observation is consistent with the phylogenetic study based on mtDNA and achieved on the whole *F. rufa* group (Goropashnaya *et al.* 2004). *F. paralugubris* and *F. aquilonia* were found to have only recently diverged. This can explain a similarity in endogenous cues used for recognition of pupae that could have led to the lack of discrimination. Both species were found to be genetically close on large scale (Goropashnaya *et al*. 2004) as well as on local scale, in the Swiss National Park where some hybrids have been found between these close species (Bernasconi *et al*., in prep.).

In our study, we observed strong differences in behaviour between *F. lugubris-X* and the three species *F. lugubris*, *F. aquilonia* and *F. paralugubris*. In particular, *F. lugubris* workers displayed a stronger discrimination when faced with *F. lugubris-X* pupae than with other *F. lugubris* pupae. Moreover, despite the three species *F. lugubris*, *F. aquilonia* and *F. paralugubris* are the genetically

closest species to *F. lugubris-X* within the *Formica rufa* group, they are genetically well separated from the latter (Bernasconi *et al.*, in prep.). In addition, *F. aquilonia* and *F. paralugubris*, which represent two separate species, showed only weak discrimination ability between their pupae in interspecific tests. Consequently, the behaviour displayed by *F. lugubris-X* workers might indicate that the separation between *F. lugubris* and *F. lugubris-X* is at least as important as between *F. aquilonia* and *F. paralugubris*. We can therefore suggest that *F. lugubris-X* is a new cryptic species of the *F. rufa* group.

To verify the hypothesis of a new cryptic species, further complementary studies should be achieved. Morphological studies permitting to distinguish *F. lugubris-X* from *F. lugubris* are currently in progress in collaboration with Dr. Bernhard Seifert (Staatliches Museum für Naturkunde, Görlitz, Germany). In complement to our study, it would also be interesting to analyse the origin of *F. lugubris-X*, by comparing it with the phylogenetic history of the whole *Formica rufa* group (Goropashnaya *et al.* 2004). The existence of prezygotic barriers between species should also be investigated, notably by analysing sexual pheromones (see Löfqvist & Bergström 1980; Walter *et al.* 1993). A chemical difference in sexual pheromones between *F. lugubris* and *F. lugubris-X* would represent a reproductive barrier, leading to genetic isolation and speciation. A study on this subject would give crucial information about the species status of *F. lugubris-X*.

As shown in this work, the pupa-carrying test alone is not sufficient to identify a species. It is although a valuable taxonomic tool, as it permits to study how different populations recognize themselves. Used as a complement to other taxonomic analyses, it can be precious to give indications on the separation existing between species.

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REFERENCES

Agrawal A.F. 2001. Kin recognition and the evolution of altruism. *Proceedings of the Royal Society of London B*, 268, 1099-1104.

Beye M., Neumann P., Chapuisat M., Pamilo P., Moritz R.F.A. 1998. Nestmate recognition and the genetic relatedness of nests in the ant *Formica pratensis*. *Behavioral Ecology and Sociobiology*, 43, 67-72.

Bonavita-Cougourdan A., Clément J.L., Lange C. 1987. Nestmate recognition – The role of cuticular hydrocarbons in the ant *Camponotus vagus* Scop.. *Journal of Entomological Science*, 22, 1-10.

Buschinger A. 1986. Evolution of social parasitism in ants. *Trends in Ecology and Evol*ution, 1, 155-160.

Carlin N.F. & Hölldobler B. 1986. The kin recognition system of carpenter ants (*Camponotus* spp.). I. Hierarchical cues in small colonies. *Behavioral Ecology and Sociobiology*, 19, 123-134.

Carlin N.F. & Hölldobler B. 1987. The kin recognition system of carpenter ants (*Camponotus* spp.). II. Larger colonies. *Behavioral Ecology and Sociobiology*, 20, 209-217.

Cherix D. 1983. Intraspecific variations of alarm pheromones between two populations of the red wood ant *Formica lugubris* Zett. (Hymenoptera, Formicidae). *Mitteilungen der Schweizerische Entomologische Gesellschaft*, 56, 57-65.

Cherix D., Devenoges D., Freitag A., Bernasconi C. & Maeder A. 2007. Premier recensement des fourmis des bois (groupe *Formica rufa*) au Parc National Suisse. *Nationalpark-Forschung in der Schweiz*, 94, 69-79.

Collingwood C.A. 1987. Taxonomy and zoogeography of the *Formica rufa* L. species group. In: Eder J. & Rembold H. (eds.), *Chemistry and biology of social insects*: 65-67. Verlag J. Peperny, München.

Cotti G. 1963. Bibliografia ragionata 1930-1961 del gruppo *Formica rufa* in Italiano, Deutsch, English. Ministero dell' agricoltura e delle foreste, Roma.

Cotti G. 1995. Bibliografia ragionata 1962-1981 del gruppo *Formica rufa* in Italiano, Français, Deutsch. Istituto di Entomologia dell'Università di Pavia, Centro Interdisciplinare di Bioacustica e di Ricerche Ambientali, Roma.

Cotti G. 1996. A bibliography of the *Formica rufa* group (Hymenoptera, Formicidae). *Insect Social Life*, 1, 133-136.

Crozier R.H. & Dix M.W. 1979. Analysis of two genetic models for the innate components of colony odor in social Hymenoptera. *Behavioral Ecology and Sociobiology*, 4, 217-224.

Czechowski W. 1993a. Hybrids in red wood ants (Hymenoptera: Formicidae). *Annales Zoologici*, 44, 43-53.

Czechowski W. 1993b. Mixed colonies of red wood ants (Hymenoptera: Formicidae). *Annales Zoologici*, 44, 27-41.

Czechowski W. 1996. Colonies of hybrids and mixed colonies; interspecific nest takeover in wood ants (Hymenoptera, Formicidae). *Memorabilia Zoologica*, 50, 1-116.

Czechowski W. & Radchenko A., 2006. Do permanently mixed colonies of wood ants (Hymenoptera: Formicidae) really exist? *Annales Zoologici*, 56, 667-673.

Devenoges A. 1999. Distribution des fourmis des bois (groupe *rufa*) au Parc National Suisse, PNS. Applications à l'aide d'un systéme d'information géographique, SIG. Diplôme de biologie, Université de Neuchâtel.

Downs S.G. & Ratnieks F.L.W. 1999. Recognition of conspecifics by honeybee guards uses nonheritable cues acquired in the adult stage. *Animal Behavior*, 58, 643-648.

Fielde A.M. 1904. Power of recognition among ants. *Biol Bull Mar Biol Lab* 7, 227-250.

Giraud T., Pedersen J.S., Keller L. 2002. Evolution of supercolonies: The Argentine ants of southern Europe. *Proceedings of the National Academy of Sciences USA*, 99, 6075-6079.

Goropashnaya A.V., Fedorov V.B., Pamilo P. 2004. Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. *Molecular Phylogeny and Evolution*, 32, 198-206.

Gösswald K. 1989. Die Waldameise. Band 1: Biologische Grundlagen, Ökologie und Verhalten AULA-Verlag, Wiesbaden.

Gösswald K. 1990. Die Waldameise. Band 2: Die Waldameise in Ökosystem Wald, ihr Nutzen und ihre Hege. AULA-Verlag, Wiesbaden.

Hamilton W.D. 1964. The genetical evolution of social behaviour. *Journal of Theoretical Biology*, 7, 1-52.

Howard D.J. & Blomquist G.J. 2005. Ecological, behavioural, and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology*, 50, 371-393.

Kutter H. 1957. Eine neue Form der Sklavengewinnung bei Ameisen. *Umschau*, 327-329.

Kutter H. 1967. Variationsstatische Erhebungen an Weibchen von *Formica lugubris* Zett. *Bulletin de la Société Entomologique Suisse*, 40, 63-77.

Kutter H. 1969. *Die sozialparasitischen Ameisen der Schweiz*. Neujahrsbl. Viertel. Naturforsh. Ges. Zürich.

Kutter H. 1977. Formicidae-Hymenoptera. Insecta Helvetica. Entomologisches Institut der ETH.

Lahav S., Soroker V., Hefetz A., Vander Meer R.K. 1999. Direct behavioural evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften*, 86, 246-249.

Le Moli F. & Mori A. 1989. Field experiments on environmental sources of nestmate recognition in two species of the *Formica rufa* group (Hymenoptera Formicidae). *Ethology, Ecology & Evolution*, 1, 329-339.

Lenoir A. 1984. Brood-colony recognition in *Cataglyphis cursor* worker ants (Hymenoptera: Formicidae). *Animal Behavior*, 32, 942-944.

Lenoir A., D'Ettorre P., Errard C., Hefetz A. 2001. Chemical ecology and social parasitism in ants. *Annual Review of Entomology*, 46, 573-599.

Löfqvist J. & Bergström G. 1980. Volatile communication substances in Dufour's gland of virgin females and old queens of the ant *Formica polyctena*. *Journal of Chemical Ecology*, 6, 309-320.

Maeder A. & Cherix D. 2001. Problèmes posés par *Formica paralugubris* une nouvelle espèce de fourmi des bois. *Actes Colloques Insectes Sociaux*, 14, 21-25.

Maeder A., Freitag A. & Cherix D. 2005. Species- and nestmate brood discrimination in the sibling wood ant species *Formica paralugubris* and *Formica lugubris*. *Annales Zoologici Fennici*, 42, 201-212.

Maeder A. 2006. Contribution to the conservation biology of the wood ant *Formica lugubris* (Hymenoptera, Formicidae) in Switzerland. PhD Thesis, University of Lausanne.

Mori A. & Le Moli F. 1993. The aggression test as a taxonomic tool - Evaluation in sympatric and allopatric populations of wood-ant species. *Aggressive Behavior* ,19, 151-156.

Mori A., Grasso D.A., Le Moli F. 2000. Raiding and foraging behavior of the blood-red ant, *Formica sanguinea* Latr. (Hymenoptera, Formicidae). *Journal of Insect Behavior*, 13, 421-438.

Obin M.S. & Vander Meer R.K. 1988. Sources of nestmate recognition cues in the imported fire ant *Solenopsis invicta* Buren (Hymenoptera: Formicidae). *Animal Behavior*, 36, 1361-1370.

Pamilo P, Chautems D., Cherix D. 1992. Genetic differentiation of disjunct populations of the ants *Formica aquilonia* and *Formica lugubris* in Europe. *Insectes Sociaux*, 39, 15-29.

Panek L.M. & Gamboa G.J. 2000. Queens of the paper wasp *Polistes fuscatus* (Hymenoptera: Vespidae) discriminate among larvae on the basis of relatedness. *Ethology*, 106, 159-170.

Richard F.J., Hefetz A., Christides J.P., Errard C. 2004. Food influence on colonial recognition and chemical signature between nestmates in the fungus-growing ant *Acromyrmex subterraneus subterraneus*. *Chemoecology*, 14, 9-16.

Rosengren R. & Cherix D. 1981. The pupa-carrying test as a taxonomic tool in the *Formica rufa* group. In: Howse P.E. & Clément J.-L. (eds.), *Biosystematics of social insects (Systematics Association Special Volume No.19)*: 263-281. Academic Press, London.

Rosengren R., Chautems D., Cherix D., Fortelius W., Keller L. 1994. Separation of two sympatric sibling species of *Formica* L. ants by a behavioural choice test based on brood discrimination. *Memorabilia Zoologica*, 48, 237-249.

Seifert B. 1991. The phenotypes of the *Formica rufa* complex in East Germany. *Abhandlungen und Berichte des Naturkundemuseum Görlitz*, 65,1-27.

Seifert B. 1996a. Ameisen beobachten, bestimmen. Naturbuch Verlag, Augsburg.

Seifert B. 1996b. *Formica paralugubris* nov. spec. – a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). *Reichenbachia Mus Tierkd Dresden*, 31, 193-201.

Seifert B. & Goropashnaya A. 2004. Ideal phenotypes and mismatching hyplotypes – errors of mtDNA treeing in ants (Hymenoptera: Formicidae) detected by standardized morphometry. *Organisms, Diversity & Evolution*, 4, 295-305.

Seifert B. 2007. *Die Ameisen Mittel- und Nordeuropas*. Lutra Verlags- und Vertriebsgesellschaft, Görlitz.

Silverman J. & Liang D. 2001. Colony disassociation following diet partitioning in a unicolonial ant. *Naturwissenschaften* 88, 73-77.

Singer T.L. 1998. Roles of hydrocarbons in the recognition systems of insects. *American Zoologist* 38, 394-405.

Stuart R.J. 1987. Transient nestmate recognition cues contribute to a multicolonial population structure in the ant, *Leptothorax curvispinosus*. *Behavioral Ecology and Sociobiology*, 21, 229-235.

Tschinkel W.R. 1992a. Brood raiding and the population dynamics of founding and incipient colonies of the fire ant, *Solenopsis invicta*. *Ecological Entomology*, 17, 179-188.

Tschinkel W.R. 1992b. Brood raiding in the fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae): laboratory and field observations. *Annals of the Entomological Society of America*, 85, 638-646.

Vander Meer R.K. & Alonso L.E. 1998. Pheromone directed behaviour in ants. In: Vander Meer R.K., Breed M., Winston M., Espelie K.E. (eds.), *Pheromone communication in social insects*: 159-192. Westview Press, Boulder.

Vander Meer R.K. & Morel L. 1998. Nestmate recognition in ants. In: Vander Meer R.K., Breed M., Winston M., Espelie K.E. (eds.), *Pheromone communication in social insects*: 79-103. Westview Press, Boulder.

Vepsäläinen K. & Pisarski B. 1981. The taxonomy of the *Formica rufa* group: chaos before order. In: Howse P.E. & Clément J.L. (eds.), *Biosystematics of social insects. – Systematics Association Special Volume* 19:27-36. Academic Press, London.

Walter F., Fletcher D.J.C., Chautems D., Cherix D., Keller L., Francke W., Fortelius W., Rosengren R., Vargo E.L. 1993. Identification of the sex-pheromone of an ant, *Formica lugubris* (Hymenoptera, Formicidae). *Naturwissenschaften*, 80, 30-34.

Table 1. Location, identity and putative species of the sampled nests. Pupae and workers were sampled two times in nests marked by *; SCU2b substitutes SCU nest because of the lack of pupae in this latter nest during the second sampling (**).

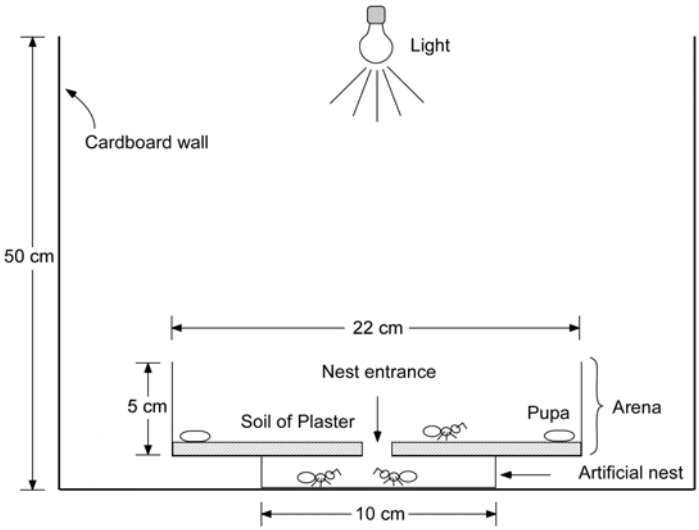
Species / Population	Study region	Nest ID	Coordinates	
Formica aquilonia	Il Fuorn	SCR3	46°38'57N, 10°10'52E	
	Il Fuorn	SCR6	46°38'52N, 10°10'44E	
	S-Charl / *Val Mingèr*	MIN1	46°43'47N, 10°18'16E	
	S-Charl / *Val Mingèr*	MIN3	46°43'44N, 10°18'15E	
	S-Charl / *Val Mingèr*	MIN4	46°43'43N, 10°18'14E	
F. paralugubris	Il Fuorn	P5A	46°39'46N, 10°12'21E	*
	Il Fuorn	P5B	46°39'44N, 10°12'23E	*
	Il Fuorn	CHP15	46°40'08N, 10°11'20E	
	S-Charl / *Val Sesvenna*	SES1	46°43'08N, 10°20'23E	
	S-Charl / *Val Sesvenna*	SES2	46°43'11N, 10°20'27E	
F. lugubris	Il Fuorn	P9B	46°39'03N, 10°15'38E	
	Il Fuorn	FUO1	46°39'57N, 10°12'21E	
	S-Charl / *Val S-Charl*	SCH2	46°42'25N, 10°21'00E	
	S-Charl / *Val S-Charl*	SCH3	46°42'04N, 10°21'21E	
	S-Charl / *Val S-Charl*	SCU2	46°46'00N, 10°17'54E	
	S-Charl / *Val S-Charl*	SCU2b	46°45'57N, 10°17'49E	**
F. lugubris-X	S-Charl / *Val Mingèr*	MIN8	46°43'18N, 10°17'56E	
	S-Charl / *Val Mingèr*	MIN15	46°43'08N, 10°17'43E	
	S-Charl / *Val Mingèr*	MIN17	46°43'06N, 10°17'38E	
	S-Charl / *Val Mingèr*	MIN18	46°43'03N, 10°17'33E	
	S-Charl / *Val Mingèr*	MIN20	46°42'56N, 10°17'14E	

Table 2. Pupa-carrying tests results. *N*: number of replicates; S: number of tests with a significant preference for homocolonial or conspecific pupae; NS: number of tests with no significant preference. The observed frequencies of replicates with a significant preference for one kind of pupae were tested against the expected frequencies under a binomial distribution with a probability parameter of 0.5 (Sign.: significativity; D: significant discrimination for homocolonial or conspecific pupae; ND: significant non-discrimination between pupae). The mean discrimination index was calculated for each combination (DI: mean discrimination index; S.E.: standard error)

Combination	Heterocolonial or heterospecific pupae	Workers	*N*			Binomial test		DI ± S.E.
			S		NS	Sign.	*p*-value	
1	*F. lugubris-X*	*F. lugubris-X*	0	(15)	15	ND	< 0.001	0.3 ± 0.6
2		*F. lugubris*	14	(15)	1	D	0.001	7.9 ± 0.5
3		*F. aquilonia*	13	(15)	2	D	0.007	8.5 ± 0.6
4		*F. paralugubris*	14	(15)	1	D	0.001	8.9 ± 0.6
5	*F. lugubris*	*F. lugubris-X*	3	(15)	12	ND	0.035	3.1 ± 0.9
6		*F. lugubris*	5	(10)	5	-	1.000	6.2 ± 1.2
7		*F. aquilonia*	10	(10)	0	D	0.002	8.8 ± 0.5
8		*F. paralugubris*	8	(10)	2	-	0.109	7.3 ± 0.8
9	*F. aquilonia*	*F. lugubris-X*	7	(15)	8	-	1.000	5.1 ± 0.8
10		*F. lugubris*	8	(10)	2	-	0.109	8.1 ± 1.0
11		*F. aquilonia*	1	(10)	9	ND	0.021	2.4 ± 1.0
12		*F. paralugubris*	7	(15)	8	-	1.000	4.5 ± 1.0
13	*F. paralugubris*	*F. lugubris-X*	4	(15)	11	-	0.119	4.3 ± 0.8
14		*F. lugubris*	8	(10)	2	-	0.109	8.2 ± 0.8
15		*F. aquilonia*	4	(15)	11	-	0.119	4.3 ± 0.9
16		*F. paralugubris*	2	(10)	8	-	0.109	2.0 ± 1.0

Figure 1. A: Experimental device used for the pupa-carrying test. **B:** Arena with 20 sectors filled with ten homocolonial or conspecific pupae and ten heterocolonial or heterospecific pupae (adapted from Maeder *et al.* 2005).

A



B

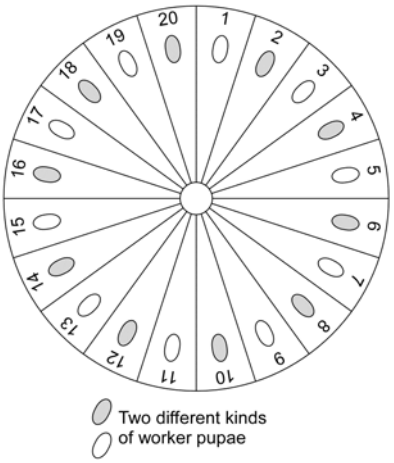
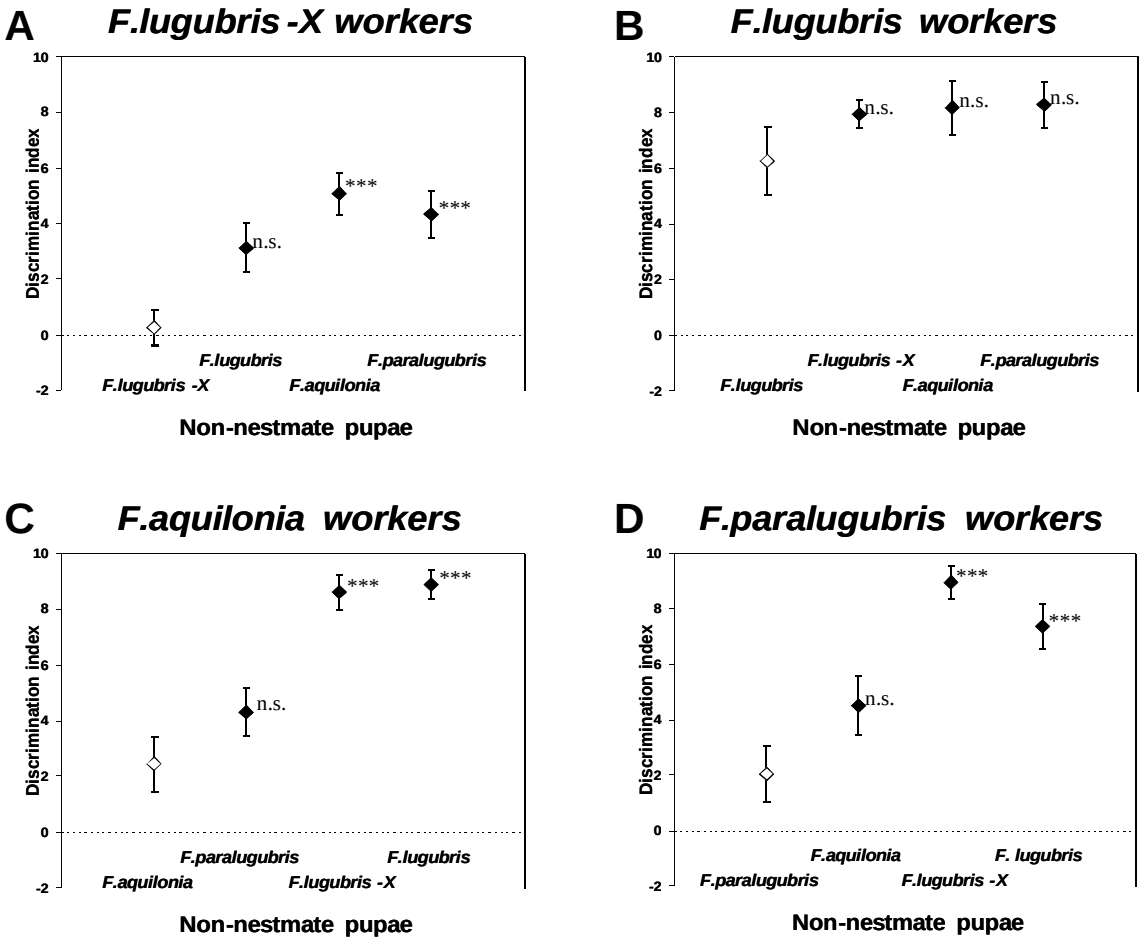


Figure 2. Matrix used for the statistical "worker choice test". The statistically significant area ($p \leq 0.05$) corresponds to the shaded area which indicates a preference for the homocolonial or conspecific pupae (no case of statistical preference for heterocolonial or heterospecific pupae was observed); x = starting point of the experiment. Each letter corresponds to choice event: A is a choice of a homocolonial or conspecific pupa, leading to one step up in the matrix, and B is a choice of a heterocolonial or heterospecific pupa, leading to one step to the right. The numbers in the upper row and last column corresponds to the "discrimination index" (DI). Bold sequence: the workers show no preference and the discrimination index attributed to this test is -2. Italic sequence: the workers show a statistically significant preference for homocolonial or conspecific pupae, with a discrimination index of 7 (adapted from Maeder *et al*. 2005).

Homocolonial/Conspecific retrieved pupae											
10th	**10**	**9**	**8**	*A*	**6**	**5**	**4**	**3**	**2**	**1**	**0**
9th				*A*							**-1**
8th			*A*	*B*					**A**	**B**	**B**
7th			*A*					**A**	**B**		**-3**
6th			*A*					**A**			**-4**
5th			*A*			**A**	**B**	**B**			**-5**
4th		*A*	*B*		**A**	**B**					**-6**
3rd	*B*	*A*			**A**						**-7**
2nd	*A*				**A**						**-8**
1st	*A*		**A**	**B**	**B**						**-9**
	x	**B**	**B**								**-10**
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th

Heterocolonial/Heterospecific retrieved pupae

Figure 3. Mean discrimination indexes (± S.E.) obtained in the pupa-carrying test. Results of each combination of tests are sorted by worker species or population. A and B: for *F. lugubris-X* and *F. lugubris*, differences in discrimination index between the intraspecific tests with pupae of the same population and the others tests (interspecific and intraspecific with a different population) were tested by Tukey HSD. C and D: For *F. aquilonia* and *F. paralugubris*, differences in discrimination index between intraspecific tests interspecific ones were tested by Tukey HSD. ***: $p \leq 0.05$; n.s.: $p > 0.05$.



CHAPTER 5

Sex pheromones identification in red wood ants (*Formica rufa* group)

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Pekka Pamilo & Daniel Cherix

ABSTRACT

Mound building red wood ants (species of the *Formica rufa* group) have a massive impact in forests in which they live and are protected by law in a number of European countries. In addition, they are considered among the most suitable insects for monitoring forest ecosystems. Nevertheless, the taxonomy of the group has been much debated and is still often neglected, mainly because of high intraspecific and geographic variability. At present time, the group is considered to count six species. However, our recent investigations based on genetic markers and behaviour revealed the existence of a new potential cryptic species within the Swiss Alps, which is morphologically similar to *F. lugubris*, but differs genetically and behaviourally from it. This putative cryptic species has been called *F. lugubris-X*. Considering that the description of a new species could influence future management plans in favour of these protected ants, we wanted to verify our hypothesis by a chemotaxonomical approach. We therefore studied the Dufour gland content (sex pheromones) produced by virgin queens of the entire *F. rufa* group, including *F. lugubris-X*.
Results confirm that sex pheromones are very useful for discriminating between red wood ant species. Moreover, our data show that *F. lugubris-X* produces significantly different sex pheromones than the other species. This indicates that *F. lugubris-X* developed a prezygotic barrier, which should prevent queens to mate with males of the other species. Present data agree with our previous genetic and behavioural observations. Consequently, we present substantial arguments indicating that *F. lugubris-X* represents a new cryptic species of red wood ant within the Swiss Alps.

KEYWORDS: Sex pheromones, Dufour gland, chemotaxonomy, *Formica rufa* group, cryptic species.

INTRODUCTION

Mound building red wood ants (species of the *Formica rufa* group) have been the topic of numerous research in the recent past (see Cotti 1963, 1995, 1996). Nevertheless, the taxonomy of the group has often been considered as confused (Vepsäläinen & Pisarski 1981, Collingwood 1987, Seifert 1991), mainly because of high intraspecific and geographic variability. In addition, these ants are morphologically similar and sometimes able to hybridize (Seifert & Goropashnaya 2004) or to form mixed colonies (Seifert 1991, Czechowski 1996). The phylogenetic study conducted by Goropashnaya *et al*. (2004) revealed that to date the group is formed by six species in Europe: *F. rufa* LINNAEUS, 1761, *F. polyctena* FÖRSTER, 1850, *F. lugubris* ZETTERSTEDT, 1838, *F. paralugubris* SEIFERT, 1996, *F. aquilonia* YARROW, 1955 and *F. pratensis* RETZIUS, 1783. However, our recent investigations based on genetic markers (Bernasconi *et al*. in prep) as well as on behaviour (Fleury *et al*. in prep.), revealed the existence of a new potential cryptic species within the Swiss Alps. This putative cryptic species, morphologically identified as *F. lugubris*, has been called *F. lugubris-X* (Bernasconi *et al*. in prep.; Fleury *et al*. in prep.).

Due to their huge impact in forests in which they live (Pavan 1959, 1981; Domisch *et al*. 2008; Jurgensen *et al*. 2008), these ants are protected by law in many European countries. Moreover, red wood ants are seen among the most promising insects for monitoring forest ecosystems (Gösswald 1990). Considering that the description of a new species would have a strong influence on management plans in favour of these protected ants, it is now necessary to validate the hypothesis of a new species as much as we can. Many authors recently suggested to use an integrative taxonomical approach for defining species boundaries (Dayrat 2005; Will *et al.* 2005; Valdecasas *et al*. 2008) and such studies already proved their utility in ants (Lucas *et al*. 2002; Steiner *et al*. 2005, 2006; Schlick-Steiner *et al*. 2006a, 2006b; Seifert 2009). We therefore decided to complete our molecular (Bernasconi *et al*. in prep.) and behavioural data (Fleury *et al*. in prep.) with chemical analyses.

Chemotaxonomy in ants is often based on cuticular hydrocarbons and previous studies on red wood ants showed that these chemical compounds could be successfully employed for species discrimination (Maeder 2006; Martin *et al*. 2008a, b). But the analysis of glandular contents is another powerful approach in chemotaxonomical studies (e.g. Keegans *et al*. 1992; Gökçen *et al*. 2002; Co *et al*. 2003; Dahbi *et al*. 2008). Ants have indeed numerous exocrine glands, which produce chemical substances and from which these compounds can be emitted (e.g. Morgan 2008). The Dufour gland is one of the principal

pheromone-producing glands present in all Formicidae (Dufour 1841). It is a small gland located in the tip of ant abdomen and its compounds can play different roles such as alarm, recruitment and sex attraction (Ali *et al*. 1988a; Walter *et al*. 1993; Morgan 2008).

It has been shown that the Dufour gland of some myrmicine and formicine ants produces a mix of straight-chain hydrocarbons from about C_9 to C_{27}. Generally, pentadecane or heptadecane are the most widespread alkanes in myrmicines, while undecane and tridecane are the most common alkanes in formicine (Walter *et al*. 1993; Morgan 2008). Moreover, some authors pointed out that the Dufour gland contents are often species-specific (e.g. Ali *et al*. 1987a, 1987b, 1988b; Bagnères *et al*. 1991; Hefetz 1993; Dahbi *et al*. 2008), making them suitable candidates for chemotaxonomical studies.

Dufour gland contents have been chemically investigated in a number of ant genera (e.g. Ali *et al*. 1987a, 1988a, 1988b, 1989; Keegans *et al*. 1992; Gökçen *et al*. 2002; Co *et al*. 2003; Dhabi *et al*. 2008; Morgan 2008) and several studies have also been conducted on *Formica* species (Bergström & Löfqvist 1973, Löfqvist & Bergström 1980; Ali *et al*. 1987b; Lanne *et al*. 1988; Bagnères *et al*. 1991). In *Formica rufa* group species, it has been shown that the Dufour gland products act as sex pheromones (Löfqvist & Bergström 1980; Cherix 1983; Walter *et al*. 1993). Virgin queens in red wood ants have indeed an enlarged gland compared to workers and old queens, because their glands produce sex pheromones that are used to attract males on mating places (Cherix *et al*. 1993; Walter *et al*. 1993) and therefore act as prezygotic barriers. These substances have been identified in *F. polyctena* (Löfqvist & Bergström 1980) and *F. paralugubris* (Walter *et al*. 1993), but their composition is still unknown in other red wood ant species. On the basis of these studies, we want to extend our researches on the Dufour gland contents of the entire *F. rufa* group, including *F. lugubris-X*.

The aim of the present study is thus to verify the hypothesis of the potential new cryptic species in the Swiss Alps by using the Dufour gland contents and to compare sex pheromones of the entire *F. rufa* group on a local scale.

METHODS

Sampling

Opportunities to collect virgin queens to analyze sex pheromones occur only when sexuals are ready for mating and come out on nest surface before the mating flight. Such chances are strictly limited to few days only, during the 2-3 weeks in spring/early summer when mating flights normally occur. In order to

collect virgin queens of all wood ant species, nests were repeatedly checked until virgin females came out for flying.
From May to August 2008 virgin alated queens were collected from 15 nests (2-3 nests per species) (Figure 1, Appendix 1) within the Swiss National Park and surrounding area (Engadin, Canton of Grisons). Nests belong to the six *F. rufa* group species and to *F. lugubris-X*. *F. polyctena* is quite rare in Engadin and the few known nests did not produce any queen in 2008. Furthermore, we did not find other *F. polyctena* nests in the same area. As a consequence, virgin queens of this species were sampled in another known population (M. Kaiser-Benz, unpublished data) located 35 km away from the Swiss National Park (Figure 1).
Species identification was assessed on the basis of the morphological criteria according to Seifert (1996a, b, 2007) by measuring morphological traits in workers of the same nests (Seifert 1996a, b, 2007; B. Seifert pers. com.) and also by comparing morphological traits in workers and queens with reference material already deposited at the Museum of Zoology of Lausanne (Switzerland). Species identification was also confirmed by genetic analyses conducted in our previous study (Bernasconi *et al*., in prep.).
Virgin queens were collected on the nest surface before the mating flight and about 30 individuals per nest were captured. Individuals were kept alive in separate plastic boxes with nest material and were fed with honey solution until dissection. The time between sampling and dissection varied from 2 to 8 days. Dissections were done in dH_20 and under microscope. Dufour glands were excised and put in glass vials with 600 microlitres of deionised water for GC-MS analysis. Samples were used immediately for chemical analyses (within 24 hours after dissection) or were frozen and analyzed later on. Comparative analyses indicated that freezing the samples has no influence on results.

GCMS

The analyses were carried out using an Agilent GC–MS system (Palo Alto, CA, USA) consisting of a 6890A GC, a 5973N Mass Detector and a CTC Combi-Pal autosampler. The headspace autosampler conditions were: incubation temperature 80 °C; incubation time 20 min; headspace syringe temperature 85 °C; agitation speed 250 rpm; injection volume 2.5 ml; fill speed 500 µL/s; syringe pull-up delay 500 ms; injection speed 500 µL/s; pre-injection delay 500 ms; post injection delay 500 ms; syringe flush 2.5 min with nitrogen. An J&W DB-624 GC column (30 m × 0.32 mm i.d. × 1.8 µm) was used for the separation. The oven temperature gradient started at 70 °C for 1 min and then ramped at 40 °C/min to 230 °C, held for 3 min. 2.5 ml headspace volume were

injected in split mode (10:1) into the GC-inlet containing a 4 mm i.d. liner at an injector temperature of 200 °C. Helium was used as carrier gas with a constant flow rate of 3.8 mL/min. The mass detector was operated in electron impact mode (70 eV). The MSD transfer line, source and quadrupole temperatures were set to 240, 230 and 150 °C, respectively. The MS data were collected in Full-Scan mode between m/z 10 and 400.

Substances identification

Identification of volatile compounds was carried out by means of GC–MS on the basis of comparison with known mass spectra from the mass spectra libraries Wiley and NIST and by gas chromatographic retention times upon co-injection of synthetic reference material. A standard solution containing all n-alkanes from C8 to C20 in concentrations of 0.006 % in n-hexane (Sigma-Aldrich, Cat-No 04070, Buchs, Switzerland) was used to establish Kovats retention indices for the used capillary column in order to undermine substance identification. In absence of commercially available reference material for the methyl-branched hydrocarbons, these compound have been identified by use of mass spectra and Kovats indices, only.

Statistical analyses

The relative amounts of substances per individuals and per species (average of all individuals of each species) have been inferred directly from original GC-MS output (peak areas). Differences of sex pheromone composition (relative proportion of each substance) between species were explored by discriminant analysis with SPSS v.16 software. The discriminant analysis was preferred to principal component analysis because of its stronger statistical power. However, principal component analysis was also done in a first exploratory analysis of data and gave similar results than the discriminant analysis.
We then used the mean relative percentage of all compounds for each species to calculate a single linkage dendrogram. The clustering analysis was done with SPSS v.16 and by using the Ward's method and Euclidian distances.

RESULTS

In total 150 virgin queens were dissected and analyzed. Of all these individuals, 76 queens (9 to 12 per species) showing the best GC-MS chromatograms were selected for statistical analyses (Table 1). A total of 20 compounds were found in the Dufour glands of the whole *F. rufa* group. Most of them were principally n-

alkanes ranging from nonane to docosane. We also found three alkenes and three substances to which we assigned putative names in according to our database (Table 1). The major compounds present in all species are undecane and tridecane. Eleven substances are common to all species. Seventeen substances were found in *F. rufa*, 16 in *F. polyctena*, *F. aquilonia*, *F. paralugubris* and *F. pratensis*, 15 in *F. lugubris*, while only 12 were found in *F. lugubris-X* (Table 1).

The discriminant analysis run on the entire dataset revealed that our samples form 4 main groups (Figure 2). One containing *F. rufa* and *F. polyctena*, one formed by *F. pratensis*, and another one formed by *F. lugubris*, *F. aquilonia* and *F. paralugubris*. The putative cryptic species *F. lugubris-X* is well separated from the other species, in particular from *F. lugubris*, and forms a different cluster. Samples are discriminated by 19 of the 20 substances with 98.7% of the original grouped observations correctly classified. Two functions explained 77.3% of the total variance, with 55.6% explained by function 1 and 21.7% explained by function 2 (Test of function 1: Wilk's Lambda = 0, Chi-square = 686.938, df = 114, Significativity = 0; Test of function 2: Wilk's Lambda = 0.001, Chi-square = 466.956, df = 90, Significativity = 0) (Table 2, Figure 2).

Individuals belonging to the same species are well grouped together. Few overlaps were observed between species groups *F. lugubris* - *F. aquilonia* - *F. paralugubri*s and *F. rufa* - *F. polyctena*. *Formica lugubris-X* is clearly separated from *F. lugubris* and from the other species. Samples of these species are located marginally and outside the whole *F. rufa* group (Figure 2).

To disentangle differences between overlapping species we then divided our dataset in two groups containing respectively *F. lugubris*, *F. aquilonia* and *F. paralugubris* in the first and *F. rufa* and *F. polyctena* in the second. We then run a discriminant analysis independently on each subset.

F. lugubris, F. paralugubris, F. aquilonia

A total of 15 substances allow the discrimination of the three species with 97.1% of the original grouped observations correctly classified. Two functions explained 100% of the total variance each function explaining 69% and 31% respectively (Test of function 1: Wilk's Lambda = 0.038, Chi-square = 81.916, df = 30, Significativity = 0; Test of function 2: Wilk's Lambda = 0.268, Chi-square = 32.930, df = 14, Significativity = 0.003) (Table 3, Figure 3).

As shown in figure 3, the three species are close, but well separated and with no overlaps.

F. rufa, F. polyctena

A total of 14 substances allow the discrimination of the two species with 100% of the original grouped observations correctly classified. A single function explained 100% of the total variance (Test of function 1: Wilk's Lambda = 0.024, Chi-square = 48.564, df = 14, Significativity = 0) (Figure 4, Table 4). *F. rufa* and *F. polyctena* are well separated and show no overlaps.

Clustering analysis

The dendrogram obtained with the clustering analysis (Figure 5) is in agreement with the results of the discriminant analysis and show that our samples form the same four groups already observed in figure 2: one containing *F. rufa* and *F. polyctena*, one with *F. pratensis*, and another one formed by *F. lugubris*, *F. aquilonia* and *F. paralugubris*. Within this latter, *F. aquilonia* and *F. paralugubris* are really close. The putative cryptic species *F. lugubris-X* is well separated from the other species, in particular from *F. lugubris*, and forms a different cluster.
The clear distinction between *F. pratensis* and the other species is mainly due to the presence of bergamotene and farnesene. These substances, produced by all *F. pratensis* queens, were never detected in the other species. The group formed by *F. rufa* and *F. polyctena* is mainly characterized by the presence of 3-methylnonane and 2-methyldodecane, which are absent in all the other species. The separation between *F*. *lugubris-X* and the other species, and *F. lugubris* in particular, is mainly due to the lack of substances found in the other species, rather than new substances synthesized by samples of these potential cryptic species (Table 1).

DISCUSSION

Our results show that the Dufour gland contents are a powerful tool for species discrimination in red wood ants. All species produce a species-specific mix of substances, which should prevent queens to mate with males of the other species. Similar conclusions have already been found in other ant species, in which authors observed that Dufour gland composition is species-specific (Ali *et al*. 1987, 1988b; Bagnères *et al*. 1991; Dahbi *et al*. 2008). But our results are very important because for the first time we highlighted mechanisms of reproductive isolation between species of the entire *F. rufa* group. In the future it might be interesting to check the specificity of these substances by

behavioural essays on the field (see Maeder 2006 for tests on mating behaviour).

Twenty substances have been found within the entire *F. rufa* group in the present study. Undecane and tridecane are the two major compounds observed in all the species. These findings correspond to previous studies in which high concentrations of undecane and tridecane have already been observed in *F. paralugubris* (Walter *et al*. 1993), *F. polyctena* (Löfqvist and Bergström 1980) and in other formicine ants (e.g. Morgan 2008). The high percentage of undecane and tridecane and their presence in all red wood ant species indicates, in agreement with Walter *et al*. (1993), that the major role of these substances is to attract males on mating places. On the other hand, the specificity of the sex pheromones is mainly due to the presence/absence and to the concentration variations of the other compounds.

The higher number of substances found in this work compared to Walter *et al*. (1993), can be explained by the different techniques employed. Walter *et al*. (1993) indeed analyzed the ventilated compounds emitted by virgin queens and collected on a charcoal filter. In that sense we should rather compare our results to the work of Löfqvist and Bergström (1980), which analyzed the entire Dufour gland and found 32 different substances in glands of *F. polyctena* virgin queens. Nevertheless, their study and the present one differ greatly in GCMS conditions.

The discriminant analysis conducted on the entire dataset divided our samples in four major groups.

Formica pratensis is well separated from the other species, mainly due to the presence of two substances (bergamotene and farnesene) never observed in the other species.

F. rufa and *F. polyctena* are characterised by the presence of 3-methylnonane and 2-methyldodecane, which are absent in all other species. Even if their Dufour gland contents are separated by the discriminant analysis (Figure 4), these species share similar sex pheromones (Table 1, Figure 2), which explains why they do hybridize in some localities in Europe (Seifert 1991; Czechowski 1996; Gyllenstrand *et al.* 2004).

As in Goropashnaya *et al*. (2004), *F. lugubris*, *F. aquilonia* and *F. paralugubris* cluster in a third group. The three species produce species-specific sex pheromones but within this group *F. aquilonia* and *F. paralugubris* share very similar substances, as shown by the dendrogram (Figure 5). The vicinity of these two species has already been observed at different levels: morphologically, genetically (Bernasconi *et al*. in prep.) and behaviourally (Fleury *et al*. in prep.). As for the species pair *F. rufa* and *F. polyctena*, a similar sex pheromones composition explains the occurrence of hybrids between *F.*

aquilonia and *F. paralugubris* within the Swiss National Park (Bernasconi *et al.* in prep.). In addition, these findings support the hypothesis of a recent divergence between these two species.

The analysis of the Dufour gland contents also revealed that *F. lugubris-X* produces significantly different sex pheromones than the other red wood ant species, indicating that *F. lugubris-X* already developed a prezygotic barrier, which should prevent queens to mate with males of the other species. Present data agree with our previous findings based on microsatellites and behaviour (Bernasconi *et al*. in prep.; Fleury *et al*. in prep.). Therefore, we provided convincing data indicating that *F. lugubris-X* represents a new cryptic species of red wood ant within the Swiss Alps.

As the alpha taxonomy is still essential for assigning valid taxonomic names to the putative cryptic species (Schlick-Steiner *et al*. 2007), more detailed morphological investigations are already in progress in order to describe *F. lugubris-X* (in collaboration with Dr. Bernhard Seifert, Staadtliches Museum für Naturkunde, Görlitz).

The existence of a new red wood ant species within the Swiss National Park is a fundamental result in terms of biodiversity and may have some influence on future strategies for the conservation of these protected ants. More precisely, we should now consider that *F. lugubris*, which is listed as near threatened by the International Union for the Conservation of Nature, is composed of at least two species. Each red wood ant species has its own ecological preferences and it is necessary to have a precise knowledge of the ecology of each species to better protect them (Maeder 2006). In addition, the small effective population size and the low dispersal rate of some ants, make several species more easily threatened (Pamilo & Crozier 1997). It is therefore essential to improve our knowledge on the ecological needs of *F. lugubris-X*. Studies should also be conducted to elucidate its geographical distribution and to understand whether *F. lugubris-X* is well distributed or endangered. At the same time, the European distribution of *F. lugubris* should be re-analyzed too, as it is probably more fragmented than previously thought.

We also believe that a phylogenetic revision of the *F. rufa* group is necessary to better understand the origin of *F. lugubris-X*. Sex pheromones seem to be a powerful tool for phylogenetic reconstruction in red wood ants. In fact, the dendrogram based on sex pheromones shows the same phylogenetic groups observed by Goropashnaya *et al*. (2004) (Figure 5, Figure 6) excepting *F. lugubris-X* (see Bernasconi *et al*. in prep). Looking at the present dendrogram, *F. lugubris-X* could seem phylogenetically distant from the other red wood ant species. In favour of this hypothesis, chemical and microsatellites data always located *F. lugubris-X* marginally and outside the *F. rufa* group. In addition, sex

pheromones composition of *F. lugubris-X* is very similar to those of *Raptiformica sanguinea* and *F. truncorum*, two species that do not belong to the *F. rufa* group and which also have less substances compared to red wood ant species (C. Bernasconi, unpublished data). Moreover, in chapter 4, we have seen that *F. lugubris-X* workers often get back heterospecific pupae to their nest, a behaviour that is similar to that of the slave-making ant *Raptiformica sanguinea* (M. Fleury, unpublished data).

However, this scenario is in contrast with data based on mtDNA sequences, which clustered *F. lugubris-X* within the *F. aquilonia* - *F. paralugubris* clade (Bernasconi *et al*., in prep.). In fact, results based on one *F. lugubris-X* mt-DNA sequence showed that *F. lugubris-X* is phylogenetically close to *F. paralugubris* and *F. aquilonia* and could have originated through past hybridization between *F. lugubris* and either *F. aquilonia* of *F. paralugubris* (Bernasconi *et al*. in prep). The phylogenetic reconstruction based on mtDNA seems more likely than the one based on sex pheromones composition. Hybridization is indeed considered a mechanism leading to speciation in ants (Helms Cahan & Keller 2003; Nonacs 2006a,b; Schwander *et al*. 2008) and in other animals (Mavàrez *et al*. 2006; Mallet 2007; Mavàrez & Linares 2008) and is known to have played a role in the evolution of the *Formica rufa* group (Seifert 1991; Czechowski 1996; Seifert & Goropashnaya 2004). In addition, sex pheromones have to be species specific to guarantee species integrity and strong selective pressures for creating new chemical combinations exist (Hefetz 1993). The selection for chemical diversity is therefore accentuated in closely related sympatric species and this may result in two sympatric species that might be phylogenetically related, but display different secretionary compositions. Nevertheless, although the hypothesis of past hybridization seems to better fit the origin of *F. lugubris-X*, more samples from the alpine regions, including *F. lugubris-X* individuals, should be analyzed and added to the phylogenetic tree obtained by Goropashnaya *et al*. (2004).

The present situation is very similar to that which opened the way to the description of *F. paralugubris* few years ago (Seifert 1996a), when *F. lugubris* and *F. paralugubris* were still identified as a single species. At that time, studies on alarm pheromones (Cherix 1983), allozymes (Pamilo *et al*. 1992) and behaviour (Rosengren & Cherix 1981; Rosengren *et al*. 1994) highlighted the existence of two different *F. lugubris* types in the Swiss Jura Mountains. Interestingly, few years before Kutter (1967, 1977) already described two forms of *F. lugubris* queens. One of these two groups was finally described as *F. paralugubris*, a sibling species of *F. lugubris* (Seifert 1996b). As it has been the case for *F. paralugubris*, gathering data with different techniques is a very useful way to guarantee correct species identification and to perform

taxonomical revisions. Therefore, such studies should be promoted in conservation biology, especially considering the actual threat of biodiversity loss.

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REFERENCES

Ali M.F., Attygalle A.B., Morgan E.D. and Billen J. 1987a. Comparison of Dufour gland secretions of two species of *Leptothorax* ants (Hymenoptera: Formicidae). *Zeitschrift für Naturforschung C-A Journal of Biosciences*, 42, 955-960.

Ali M.F., Attygalle A.B., Morgan E.D. and Billen J. 1987b. The Dufour gland substances of the workers *Formica fusca* and *Formica lemani* (Hymenoptera: Formicidae). *Comp. Biochem. Physiol*., 88B, 59-63.

Ali M.F., Attygalle A.B., Morgan E.D. and Billen J. 1988a. Contents of Dufour glands in some formicine ants: queens and workers of *Camponotus aethiops* and re-examination of *Lasius fuliginosus*. *Entomol. Exp. Appl.*, 46, 109-115.

Ali M.F., Billen J.P.J., Jackson B.D. & Morgan E.D. 1988b. Secretion of the Dufour glands of two African desert ants, *Camponotus aegyptiacus* and *Cataglyphis savignyi* (Hymenoptera: Formicidae). *Biochemical Systematics and Ecology*, 16, 647-654.

Ali M.F., Billen J.P.J., Jackson B.D. & Morgan E.D. 1989. The Dufour gland contents of three species of Euro-African *Messor* ants and a comparison with those of North American *Pogonomyrmex* (Hymenoptera: Formicidae). *Biochemical Systematics and Ecology*, 17, 469-477.

Bagnères A.G., Morgan E.D. & Clément J-L. 1991. Species-specific secretions of the Dufour glands of three species of Formicine ants (Hymenoptera: Formicidae). *Biochemical Systematics and Ecology*, 19, 25-33.

Bergström G. and Löfqvist J. 1973. Chemical congruence of the complex odoriferous secretions from Dufour's gland in three species of ants of the genus *Formica*. *Journal of Insect Physiology*, 19, 877-907.

Cherix D. 1983. Intraspecific variations of alarm pheromones between two populations of the red wood ant *Formica lugubris* Zett. (Hymenoptera, Formicidae). *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, 56, 57-65.

Cherix D., Fletcher D.J.C., Chautems D., Fortelius W., Gris G., Keller L., Rosengren R., Vargo E.L. and Walter F. 1993. Attraction of sexes in *Formica lugubris* Zett (Hymenoptera: Formicidae). *Insectes Sociaux*, 40, 319-324.

Co J.E., Jones T.H., Hefetz A., Tinaut A., Snelling R.R. 2003. The comparative exocrine chemistry of nine Old World species of *Messor* (Formicidae: Myrmicinae). *Biochemical Systematics and Ecology*, 31, 367-373.

Collingwood C. A. 1987. Taxonomy and zoogeography of the *Formica rufa* L. species group. In: *Chemistry and Biology of Social Insects* (J. Eder and H. Rembold, Eds), München: Verlag J. Peperny. pp. 65-67.

Cotti G. 1963. Bibliografia ragionata 1930-1961 del gruppo *Formica rufa* in Italiano, Deutch, English. Ministero dell'agricoltura e delle foreste. Roma. 413pp.

Cotti G. 1995. Bibliografia ragionata 1962-1981 del gruppo *Formica rufa* in Italiano, Français, Deutsch. Istituto di Entomologia dell'Università di Pavia, Centro Interdisciplinare di Bioacustica e di Ricerche Ambientali. Roma, 520 pp.

Cotti G. 1996. A bibliography of the *Formica rufa* group (Hymenoptera, Formicidae). *Insect Social Life*, 1, 133-136.

Czechowski W. 1996. Colonies of hybrids and mixed colonies, interspecific nest takeover in wood ants (Hymenoptera, Formicidae). *Memorabilia Zool*ogica, 50, 1-116.

Dahbi A., Hefetz A. and Lenoir A. 2008. Chemotaxonomy of some *Cataglyphis* ants from Morocco and Burkina Faso. *Biochemical Systematics and Ecology*, 36, 564-572.

Dayrat B. 2005. Towards integrative taxonomy. *Biological Journal of the Linnean Society*, 85, 407-415.

Domisch T., Ohashi M., Finer L., Risch A.C., Sundstrom L., Kilpelainen J. & Niemela P. 2008. Decomposition of organic matter and nutrient mineralisation in wood ant (*Formica rufa* group) mounds in boreal coniferous forests of different age. *Biology and Fertility of Soils*, 44, 539-545.

Dufour M.L. 1841. Recherches anatomiques et physiologiques sur les Orthoptères, les Hyménoptères et les Nevroptères. *Mem. Pres. Div. Sav. Acad. Sci. Inst. Fr.*, 7, 265-647.

Goropashnaya A.V., Fedorov V.B., Pamilo P. 2004. Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. *Molecular Phylogenetics and Evolution*, 32, 198-206.

Gökçen O.A., Morgan E.D., Dani F.R., Agosti D. and Wehner R. 2002. Dufour gland contents of ants of the *Cataglyphis bicolor* group. *Journal of Chemical Ecology*, 28, 71-87.

Gösswald K. 1990. Die Waldameise. Band 2: Die Waldameise in Ökosystem Wald, ihr Nutzen und ihre Hege. AULA-Verlag Wiesbaden.

Hefetz A. 1993. Hymenopteran exocrine secretions as a tool for chemosystematic analysis – possibilities and constraints. *Biochemical Systematics and Ecology*, 21, 163-169.

Helms Cahan S. & Keller L. 2003. Complex hybrid origin of genetic caste determination in harvester ants. *Nature*, 424, 306-309.

Jurgensen M.F., Finer L., Domisch T., Kilpelainen J., Punttila P., Ohashi M., Niemela P., Sundstrom L., Neuvonen S. & Risch A.C. 2008. Organic mound-building ants: their impact on soil properties in temperate and boreal forests. *Journal of Applied Entomology*, 132, 266-275.

Keegans S.J., Morgan E.D., Agosti D. and Wehner R. 1992. What do glands tell us about species? A chemical case study of *Cataglyphis* ants. *Biochemical Systematics and Ecology*, 20, 559-572.

Kutter H. 1967. Variationsstatische Erhebungen an Weibchen von *Formica lugubris* Zett. *Bulletin de la Société Entomologique Suisse*, 40, 63-77.

Kutter H. 1977. Formicidae-Hymenoptera. Insecta Helvetica. Entomologisches Institut der ETH.

Lanne B.S., Bergström G. and Löfqvist J. 1988. Dufour gland alkenes from the four ant species: *F. polyctena, F. lugubris, F. truncorum* and *F. uralensis. Comp. Biochem. Physiol*., 19, 729-734.

Löfqvist J. & Bergström G. 1980. Volatile communication substances in Dufour's gland of virgin females and old queens of the ant *Formica polyctena*. *Journal of Chemical Ecology*, 6, 309-320.

Lucas C., Fresneau D., Kolmer K., Heinze J., Delabie J.H.C. & Pho D.B. 2002. A multidisciplinary approach to discriminating different taxa in the species complex *Pachycondyla villosa* (Formicidae). *Biological Journal of the Linnean Society*, 75, 249-259.

Maeder A. 2006. Contribution to the conservation biology of the wood ant *Formica lugubris* (Hymenoptera, Formicidae) in Switzerland. PhD Thesis, University of Lausanne.

Mallet J. 2007. Hybrid speciation. *Nature*, 446, 279-283.

Martin S.J., Helanterä H. and Drijfhout F.P. 2008. Colony-specific hydrocarbons identify nest mates in two species of *Formica* ants. *Journal of Chemical Ecology*, 34, 1072-1080.

Martin S.J., Helanterä H. and Drijfhout F.P. 2008. Evolution of species-specific cuticular hydrocarbon patterns in *Formica* ants. *Biological Journal of the Linnean Society*, 95, 131-140.

Mavarez J., Salazar C., Bermingham E., Salcedo C., Jiggins C.D. & Linares M. 2006. Speciation by hybridization in *Heliconius* butterflies. *Nature*, 441, 868-871.

Mavarez J. & Linares M. 2008. Homoploid hybrid speciation in animals. *Molecular Ecology*, 17, 4181-4185.

Morgan E.D. 2008. Chemical sorcery for sociality: Exocrine secretions of ants (Hymenoptera: Formicidae). *Myrmecological News*, 11, 79-90.

Nonacs P. 2006a. The ecology and evolution of hybridization in ants. *Ecology*, 87, 2141-2142.

Nonacs P. 2006b. Interspecific hybridization in ants: at the intersection of ecology, evolution and behavior. *Ecology*, 87, 2143-2147.

Pamilo P., Chautems D., Cherix D. 1992. Genetic differentiation of disjunct populations of the ants *Formica aquilonia* and *Formica lugubris* in Europe. *Insectes Sociaux*, 39, 15-29.

Pamilo P. & Crozier R.H. 1997. Population biology of social insect conservation. *Memoirs of the Museum of Victoria*, 56, 411-419.

Pavan M. 1959. Attività italiana per la lotta biologica con formiche del gruppo *Formica rufa* contro gli insetti dannosi alle foreste. Ministero dell'agricoltura e delle foreste, Roma. Collana verde 4, 1-70.

Pavan M. 1981. Utilizzazione delle formiche del gruppo *Formica rufa* nella difesa biologica delle foreste. In : Studi sulle formiche utili alle foreste, Ministero dell'agricoltura e delle foreste, Roma. Collana verde 59.

Rosengren R., Cherix D. 1981. The pupa carrying test as a taxonomic tool in the *Formica rufa* group. In: Biosystematics of Social Insects. Systematics Association Special Volume No.19 (P.E Howse and J.L. Clément, Eds), London: Academic Press. pp 263-281.

Rosengren R., Chautems D., Cherix D., Fortelius., Keller L. 1994. Separation of two sibling species of *Formica* L. ants by a behavioural choice test based on brood discrimination. *Memorabilia Zoologica*, 48, 237-249.

Schlick-Steiner B.C., Steiner F.M., Konrad H., Marko B., Csösz S., Heller G., Ferencz B., Sipos B., Christian E., Stauffer C. 2006a. More than one species of *Messor* harvester ants (Hymenoptera: Formicidae) in Central Europe. *European Journal of Entomology*, 103, 469-476.

Schlick-Steiner B.C., Steiner F.M., Moder K., Seifert B., Sanetra M., Dyreson E., Stauffer C., Christian E. 2006b. A multidisciplinary approach reveal cryptic diversity in Western Palearctic *Tetramorium* ants (Hymenoptera: Formicidae). *Molecular Phylogenetics and Evolution*, 40, 259-273.

Schlick-Steiner B., Seifert B., Stauffer C., Christian E., Crozier R.H. & Steiner F.M. 2007. Without morphology, cryptic species stay in taxonomic crypsis following discovery. *Trends in Ecology and Evolution*, 22, 391-392.

Schwander T., Suni S.S., Cahan S.H and Keller L. 2008. Mechanisms of reproductive isolation between an ant species of hybrid origin and one of its parents. *Evolution*, 62, 1635-1643.

Seifert B. 1991. The phenotypes of the *Formica rufa* complex in East Germany. *Abhandlungen und Berichte des Naturkundemuseum Görlitz,* 65, 1-27.

Seifert B. & Goropashnaya A. 2004. Ideal phenotypes and mismatching haplotypes – errors of mtDNA treing in ants (Hymenoptera, Formicidae) detected by standardized morphometry. *Organism, Diversity & Evolution*, 4, 295-305.

Seifert B. 1996a. *Formica paralugubris* nov. spec. – a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). *Reichenbachia Museum Tierkunden*. Dresden 31, 193-201.

Seifert B. 1996b. Ameisen beobachten, bestimmen. Naturbuch Verlag, Augsburg.

Seifert B. 2007. Die Ameisen Mittel- und Nordeuropas. Lutra Verlags- und Vertriebsgesellschaft, Görlitz.

Seifert B. 2009. Cryptic species in ants (Hymenoptera: Formicidae) revisited: we need a change in the alpha-taxonomic approach. *Myrmecological News* 12: 149-166.

Steiner F.M., Schlick-Steiner B.C., Sanetra M., Ljubomirov T., Antonova V., Christian E., Stauffer C. 2005. Towards DNA-aided biogeography: An example from *Tetramorium* ants (Hymenoptera, Formicidae). *Annales Zoologici Fennici*, 42, 23-35.

Steiner F.M., Schlick-Steiner B.C., Konrad H., Moder K., Christian E., Seifert B., Crozier R.H., Stauffer C. & Buschinger A. 2006. No sympatric speciation here: multiple data sources show that the ant *Myrmica microrubra* is not a separate species but an alternate reproductive morph of *Myrmica rubra*. *Journal of Evolutionary Biology*, 19, 777-787.

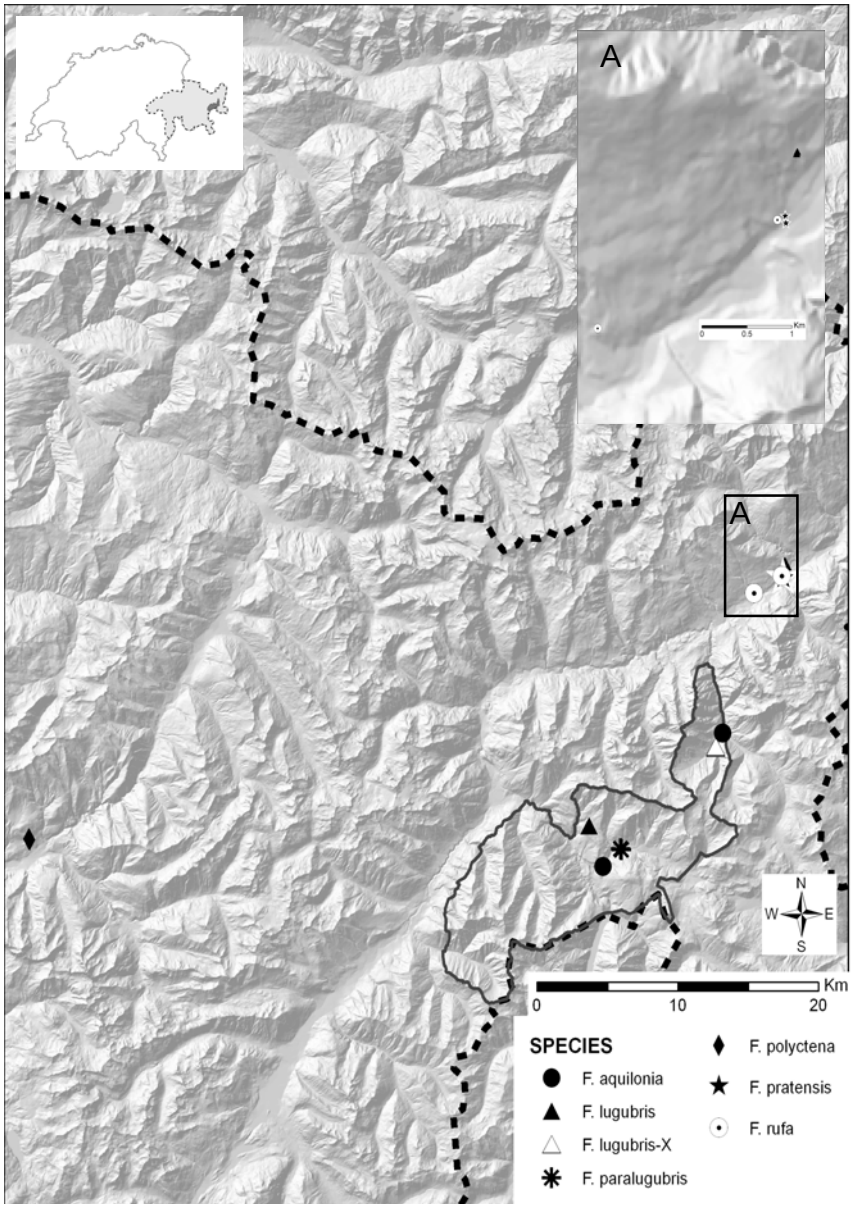
Valdecasas A.G., Williams D. and Wheeler Q.D. 2008. 'Integrative taxonomy' then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society*, 93, 211-216.

Vespäläinen K., Pisarski B. 1981. The taxonomy of the *Formica rufa* group: chaos before order. In: Biosystematics of Social Insects. Systematics Association Special Volume No.19 (P.E Howse and J.L. Clément, Eds), London: Academic Press. pp 27-36.

Walter F., Fletcher D.J.C., ChautemsD., Cherix D., Keller L. Franche W., Fortelius W., Rosengren R. and Vargo E.L. 1993. Identification of the sex pheromone of an ant, *Formica lugubris* (Hymenoptera, Formicidae). *Naturwissenschaften*, 80, 30-34.

Will K.W., Mishler B.D, Wheeler QD. 2005. The perils of DNA barcoding and the need for integrative taxonomy. *Systematic Biology*, 54, 844-851.

Figure 1. Location of the sampled nests in the Swiss National Park area. Black line: limits of the Swiss National Park. Dotted line: Swiss border. Geographical coordinates of each nest are shown in appendix 1.



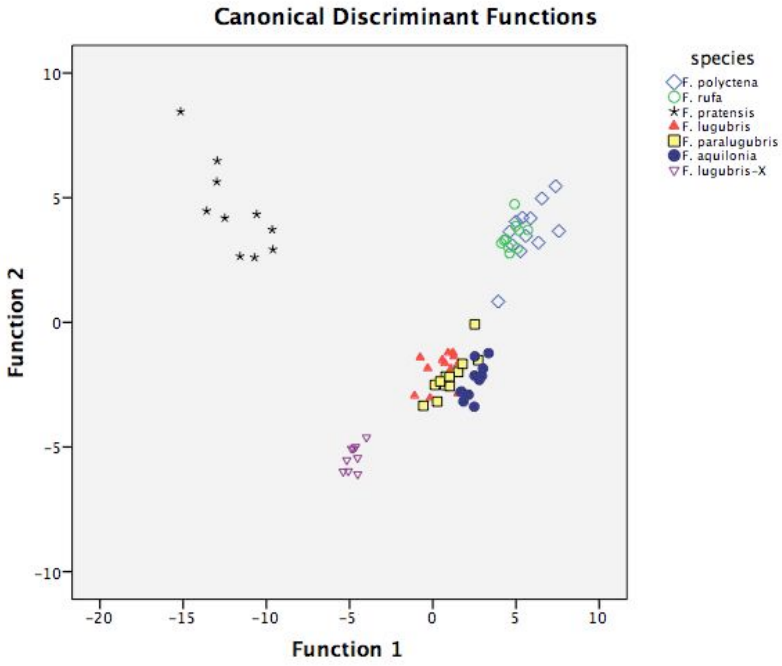


Figure 2. Cluster diagram of red wood ant virgin queens as differentiated by canonical discriminant analysis of the Dufour gland components (N=76).

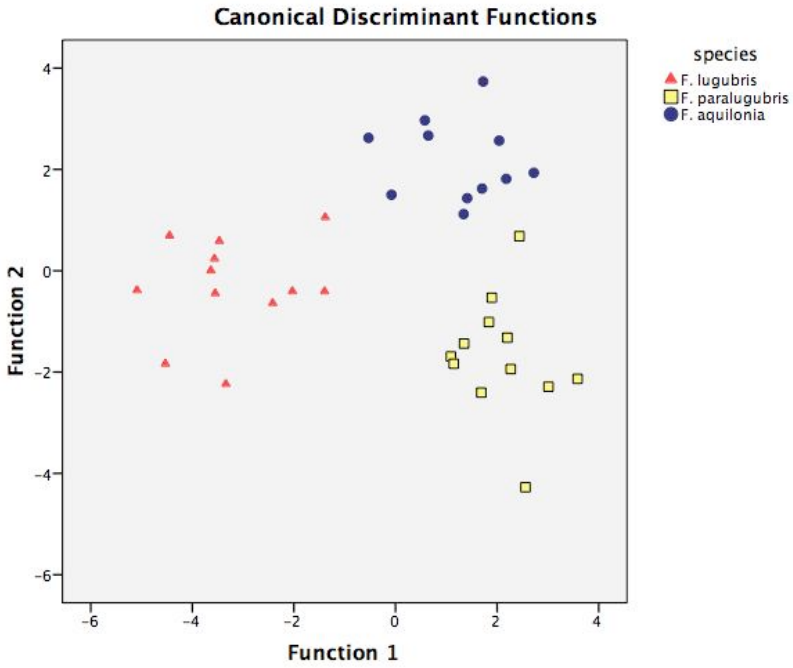


Figure 3. Cluster diagram of *F. lugubris* (N=12), *F. paralugubris* (N=12) and *F. aquilonia* (N=11) virgin queens as differentiated by canonical discriminant analysis of the Dufour gland components.

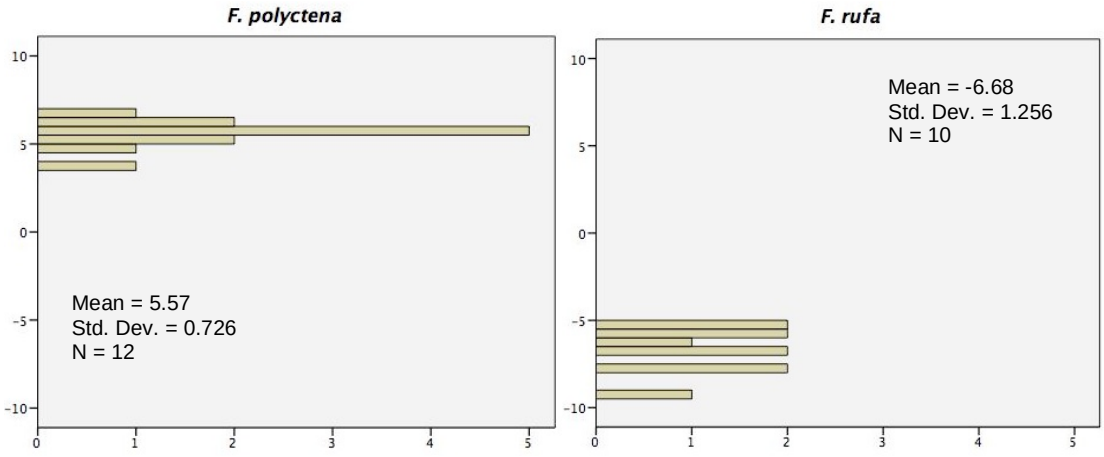


Figure 4. Histogram of *F. polyctena* and *F. rufa* virgin queens as differentiated by canonical discriminant analysis of 14 Dufour gland compounds.

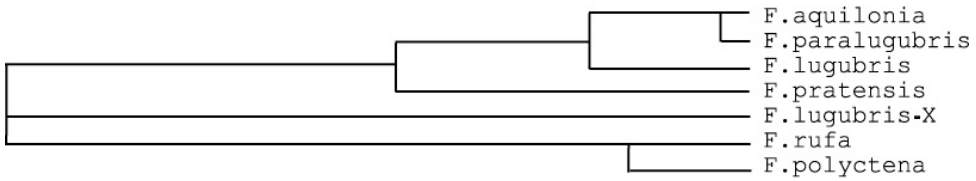


Fig. 5. Cluster analysis (Ward method) on the Dufour gland content (mean relative percentage of each component per species) of red wood ant species including *F. lugubris-X*.

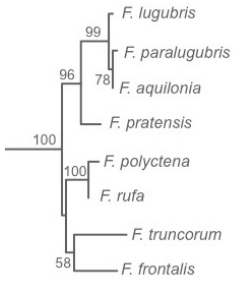


Fig. 6. Phylogeny of the *F. rufa* group species, based on mtDNA (adapted from Goropashnaya *et al*. 2004)

Table 1. Substances found in the Dufour glands of virgin queens of red wood ant species and of the cryptic species *F. lugubris-X*. « % » = relative percentage ; « SD » = Standard Deviation. N=number of virgin queens taken into account for statistical analyses. Empty cells (-) when the substance was not detectable. * = Putative compounds as indicated by our reference database.

Peak	Retention Time (min)	Compounds	*F. rufa* (N=10)		*F. polyctena* (N=12)		*F. pratensis* (N=10)		*F. aquilonia* (N=11)		*F. paralugubris* (N=12)		*F. lugubris* (N=12)		*F. lugubris-X* (N=9)	
			%	SD	%	SD	%	SD	%	SD	%	SD	%	SD	%	SD
1	3.186	Nonane	2.30	0.23	2.24	0.44	1.06	0.29	1.10	0.11	0.81	0.35	1.18	0.30	0.71	0.37
2	3.564	3-Methylnonane	0.06	0.01	0.08	0.04	-	-	-	-	-	-	-	-	-	-
3	3.696	Decane	3.78	0.64	3.26	0.36	2.12	0.41	2.51	0.25	2.25	0.51	2.77	0.77	2.07	0.69
4	4.163	Undecane	77.25	5.72	70.00	7.07	67.68	5.16	66.06	3.49	66.39	5.74	72.76	5.76	85.95	3.82
5	4.401	5-Methylundecane	1.08	0.44	0.98	0.59	0.19	0.10	0.40	0.26	0.41	0.17	0.35	0.24	0.18	0.09
6	4.468	3-Methylundecane	2.46	1.03	2.65	1.64	0.35	0.17	0.83	0.51	0.84	0.36	0.77	0.57	0.43	0.19
7	4.583	Dodecane	0.89	0.39	1.06	0.43	0.57	0.18	0.76	0.19	0.76	0.28	0.60	0.25	0.35	0.10
8	4.836	2-Methyldodecane	0.05	0.05	0.06	0.08	-	-	-	-	-	-	-	-	-	-
9	4.975	Tridecane	8.66	3.10	15.01	4.90	18.68	3.58	21.83	2.74	21.39	4.38	16.74	4.41	9.46	3.75
10	5.183	5-Methyltridecane	0.05	0.06	0.12	0.09	-	-	0.06	0.07	0.08	0.07	-	-	-	-
11	5.257	3-Methyltridecane	0.18	0.05	0.39	0.22	-	-	0.18	0.10	0.22	0.09	0.06	0.08	-	-
12	5.477	Tetradecane	0.15	0.12	0.23	0.17	0.07	0.10	0.27	0.14	0.22	0.09	0.22	0.11	-	-
13	5.685	Dodecanol	1.58	0.31	1.70	0.58	1.67	0.25	2.21	0.45	2.07	0.47	1.62	0.43	0.02	0.07
14	5.787	Trans-7-Pentadecene	0.02	0.02	-	-	0.15	0.07	0.40	0.19	0.30	0.08	0.14	0.08	0.03	0.08
15	5.825	Penatdecane	0.95	0.15	1.43	0.52	2.05	0.58	1.71	0.33	1.95	0.29	1.23	0.32	0.70	0.55
16	5.892	Bergamotene *	-	-	-	-	0.38	0.21	-	-	-	-	-	-	-	-
17	5.993	Farnesene *	-	-	-	-	2.22	0.56	-	-	-	-	-	-	-	-
18	6.981	8-Heptadecane	0.11	0.07	0.13	0.08	0.07	0.12	0.44	0.12	0.34	0.08	0.23	0.14	0.03	0.10
19	7.072	Docosane	0.45	0.09	0.67	0.18	0.61	0.19	0.47	0.10	0.60	0.08	0.50	0.25	0.06	0.19
20	7.262	Nonadecene *	-	-	-	-	2.12	0.44	0.79	0.35	1.36	0.38	0.85	0.42	-	-

Table 2. Results of the discriminant analysis of the six red wood ant species and *F. lugubris-X* made by using the relative proportions of Dufour gland compounds. * = Fisher's linear discriminant function ; **= Unstandardized canonical discriminant function coefficients.

	*Classification function coefficients**							*Canonical discriminant function coefficients***	
Compounds	*F. polyctena*	*F. rufa*	*F. pratensis*	*F. lugubris*	*F. paralugubris*	*F. aquilonia*	*F. lugubris-X*	Function 1	Function 2
Nonane	879.820	878.386	849.545	856.985	852.364	864.421	842.134	1.826	2.251
3-Methylnonane	19358.765	19290.585	18426.299	18795.564	18879.584	18986.523	18552.900	52.747	32.455
Decane	2403.332	2399.638	2367.816	2388.193	2384.192	2390.942	2394.479	1.618	-0.159
Undecane	2080.079	2074.437	2045.612	2060.109	2058.615	2067.310	2062.229	1.660	0.499
5-Methylundecane	2064.671	2138.263	1992.644	2048.081	2048.357	2080.753	1943.863	7.481	6.091
3-Methylundecane	2228.413	2205.888	2222.950	2226.228	2214.015	2216.940	2255.170	-0.892	-1.766
Dodecane	730.916	692.040	680.297	656.205	673.168	661.282	706.418	0.917	2.820
2-Methyldodecane	3971.182	3923.410	3842.224	3886.163	3910.980	3930.210	3925.027	5.438	-0.766
Tridecane	2086.816	2081.967	2053.833	2069.759	2067.008	2076.712	2070.148	1.626	0.344
5-Methyltridecane	3999.709	3980.541	3900.607	3943.666	3964.093	3965.358	3987.120	4.167	-1.604
3-Methyltridecane	1514.853	1460.588	1473.036	1471.110	1511.057	1514.715	1491.185	1.318	-1.680
Tetradecane	2885.409	2867.507	2740.687	2816.481	2836.229	2858.272	2744.200	8.876	3.563
Dodecanol	3132.885	3126.630	3054.474	3095.171	3098.493	3114.876	3064.916	4.642	1.930
Trans-7-Pentadecene	2240.380	2221.602	2218.546	2226.166	2245.056	2261.673	2224.993	1.248	-1.370
Penatdecane	2379.903	2373.310	2346.639	2347.502	2353.563	2356.850	2360.323	1.429	1.360
Bergamotene (putative)	5124.184	5110.707	4953.951	5073.435	5068.612	5060.797	5094.916	7.808	-2.774
Farnesene (putative)	1926.145	1922.333	1993.514	1915.151	1912.673	1924.758	1931.365	-3.745	3.642
8-Heptadecane	1651.798	1659.758	1625.917	1658.084	1655.720	1683.991	1667.505	1.613	-3.025
Docosane	2848.106	2832.170	2775.741	2803.700	2790.179	2791.635	2771.460	3.702	4.274
(Constant)	-1.049E5	-1.043E5	-1.014E5	-1.028E5	-1.027E5	-1.036E5	-1.030E5	-170.191	-55.356

Table 3. Results of discriminant analysis of *F. lugubris*, *F. paralugubris* and *F. aquilonia* samples by using Dufour gland compounds relatives proportions. * = Fisher's linear discriminant function ; **= Unstandardized canonical discriminant function coefficients

	*Classification function coefficients**			*Canonical discriminant function coefficients***	
Compounds	*F. lugubris*	*F. paralugubris*	*F. aquilonia*	*Function 1*	*Function 2*
Nonane	-680.983	-678.817	-671.439	0.951	2.117
Decane	2957.893	2946.977	2957.831	-1.407	2.503
Undecane	1978.444	1978.809	1986.601	0.621	2.153
5-Methylundecane	5852.309	5784.531	5834.775	-9.947	10.842
3-Methylundecane	894.770	892.786	896.706	-.0119	0.989
Dodecane	-1287.432	-1219.983	-1264.839	10.261	-9.379
Tridecane	1995.559	1992.354	2002.359	0.067	2.605
5-Methyltridecane	6597.317	6599.416	6632.307	2.734	9.110
3-Methyltridecane	-1762.657	-1659.845	-1687.897	18.481	-3.240
Tetradecane	3757.792	3802.603	3804.858	9.075	2.559
Dodecanol	3588.262	3584.203	3604.164	0.598	5.298
Trans-7-Pentadecene	1853.707	1884.868	1879.243	5.804	-.0193
Penatdecane	2330.580	2362.493	2353.648	5.727	-1.043
8-Heptadecane	2284.915	2248.407	2298.887	-3.709	12.261
Docosane	2355.950	2329.521	2330.191	-5.211	-0.961
(Constant)	-99018.927	-99025.826	-99822.187	-57.818	-218.861

Table 4. Results of discriminant analysis of *F. polyctena* and *F. rufa* samples by using relative proportions of Dufour gland compounds. * = Fisher's linear discriminant function ; **=Unstandardized canonical discriminant function coefficients

	*Classification function coefficients**		*Canonical discriminant function coefficients***
Compounds	*F. polyctena*	*F. rufa*	*Function 1*
Nonane	-1737.166	-1522.548	-17.522
3-Methylnonane	53019.253	50515.171	204.435
Decane	3402.099	3298.539	8.455
Undecane	2221.404	2192.706	2.343
5-Methylundecane	5662.065	5693.030	-2.528
3-Methylundecane	2278.487	2158.708	9.779
Dodecane	-7869.721	-7510.086	-29.361
2-Methyldodecane	4439.240	4348.666	7.395
Tridecane	3198.649	3134.451	5.241
5-Methyltridecane	12852.842	12297.208	45.363
3-Methyltridecane	-3104.958	-2797.400	-25.109
Tetradecane	-235.163	-104.538	-10.664
Dodecanol	783.923	814.866	-2.526
Trans-7-Pentadecene	-22140.193	-20923.953	-99.295
(Constant)	-1.100E5	-1.072E5	-223.092

Appendix 1. Identity, sampling location, latitude, longitude and altitude of the nests on which the analysed virgin queens have been collected.

Species	Nest identity	Locality	Latitude	Longitude	Altitude (m)
F. lugubris-X	MIN13	Scuol	46.72014	10.297102	1797
F. lugubris-X	MIN36	Scuol	46.720859	10.297153	1796
F. lugubris	SEN33	Sur-En	46.825643	10.367247	1267
F. lugubris	SEN32	Sur-En	46.825838	10.367349	1259
F. lugubris	C3	Zernez	46.677115	10.177239	2050
F. aquilonia	MIN4	Scuol	46.728815	10.304042	1689
F. aquilonia	SCR1	Zernez	46.653018	10.188852	1713
F. paralugubris	P5B	Zernez	46.662485	10.206422	1818
F. paralugubris	P5A	Zernez	46.662804	10.20585	1798
F. pratensis	SEN1	Sur-En	46.819977	10.365273	1134
F. pratensis	SEN2	Sur-En	46.819327	10.365342	1112
F. rufa	SEN18	Sur-En	46.819593	10.364124	1156
F. rufa	SEN22	Sur-En	46.810246	10.33742	1250
F. polyctena	ALV1	Alvaneu	46.680696	9.656256	1246
F. polyctena	ALV3	Alvaneu	46.680106	9.656964	1241

CONCLUSION

With the growing biodiversity loss, studies in conservation biology become essential and one of the major tasks in such works is to guarantee a correct species identification.

Given their importance in forested habitat, European red wood ants are considered among the most suitable species for monitoring forest ecosystems. Nevertheless, the classical method for the identification of these protected ants relies on morphological characters and, unfortunately, it requires lots of time and experience (Seifert 1996, 2007). Consequently, their correct taxonomy is sometimes ignored. In this work, we therefore employed a multidisciplinary approach to study the taxonomy of European red wood ants and to provide new tools for their identification. Our data, in agreement with Goropashnaya *et al*. (2004), confirm the species status of the six species known so far. In addition, our results revealed the existence of a new cryptic species within the Eastern Swiss Alps.

In the first chapter, published in *Myrmecological News* (Bernasconi *et al*. 2006), we analyzed the distribution of *F. lugubris* and *F. paralugubris* in the Italian Alps by collecting new samples on the field and by morphologically examining one of the major red wood ant collections, which is deposited at the University of Pavia, Italy. The collection was initiated by Professor Mario Pavan and Professor Giovanni Ronchetti and consists of about 2860 samples that were collected from about 500 stations within the Italian Alps (Pavan 1959, 1981, Ronchetti & Groppali 1995). Our work pointed out the importance of reference collections deposited in Natural History Museums and at the same time it confirmed that *F. paralugubris* is well distributed and often lives in sympatry with *F. lugubris* within the Italian Alps. Our data are very important, because the existence of the former species is unfortunately too often neglected. This is mainly due to the complexity of the identification method based on morphological characters (Seifert 1996).

In chapter two (submitted to *Systematic Entomology*), we therefore decided to develop a new taxonomic tool for helping in the discrimination of *F. lugubris* and *F. paralugubris*. The method is based on the mitochondrial COI gene and restriction enzyme, and its efficacy was confirmed with microsatellites. Our data showed that this method is highly reliable and allows rapid discrimination between these two sibling species. If we consider that *F. lugubris* and *F. paralugubris*, along with *F. aquilonia*, are the most abundant species in alpine coniferous forests, our work represents a clear breakthrough in red wood ant

species recognition and will be really helpful in future monitoring of these protected species. We recommend to employ this new tool whenever possible.
In accordance with chapter two, molecular markers have also proven their utility in species identification of various organisms and in biodiversity monitoring projects (e.g. Sharley *et al*. 2004; Smith *et al*. 2005; Ellis *et al*. 2006; Schwartz *et al*. 2006; Pfenninger *et* al. 2007). For that reason, we employed nine microsatellite loci and mitochondrial DNA to compare the species of the *F. rufa* group within the Swiss National Park region in chapter 3. We analyzed 83 nests belonging to all red wood ant species. According to Goropashnaya *et al*. (2004), genetic data indicated that these species represent different genetic pools. Moreover, results showed that *F. aquilonia* and *F. paralugubris* 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 the Mingèr Valley and morphologically identified as *F. lugubris*, is genetically different to all other analyzed *F. lugubris* populations found within the same area and to other red wood ant species. These findings suggest the existence of a new cryptic species within the Eastern Swiss Alps. This putative cryptic species has been named *F. lugubris-X* in chapter 3 and throughout the rest of this study.
As the existence of a new species can have a great influence on future conservation plans of these protected ants - and consequently on forested habitat - we decided to verify and complete molecular data by further analyses. In chapter 4, we used a behavioural test (called pupae carrying test) based on the capacity of workers to recognize pupae of their own species when compared to those of another species. In accordance to previous findings, behavioural data show that *F. lugubris-X* is different from *F. lugubris*, *F. aquilonia* and *F. paralugubris*. Then, as ultimate verification of our hypothesis, we conducted a chemical study on sex pheromones, produced by virgin queens of the entire *F. rufa* group, including *F. lugubris-X* (chapter 5). It was the first time that a chemical comparison was conducted on the whole group and on a local scale. Chemical data confirmed previous findings: the six red wood ant species known so far produce different sex pheromones. Our results are very important because they highlight for the first time mechanisms of reproductive isolation between species of the entire *F. rufa* group. These findings might promote further studies to check the specificity of these substances by behavioural essays on the field or to investigate on mating behaviour of red wood ants (see Maeder 2006).
In addition, sex pheromones of *F. lugubris-X* are significantly different from other red wood ant species. We therefore confirm that *F. lugubris-X* represents a new cryptic species of red wood ant.

The morphology-based alpha-taxonomy is still essential for assigning valid names to cryptic species (Schlick-Steiner *et al*. 2007). For that reason, in order to provide a morphological description of the new species, more detailed investigations are currently in progress in collaboration with Dr. Bernhard Seifert (Staatliches Museum für Naturkunde, Görlitz, Germany). Preliminary results based on discriminant analysis of morphological characters in queens are quite promising. Some morphological differences were indeed highlighted at least between queens of these two species (B. Seifert, pers. comm.).

The present situation is very similar to that which conducted to the description of *F. paralugubris* few years ago (Seifert 1996), when *F. lugubris* and *F. paralugubris* were still identified as a single species. After the discovery of two distinct morphotypes among *F. lugubris* queens (Kutter 1967, 1977), studies on alarm pheromones (Cherix 1983), allozymes (Pamilo *et al*. 1992) and behaviour (Rosengren & Cherix 1981; Rosengren *et al*. 1994) highlighted the existence of two different *F. lugubris* types in the Swiss Jura Mountains. One of these two groups was finally described as *F. paralugubris*, a sibling species of *F. lugubris* (Seifert 1996). As it has been the case for *F. paralugubris*, gathering data with different techniques is a very useful way to guarantee correct species identification and to perform taxonomical revisions. Such as studies should therefore be promoted in conservation biology, especially considering the ongoing biodiversity loss.

The presence of a new red wood ant species within the Swiss National Park is a fundamental result in terms of biodiversity and gives important information for future conservation plans. More precisely, we should now consider that *F. lugubris*, which is already listed as near threatened by the International Union for the Conservation of Nature, is indeed composed of at least two species. It is known that each species of the *Formica rufa* group has its own ecological preferences and it is necessary to have a precise knowledge of the ecology of each species for a better protection (Maeder 2006). In addition, the small effective population size and the low dispersal rate of some ants, make several species more easily threatened (Pamilo & Crozier 1997). It is therefore essential to improve our knowledge on the ecological needs of *F. lugubris-X*.

First of all more studies should be conducted to elucidate its geographical distribution: is *F. lugubris-X* present only in the Swiss National Park or also elsewhere? Is it well distributed or endangered? Is its presence connected to the high protection of the study area? At the same time, the European distribution of *F. lugubris* should be re-evaluated too, as it is probably more fragmented than previously thought.

We also believe that a phylogenetic revision of the *F. rufa* group is necessary to elucidate the origin of the new species.

Behavioural and chemical analyses seem to indicate that *F. lugubris-X* could be phylogenetically distant from the other species of the *F. rufa* group. In favour of this hypothesis, chemical and microsatellites data always located *F. lugubris-X* marginally and outside the *F. rufa* group. In addition, its sex pheromones composition is very similar to those of *Raptiformica sanguinea* and *F. truncorum*, two species that do not belong to the *F. rufa* group and which also have less substances compared to red wood ant species (C. Bernasconi, unpublished data). Moreover, in chapter 4, we have seen that *F. lugubris-X* workers often retrieve heterospecific pupae to their nest, a behaviour that is somehow similar to that of the slave-making ant *Raptiformica sanguinea*. Further analyses are now required to verify these results. Nevertheless, these findings are in contrast with genetic data.

In fact, molecular data obtained in this study clustered the mtDNA haplotype of *F. lugubris-X* with *F. paralugubris*, but microsatellite data indicate that *F. paralugubris* is nuclearly further separated from *F. lugubris-X* than from other *F. lugubris* individuals. These results suggest that *F. lugubris-X* could have originated recently through hybridization between *F. lugubris* and *F. paralugubris* or *F. aquilonia*. The divergence between *F. lugubris-X* and *F. lugubris* is similar to that observed between *F. paralugubris* and *F. lugubris*, which diverged about 100 thousand years ago (Goropashnaya *et al*. 2004). We can therefore suggest that *F. lugubris-X* probably originated at about the same period during the last glaciation in an alpine valley, which was not covered by ice. This hypothesis seems more likely. As for the origin of *F. paralugubris* (Goropashnaya *et 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 1991; Helms Cahan & Keller 2003; Schwander *et al*. 2008) and hybridization has also been suggested as a mechanism leading to speciation in these social insects (Nonacs 2006a,b) and other animals (Mavàrez *et 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 having no reproductive success at all (Nonacs 2006b).

Considering the particular situation of *F. lugubris-X*, 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-X* individuals, to the phylogenetic tree obtained by Goropashnaya *et al*. (2004).

Species of the *F. rufa* group should be particularly analyzed in the alpine region. Some authors have indeed highlighted the existence of scattered ice-free areas located within the Alps or at their periphery during the last glacial maximum. In particular, high levels of endemism have been found in the southern, southeastern, easternmost and northeastern Alps (Tribsch 2004). Numerous alpine species persisted and developed independently in these refugia, which are now seen as centres of alpine species diversity and endemism (Stehlik 2000; Stehlik 2003; Tribsch 2004; Schönswetter *et al*. 2005; Latalowa & van der Knaap 2006; Haubrich & Schmitt 2007; Parisod & Besnard 2007; Parisod 2008; Tollefsrud *et al*. 2008). Considering this particular situation and thanks to technical advances, more cryptic species of red wood ants might be discovered in alpine valleys in the future.

Our hypothesis seems to be confirmed by very recent findings. In fact, while writing this manuscript, some morphological investigations on red wood ant samples collected in the Southern Swiss Alps, pointed out the existence of another potential new red wood ant species (B. Seifert, unpublished data). The morphology of queens and workers of one particular population, located in Leventina Valley, Canton of Ticino, does not fit to any known species and might represent another cryptic species (B. Seifert, unpublished data). The hypothesis seems corroborated by genetic data (C. Bernasconi, unpublished data), but further analyses are now necessary to verify these findings. As for the case of *F. lugubris-X*, analyses on microsatellites, behaviour and sex pheromones could be very helpful and will be conducted soon.

Results presented in this work can also promote further studies on the other species of the *F. rufa* group. For example, considering the utility and the rapidity of genetic techniques for identifying species boundaries, more molecular investigations should be conducted on *F. pratensis* and its ecomorphs. In particular, we suggest clarifying the status of *F. nigricans*. Considered as a different species in the past, *F. nigricans* is today rather regarded as an ecomorph, thanks to morphological and ecological investigations conducted by Seifert (1992). We believe that molecular, behavioural and chemical data could add further information to this intriguing question.

At the same time we should investigate the status of the extremely hairy form of *F. lugubris* occurring within the Fennoscandian regions with an integrative approach. This “Hippie Ant” is frequently suspected to represent a separate species, but was finally classified as a morph of *F. lugubris* after morphological investigations (Seifert 2003).

In this work we provided a taxonomical revision of the *F. rufa* group and illustrated new reliable tools for correct species identification. However, we hypothesize that this taxonomy will change again in the future. In fact, considering the new findings obtained in collaboration with Dr. Bernhard Seifert and other data obtained on red wood ant populations in Finland (J. Saapunki & P. Pamilo unpublished data), we believe that this will happen soon. This could be mainly due to further technical advances, to the analyses of the numerous ecomorphs present in this group and to a more detailed investigation of the alpine valleys. We therefore agree with Maeder (2006) and believe that conservation efforts should not be directed towards a particular species only, but for a better protection of red wood ant species we should rather work to maintain a maximum of diversity at different levels, such as habitats, behaviours, social and genetic structures.

This work is an integrative study not only for the complementary techniques that we used, but also for the successful collaboration between the institutions in which this work has been realized: the University of Lausanne, the Museum of Zoology of Lausanne, the University of Oulu and, for chapter 5, the Institute of Legal Medicine of Lausanne. This study is a good example of fruitful collaboration between several researchers, which provides important taxonomical information, reliable tools for species identifications and future perspectives for a consequent conservation of red wood ant species.

REFERENCES

Bernasconi C., Maeder A., Freitag A., & Cherix D. 2006. *Formica paralugubris* (Hymenoptera, Formicidae) in the Italian Alps from new data and old data revisited. *Myrmecological News*, 8, 251-256.

Cherix D. 1983. Intraspecific variations of alarm pheromones between two populations of the red wood ant *Formica lugubris* Zett. (Hymenoptera, Formicidae). *Mitteilungen der Schweizerischen Entomologischen Gesellschaft*, 56, 57-65.

Ellis J.S., Knight M.E., Carvell C. & Goulson D. 2006. Cryptic species identification: a simple diagnostic tool for discriminating between two problematic bumblebee species. *Molecular Ecology Notes*, 6, 540-542.

Goropashnaya A.V., Fedorov V.B., Pamilo P. 2004. Recent speciation in the *Formica rufa* group ants (Hymenoptera, Formicidae): inference from mitochondrial DNA phylogeny. *Molecular Phylogenetics and Evolution*, 32, 198-206.

Haubrich K. & Schmitt T. 2007. Cryptic differentiation in alpine-endemic, high altitude butterflies reveals down-slope glacial refugia. *Molecular Ecology*, 16, 3643-3658.

Helms Cahan S. & Keller L. 2003. Complex hybrid origin of genetic caste determination in harvester ants. *Nature*, 424, 306-309.

Kutter H. 1967. Variationsstatische Erhebungen an Weibchen von *Formica lugubris* Zett. *Bulletin de la Société Entomologique Suisse*, 40, 63-77.

Kutter H. 1977. Formicidae-Hymenoptera. Insecta Helvetica. Entomologisches Institut der ETH.

Latalowa M. & van der Knaap W.O. 2006. Late Quaternary expansion of Norway spruce *Picea abies* (L.) Karst. in Europe according to pollen data. *Quaternary Science Reviews*, 25, 2780-2805.

Maeder A. 2006. Contribution to the conservation biology of the wood ant *Formica lugubris* (Hymenoptera, Formicidae) in Switzerland. PhD Thesis, University of Lausanne.

Mallet J. 2007. Hybrid speciation. *Nature*, 446, 279-283.

Mavarez J., Salazar C., Bermingham E., Salcedo C., Jiggins C.D. & Linares M. 2006. Speciation by hybridization in *Heliconius* butterflies. *Nature*, 441, 868-871.

Mavarez J. & Linares M. 2008. Homoploid hybrid speciation in animals. *Molecular Ecology*, 17, 4181-4185.

Nonacs P. 2006a. The ecology and evolution of hybridization in ants. *Ecology*, 87, 2141-2142.

Nonacs P. 2006b. Interspecific hybridization in ants: at the intersection of ecology, evolution and behavior. *Ecology*, 87, 2143-2147.

Pamilo P., Chautems D., Cherix D. 1992. Genetic differentiation of disjunct populations of the ants *Formica aquilonia* and *Formica lugubris* in Europe. *Insectes Sociaux*, 39, 15-29.

Pamilo P. & Crozier R.H. 1997. Population biology of social insect conservation. *Memoirs of the Museum of Victoria*, 56, 411-419.

Parisod C. 2008. Postglacial recolonization of plants in the Western Alps of Switzerland. *Botanica Helvetica*, 118, 1-12.

Parisod C. & Besnard G. 2007. Glacial *in situ* survival in the Western Alps and polytopic autopolyploidy in *Biscutella levigata* L. (Brassicaceae). *Molecular Ecology*, 16, 2755-2767.

Pavan M. 1981. Utilizzazione delle formiche del gruppo *Formica rufa* nella difesa biologica delle foreste. In : Studi sulle formiche utili alle foreste, Ministero dell'agricoltura e delle foreste, Roma. Collana verde 59.

Pearson B. 1983. Hybridization between the ant species *Lasius niger* and *Lasius alienus*, the genetic evidence. *Insectes Sociaux*, 30, 402-411.

Pfenninger M., Nowak C., Kley C., Steinke D. and Streit B. 2007. Utiliy of DNA taxonomy and barcoding for the inference of larval community structure in morphologically cryptic *Chironomus* (Diptera) species. *Molecular Ecology*, 16, 1957-1968.

Ronchetti G. and Groppali R. 1995. Quarantacinque anni di protezione forestale con *Formica lugubris* Zett. (Hym. Formicidae). L'esperienza di monte d'Alpe (Appennino ligure in provincia di Pavia). MURST, Istituto di entomologia dell'università di Pavia.

Rosengren R., Cherix D. 1981. The pupa carrying test as a taxonomic tool in the *Formica rufa* group. In: Biosystematics of Social Insects. Systematics Association Special Volume No.19 (P.E Howse and J.L. Clément, Eds), London: Academic Press. pp 263-281.

Rosengren R., Chautems D., Cherix D., Fortelius., Keller L. 1994. Separation of two sibling species of *Formica* L. ants by a behavioural choice test based on brood discrimination. *Memorabilia Zoologica* 48: 237-249.

Schlick-Steiner B., Seifert B., Stauffer C., Christian E., Crozier R.H. & Steiner F.M. 2007. *Trends in Ecology and Evolution*, 22, 391-392.

Schönswetter P., Stehlik I., Holderegger R., Tribsch A. 2005. Moecular evidence for glacial refugia of mountain plants in the Europena Alps. *Molecular Ecology*, 14, 3547-3555.

Schwander T., Suni S.S., Cahan S.H and Keller L. 2008. Mechanisms of reproductive isolation between an ant species of hybrid origin and one of its parents. *Evolution*, 62, 1635-1643.

Schwartz M., Luikart G. and Waples R.S. 2006. Genetic monitoring as a promising tool for conservation and management. *Trends in Ecology and Evolution*, 22, 25-33.

Seifert B. 1991. The phenotypes of the *Formica rufa* complex in East Germany. *Abhandlungen und Berichte des Naturkundemuseum Görlitz,* 65, 1-27.

Seifert B. 1992. *Formica nigricans* Emery, 1909 – an ecomorph of *Formica pratensis* Retzius, 1783 (Hymenoptera, Formicidae). *Entomologica Fennica*, 2, 217-226

Seifert B. 1996. *Formica paralugubris* nov. spec. – a sympatric sibling species of *Formica lugubris* from the western Alps (Insecta: Hymenoptera: Formicoidea: Formicidae). *Reichenbachia Museum Tierkunden*. Dresden 31: 193-201.

Seifert B. 2003. The « Hippie Ant » - A case of extreme intranidal polymorphism in Fennoscandian *Formica lugubris*. *Sociobiology*, 42, 285-297.

Seifert B. 2007. Die Ameisen Mittel- und Nordeuropas. Lutra Verlags- und Vertriebsgesellschaft, Görlitz.

Sharley D.J., Pettigrove V. and Parsons Y. 2004. Molecular identification of *Chironomus* spp. (Diptera) for biomonitoring of aquatic ecosystems. *Australian Journal of Entomology*, 43, 359-365.

Smith A., Fisher B.L. and Hebert P.D.N. 2005. DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society B London*, 360, 1825-1834.

Stehlik I. 2000. Nunataks and peripheral refugia for alpine plants during quaternary glaciation in the middle part of the Alps. *Botanica Helvetica*, 110, 25-30.

Stehlik I. 2003. Resistance or emigration? Response of alpine plants to ice ages. *Taxon*, 52, 499-510.

Tollefsrud M.M., Kissling R., Gugerli F., Johnsen Ø., Skrøppa T., Cheddadi R., van der Knaap W.O., Latalowa M., Terhürne-Berson R., Litt T., Geburek T., Brochmann C. & Sperisen C. 2008. Genetic consequences of glacial survival and postglacial colonization in Norway spruce: combined analysis of mitochondrial DNA and fossil pollen. *Molecular Ecology*, 17, 4134-4150.

Tribsch A. 2004. Areas of endemism of vascular plants in the Eastern Alps in relation to Pleistocene glaciation. *Journal of Biogeography*, 31, 747-760.