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A new taxonomic status for *Iberoformica* (Hymenoptera, Formicidae) based on the use of molecular markers

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Abstract

The subgeneric subdivision of the genus *Formica* is still open. In this article, we make a phylogenetic study on several species of the genus *Formica* and of its closely related genera, *Polyergus* and *Proformica*, using sequences of nuclear satellite DNA (stDNA) and the mitochondrial *rrnL* as molecular markers. Our goal was to shed light on their phylogenetic relationships and particularly on the systematic position of *F. subrufa*. This species was first included in the subgenus *Serviformica,* but afterwards a new subgenus (*Iberoformica*) was established to include only this species. The results show that a stDNA family previously reported in *Formica* species, with a repetitive unit 129 bp long, is also found in *Polyergus rufescens* and *P. samurai* but not in *Proformica longiseta*. This is the first case of presence of a stDNA family in two different ant genera. In *F. subrufa,* this stDNA is very divergent relative to those isolated in the remaining *Formica* species and in the genus *Polyergus*. The Bayesian analysis of mitochondrial *rrnL* sequences shows three highly supported groups: *F. subrufa,* the remaining *Formica* species studied, and the genus *Polyergus,* suggesting that parasites (*Polyergus* species) and hosts (*Formica* species) are closely related but not sibling species. The combined analysis of nuclear stDNA sequences and mitochondrial *rrnL* showed their phylogenetic congruence despite their distinct evolutionary dynamics. This analysis did not discriminate between the remaining *Formica* species that were not grouped according to the subgeneric classification. According to these results, it can no longer be assumed that *F. subrufa* belongs to the subgenus *Serviformica* or of the *fusca* species group. This differentiation was also supported by previous studies based on the morphological characters, molecular and cytogenetic data. Therefore, taking into consideration these arguments and others explained in detail in this article, we propose that the taxon *Iberoformica*, formerly synonymized subgenus, be raised to a genus status. This genus would be monotypic and only composed, up to the moment, by *Iberoformica subrufa* (= *F. subrufa* Roger, 1859).

Key words: Satellite DNA – mitochondrial DNA – molecular phylogenies – *Iberoformica* new status

Introduction

The taxonomic classification of the genus *Formica* Linnaeus, 1758 is still disputed. For European *Formica* species have been distinguished four subgenera (*Raptiformica* Forel, 1913, *Coptoformica* Muller, 1923, *Serviformica* Forel, 1913, *Formica* s. str.) or five subgenera (including *Iberoformica* Tinaut, 1990) (e.g. Donisthorpe 1943; Creighton 1950; Dlussky 1967; Tinaut 1990) whereas others considered only species groups (e.g. Wheeler 1922; Collingwood 1979; Agosti 1994). The problem was whether to accept the subgenus as a valid category or use a more cautious classification with the informal species group, in which the problem of the monophyletic category does not affect the species group organization. Agosti (1994) proposed eliminating all subgenera and waiting for future systematic analyses to determine their validity. One of the most controversial issues was the position of *F. subrufa* Roger 1859, which was included in the *fusca* species group (Wheeler, 1913) or in *Serviformica* subgenus (Santschi, 1919) and finally included in a new monotypic subgenus *Iberoformica* (Tinaut, 1990).

A stDNA family was isolated in eight *Formica* species (*Formica* 129 bp satellite) and used to infer evolutionary relationships among four subgenera: *Serviformica* (*F. cunicularia* Latreille, 1798, *F. fusca* Linnaeus, 1758, *F. rufibarbis* Fabricius, 1793 and *F. selysi* Bondroit, 1918), *Raptiformica* (*F. sanguinea* Latreille, 1798), *Formica* s. str. (*F. frontalis* Santschi, 1919) and *Iberoformica* (formerly *F. subrufa*) (Lorite et al. 2004). Satellite DNA (stDNA), a highly repetitive DNA characteristic of eukaryotic genomes, is composed of a sequence unit tandemly arranged in long and abundant arrays in the constitutive heterochromatin (Palomeque and Lorite 2008). Previously, several molecular studies have demonstrated that monomer sequences indicate phylogenies similar to those found with classical markers, and particularly with ribosomal markers, as both exhibit concerted evolution (e.g. Pons and Gillespie 2004; Palomeque and Lorite 2008; Martinsen et al. 2009). The consequence of the concerted evolution is an intra-specific variability lower than the inter-specific divergence. Hence, stDNA may be used as an alternative phylogenetic nuclear marker when the sequence of the repetitive unit is conserved across related species (Meštrović et al. 2009). In all *Formica* species studied, this stDNA family was organized as tandemly repeated 129-bp monomers (Lorite et al. 2004). Phylogenetic analysis placed all stDNA sequences of *Formica* in a highly supported monophyletic group, with the exception of the sequences from *F. subrufa*. Hence, the 129-bp stDNA from *F. subrufa* was considered a subfamily of the 129-bp stDNA family from the other *Formica* species (Lorite et al. 2004).

On the contrary, a molecular phylogenetic study of 20 *Formica* species using partial mitochondrial cytochrome-b sequences clustered them according to subgeneric classifications (Goropashnaya 2003). However, other studies using another mitochondrial region, NADH dehydrogenase subunit 6 and tRNA serine (Goropashnaya et al. 2004a,b), have not agreed in some cases with those results (Seifert and

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Goropashnaya 2004). None of these molecular studies included the controversial *F. subrufa*.

Our main goal is to clarify the taxonomic position of *F. subrufa* by using a classical phylogenetic marker, the 16S mitochondrial DNA (*rrnL*). This marker has not been used in *Formica* yet although it has been used successfully in other Hymenoptera (Tanaka et al. 2001; Taylor et al. 2011). Here, we also included *rrnL* sequences of other *Formica* species and species from the genera *Polyergus* Latreille, 1804 and *Proformica* Ruzsky, 1902. Molecular studies have shown that the genus most closely related to *Formica* is *Polyergus* while *Proformica* is closely related to the *Polyergus-Formica* clade (Hasegawa et al. 2002; Brady et al. 2006; Moreau et al. 2006). We also investigated whether sequences of the stDNA family previously described in *Formica* are found in those closely related genera, and, if so, whether they could be used as nuclear phylogenetic markers. The genus *Polyergus* consists of five species (Bolton 2011), all obligatory social parasites of *Formica* species. Social parasitism is the coexistence of two species of social insects in the same nest, one of the species being parasitically dependent on the other (Tinaut and Ruano 1999; Buschinger 2009). It is widespread in the social bees, wasps and especially in ants (reviewed by D'Ettorre et al. 2002). Although the mechanism of obligatory social parasitism is not well known, it has been considered a consequence of chemical interactions between hosts and parasites (reviewed by Lenoir et al. 2001; Buschinger 2009). In Hymenopteran social parasitism, two life-history classes can be differentiated. Inquilines are social parasites that live inside a host colony but without harming adults, queens or workers of the host species, whereas slave makers (known only in ants) live in a host nest in which the queen of the host has been killed and only the host workers are left; periodically, the slave makers workers raid nearby host colonies and rob their larvae and pupae (Ruano and Tinaut 2004). Emery (1909) noted that social parasites are closely related to their host. In principle, obligate parasites may originate by sympatric speciation as parasites from their host's lineage or through a combination of allopatry and secondary sympatry (Lowe et al. 2002). The so-called loose version of Emery's rule suggests that social parasites may be close relatives of their host but not necessarily their sister species. In this case, social parasites could evolutionarily have arisen inter-specifically from a lineage differing from that of their hosts or intra-specifically from the same lineage as their hosts and subsequently could have undergone a speciation process when new host species were successfully colonized (Tinaut and Ruano 1999; Buschinger 2009).

The slave makers, as *Polyergus*, are obligatory social parasite species in which both queens and workers lack the capacity of brood rearing, foraging and colony maintenance. The host workers perform all the necessary work for the maintenance and development of the community. When the captive *Formica* larvae and pupae workers emerge in the *Polyergus* nest, they take the parasite colony for their own colony. This fact is considered as an imprinting-like effect (Mori et al. 1991; Le Moli et al. 1994). In addition, when the founding queen of *Polyergus* usurps a host *Formica* colony, the *Formica* workers are induced somehow to accept the *Polyergus* queen. *Polyergus rufescens* Latreille, 1798 parasitizes species from the genus *Formica* subgenus *Serviformica*, *F. rufibarbis* and *F. cunicularia*, which are the usual host species, and *F. selysi*, which is a non-natural host (D'Ettorre et al. 2002). Finally, *Polyergus samurai* (the Japanese slave-making ant) parasitizes *F. japonica* Motschoulsky, 1866 and *F. hayashi* Terayama & Hashimoto, 1996 (Liu et al. 2003).

Material and Methods

Material and DNA extraction

Several ant species were used in this study: *Formica cunicularia, F. fusca* and *F. subrufa* (Jaén, Spain); *F. frontalis* and *Proformica longiseta* Collingwood, 1978 (Granada, Spain); *F. rufibarbis* and *Polyergus rufescens* (Tours, France), *Formica selysi* (Morillon, France), *F. sanguinea* (Tuscany, Italy) and *Polyergus samurai* (Kyoto, Japan). A population was analysed for each species. A pull of 10–15 adult worker ants were used for genomic DNA extraction according to the technique of Heinze et al. (1994). Because the number of available individuals from *Polyergus samurai* was very low, only two workers were used for DNA extraction in this species.

Satellite-DNA isolation, cloning, sequencing and Southern blot

The sequences of the 129 stDNA family from *Formica* species used here were obtained elsewhere (Lorite et al. 2004; EMBL accession numbers from AJ238724 to AJ238728, AJ238730 to AJ238732, AJ308973 to AJ308981, AJ308985, AJ308988 and AJ508813 to AJ508878).

Polyergus rufescencs genomic DNA was restricted with a battery of endonucleases, and fragments were separated by electrophoresis on 2% agarose gels. stDNA fragments produced by the digestion of genomic DNA with *Sau*3A were eluted from agarose gel and inserted into the *Bam*HI site of pUC19 vector. The ligation mix was used to transform *E. coli* DH5 alpha competent cells that allow the blue-white selection of successful ligation of DNA into the vector. Another portion was digoxigenin labelled by random priming with the DIG system (Roche) and used as hybridization probes in plasmids screening. Plasmids were isolated from white bacterial colonies, and recombinants plasmids yielding positive hybridization signals were directly sequenced on both strands by the dideoxy sequencing method. Genomic DNA of *Proformica longiseta* was also digested with the same restriction endonucleases used in the *P. rufescens* stDNA isolation. None of these endonucleases generated a characteristic ladder of stDNA in this species.

The 129-bp stDNA was also amplified in *P. rufescens* and *P. samurai* by polymerase chain reaction (PCR) using the primers Formica-3 (5′-GCCTGTAAGTGAGATTTGCC) and Formica-4 (5′- CACGT AACTAAGTCGTTCCG), which were designed on stDNA sequences of *Formica* (Lorite et al. 2004). The same procedure was used with genomic DNA from *Proformica longiseta*. The PCRs were carried out using very different annealing conditions, even at very low annealing temperatures.

For Southern analysis, electrophoresed DNA from agarose gels was transferred onto a nitro-cellulose membrane. Hybridization of membranes was performed with the clone PORU-21 labelled with digoxigenin (20 ng ml^{-1}), and the final stringency of the hybridization was determined with a final wash in 2xSSC at 60°C (DIG-detection kit, Roche).

Satellite-DNA sequence analysis

Multiple-sequence alignment was performed using the CLUSTAL W program (Thompson et al. 1994). Nucleotide diversity and pairwise sequence divergences were estimated using the DnaSP program (Rozas and Rozas 1999). The nucleotide diversity in each species was calculated as the average number of nucleotide substitutions per site between two sequences with the Jukes and Cantor's correction, Pi (JC) (Jukes and Cantor 1969). Sequence divergences were calculated as the average nucleotide substitutions per site between species (D_{xy} value from DnaSP, Nei 1987; equation 10–20). The number of fixed differences between species (i.e. nucleotide sites at which all of the sequences in one species differ from all the sequences in the second species) was also determined in DnaSP program. Neighbour-joining (NJ) and maximum-parsimony (MP) analyses were conducted using MEGA 4.0 (Tamura et al. 2007). Support values were determined by bootstrap analyses with 1000 replicates by NJ and 100 by MP.

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Mitochondrial-DNA amplification, cloning, and sequencing

All mitochondrial sequences used in the phylogenetic analysis were obtained in this work. As indicated earlier, the DNA from a pull of 10–15 individuals (two for *P. samurai*) of the same nest was used for mitochondrial-DNA amplification. The 3′ end of the mitochondrial *rrnL* gene (16S rDNA) was amplified by PCR using the primers 16SWa (5′-CGTCGATTTGAACTCAAATC) and 16SWb (5′-CACCTGT TATCAAAAACAT), which performed successfully in several ant species (Dowton and Austin 1994, 2001). PCR amplifications were carried out using the following cycling profile: initial denaturing at 92°C (2 min); 35 cycles at 92°C (20 s), 50°C (1 min), 72°C (3 min); and a final elongation step of 72°C for 3 min. Reactions were set up in a 50-μl mixture containing 100 ng of genomic DNA, 5% DMSO, 0.5 mM dNTPs, 40 pmol of each primer, and 1 U of Taq polymerase. PCR products were eluted from the agarose gel, cloned into the pGEMT Easy vector (Promega). The ligation mix was used to transform *E. coli* DH5 alpha competent cells. Plasmids were isolated from white bacterial colonies and were directly sequenced on both strands by the dideoxy sequencing method.

Two different PCRs were carried out, and therefore, two different sequences from the mitochondrial *rrnL* gene were obtained for each species. The number of differences among the sequenced clones of each *Formica* species was low (0–0.17%), and they can be considered characteristic of each species. There is more similarity between sequences from the same species than between sequences of different species (data not shown). Only one of the obtained sequences was used in all subsequent studies. Furthermore, the results were identical regardless which of the two sequences was selected.

Mitochondrial-DNA sequence analysis

Multiple alignment of *rrnL* sequences was performed using the CLUSTAL W program but then was manually refined based on the 16S rRNA secondary-structure models (Buckley et al. 2000; Gillespie et al. 2006). The *rrnL* sequences were alternatively aligned with MAFFT (Katoh et al. 2005) using the Q-INS-i algorithm because it takes into account secondary structure to build multiple alignment. For some phylogenetic analyses, gaps were recoded as binary states in Gapcoder (Young and Healy 2003), and in others, the poorly aligned regions were removed with Gblocks (Castresana 2000). Bayesian analyses were conducted using MrBayes version 3.1 (Ronquist and Huelsenbeck 2003). The General Time Reversible model with a γ-distributed substitution rate (GTR + G) was the best evolutionary model for the *rrnL* sequences according to Modeltest and the Akaike Information Criterion. Recoded gaps were treated as variable morphological characters with binary states (presence/absence). Each Bayesian search performed two independent runs with prior default values, unlinked parameters among partitions (nucleotide data versus recoded gaps), starting random trees, and three heated and one cold Markov chains running for two million generations, sampled at intervals of 1000 generations. Burn-in and convergence of runs were assessed by examining the plot of generations against likelihood scores using the *sump* command in MrBayes, and with the program Tracer v.1.4 (Rambaut and Drummond 2007). After burning, trees from both runs were combined in a single majority consensus topology using the *sumt* command in MrBayes, and the frequencies of the nodes in a majority-rule tree were taken as *a posteriori* probabilities (Huelsenbeck and Ronquist 2001). Finally, we performed a combined analysis of the *rrnL* sequence and the consensus stDNA sequence of each species (70% majority rule) to test the congruence of their phylogenetics using Partition Bremer Support (Baker and DeSalle 1997; Baker et al. 1998).

Results

Isolation and study of satellite DNA

After the digestion of genomic DNA from *Polyergus rufescens* with *Sau*3A, a weak ladder of 130-bp stDNA was observed. The generated bands were cloned, and recombinant plasmids were named PORU. Recombinant clones yielding strong positive signals were directly sequenced in both strands. Three clones (PORU-21, -22 and -25) were selected for further studies (EMBL accession nos. AM910821 to AM910823). The genomic DNA restricted with the endonucleases, *Xba*I, *Sau*3A, *Msp*I and *Alu*I, was examined by Southern blot (Fig. 1a) using PORU-21 insert as a probe. Hybridization results showed a ladder of multimers that is characteristic of the restriction of tandemly repetitive DNAs.

The monomer sequences of *P. rufescens* had the same length (129 bp) and about 80% identity with the 129-bp stDNA isolated previously in *Formica* ant species (Lorite et al. 2004). We also isolated new stDNA sequences in *P. rufescens* by PCR using primers designed on the most conserved region of *Formica* 129-bp stDNA (Lorite et al. 2004). The primers allowed the amplification of fragments of different sizes (Fig. 1b); 260-bp fragments were constituted by two incomplete monomers and a complete monomer of the stDNA in the middle (Fig. S1). Consequently, only fragments with 260 bp or higher were cloned. Five new clones were sequenced (PORU-31, -61, -63, -72 and -101) (EMLB accession nos. AM910824 to AM910828). The same primers amplified sequences of this stDNA family in *Polyergus samurai* (Fig. 1c). Seven clones were sequenced (POSA-40, -41, -43, -51, -53, -62 and -63), and the monomers found were also 129 bp long (Fig. S1) (EMBL accession nos. AM910829 to AM910835).

On genomic DNA from *Proformica longiseta,* none of the used endonucleases generated a ladder of 130 bp. Besides this, no amplification bands were obtained for the genomic DNA of *P. longiseta* despite that very different annealing conditions were used. Both results would indicate that the 129-bp stDNA family is absent in its genome. It is probably that another stDNA family was present in the *P. longiseta* genome, but until this moment, we have been unable to found an endonuclease that cut their stDNA.

The divergences (between sequences of two different species), the nucleotide diversities (within sequences of the same species) and the number of fixed differences between the stDNA in all analysed species are showed in Table S1a. The number of fixed differences is indicative of the gradual spreading of new species-specific variants and the subsequent divergence of stDNA families of the two species (Dover 2002). The stDNA from both *Polyergus* species are very similar, and

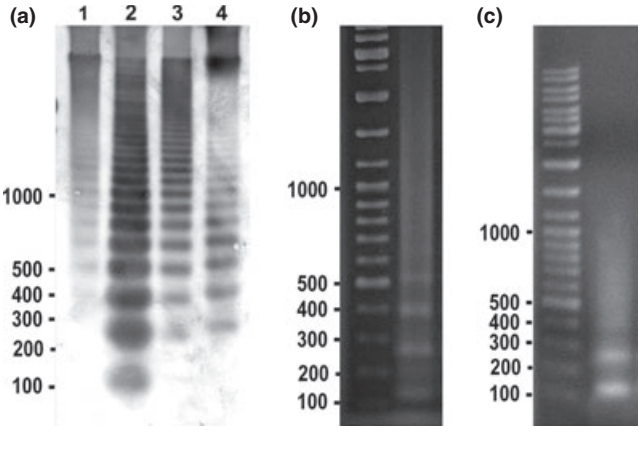

Fig. 1. (a) Southern blot of restricted *Polyergus rufescens* genomic DNA using PORU-21 insert as probe. Restriction enzymes used are *Xba*I (lane 1), *Sau*3A (lane 2), *Msp*I (lane 3) and *Alu*I (lane 4). Amplification PCR bands using the primers Formica-3 and Formica-4 on genomic DNA from *Polyergus rufescens* (b) and *P. samurai* (c)

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no fixed differences were found between them. The higher DNA divergences and the largest number of fixed differences are found in all comparisons involving *F. subrufa*. Therefore, *F. subrufa* was shown to be the most divergent one, while the rest of *Formica* ('*Formica* spp.' hereon) species turned out much closer to *Polyergus* species (Table S1a). Similar results are shown in cases in which species were compared at the group level (Table S1b).

The DNA divergences between stDNA from *P. rufescens* and stDNA from *F. cunicularia* and *F. rufibarbis*, their usual host species, were slightly higher than the DNA divergences between *P. rufescens* and the other *Formica* species considered in this article, except in relation to *F. subrufa*. The lowest DNA divergence was observed between *F. selysi* and *P. rufescens*, although *F. selysi* is considered a non-natural host of *P. rufescens*.

The topology of the NJ tree constructed using stDNA sequences is in accordance with the aforementioned data (Fig. 2). The sequences of *F. subrufa* comprise a highly supported monophyletic cluster (99% bootstrap value) that is sister to two clusters: one including all sequences of *Polyergus* with low support (54%) and the other all sequences of '*Formica* spp.' but with no support (< 50%). Within those clusters, the sequences appeared intermixed at the species level. Like topologies and supports were found using the MP method (data not shown). When the consensus sequence for each species was used to build NJ and MP trees, the same clades are observed (data not shown).

Phylogenetic study of mitochondrial *rrnL* sequences

The amplified fragments of the mitochondrial *rrnL* gene varied in size, ranging from 548 to 666 bp (EMBL accession nos. AM910836 to AM910845, FR750263, FR865866 to FR865875). In the analysed species, *rrnL* sequences were 80% AT rich, in agreement with the data reported for other arthropods (Whitfield and Cameron 1998). The manual correction of the CLUSTAL alignment based on the putative secondary structure of the mitochondrial rRNA 16S (Buckley et al. 2000; Gillespie et al. 2006) revealed that variable-length regions correspond to the RNA loops: e.g. helices H1835 and H2077 in agreement with the nomenclature of Gillespie et al. (2006). However, the most problematic segment to align was the hypervariable and AT-rich region comprising the helices H2347–H2395. In fact, several different models of secondary structure have been proposed for this region (revision in Gillespie et al. 2006) or have remained unpaired (Kambhampati et al. 1996).

Since the alignment of loops is complex, *rrnL* sequences were aligned with the Q-INS-i algorithm implemented in MAFFT because it takes into account the secondary structure to build the multiple alignment. In addition, we used several strategies to investigate the effect of those ambiguously aligned and gappy regions on the phylogenetic signal: (1) MAFFT-aligned sequences, (2) MAFFT aligned but removing poorly aligned regions with Gblocks, (3) MAFFT plus gap information also recoded as binary states and (4) the latter but removing poorly aligned regions. The recoding of gaps considers as identities (i.e. primary homologies) only those gaps coinciding at the 5′ and 3′ ends (Simmons and Ochotorena 2000).

The Bayesian analysis of *rrnL* sequences shows *Proformica* to be clearly separated from the other species according to previous molecular phylogenetic studies, showing that *Proformica* is a clade close to *Polyergus* and *Formica*, but clearly differentiated from them (Hasegawa et al. 2002; Moreau et al. 2006). The same results, with those three groups, were retrieved regardless of how gaps and ambiguous data were treated or recoded. Likewise, this topology was also obtained using stDNA sequences. However, in this case, the clades are strongly supported (> 90%): the genus *Polyergus*, '*Formica* spp.' and finally *F. subrufa,* which are sister to all the others (Fig. 3). There was also another strongly supported clade within *Formica*, with *F. fusca* and *F. rufibarbis*. The Bayesian analysis of the combined dataset of *rrnL* and the consensus stDNA sequence at the species level displayed similar topology and support values (Fig. 4). The partition Bremer support test revealed the congruence of the phylogenetic signal of both markers although stDNA sequences showed lower support levels. In addition, there was also congruence between the signal from conserved *rrn*L regions and that from loop sequences in which gaps were recoded as binary characters (presence/absence).

Discussion

In this article, we present a phylogenetic study on several species of the *Formica* genus (*F. cunicularia, F. fusca, F. rufibarbis, F. selysi F. sanguinea, F. frontalis* and *F. subrufa*), the *Polyergus* genus (*P. rufescens* and *P. samurai*) and *Proformica longiseta,* based on stDNA and mitochondrial *rrnL* sequences as molecular markers.

This study reveals that *Polyergus rufescens* and *P. samurai* share the same stDNA family with *Formica* species but not with *P. longiseta*. In addition, there is no species specificity within the genus *Polyergus* (i.e. sequences are intermixed across species), as has been observed in *Formica* and in other ant genera (Lorite et al. 2002b). Consequently, in ants, the stDNA is a poor phylogenetic marker at species level. Although there are few studies on stDNA in ants, published results suggest that stDNA is conserved only at what is taxonomically accepted as the genus level (reviewed by Palomeque and Lorite 2008). The case presented here is the first example known till now of a stDNA family shared by two different (admittedly, closely related) ant genera.

The ants belonging to the genus *Polyergus* are obligatory social parasites of *Formica* species. According to the strict version of Emery's rule (Emery 1909), slave makers and slaves should be sister taxa. However, as mentioned above, the so-called loose version of Emery's rule suggests that social parasites may be close relatives of their host but not necessarily their sister species. The stDNA data show that the nucleotide divergence between *Polyergus rufescens* and its usual host species (*F. rufibarbis* and *F. cunicularia*) is not greater than the divergence with other species of *Formica* studied (e.g. *F. selysi*, which it is considered a non-natural host) in accordance with the loose version of Emery's rule. Similar results have been obtained by different authors (Hasegawa et al. 2002).

The most unexpected result is that there are more differences between the stDNA of *F. subrufa* and the remaining *Formica* species (referred as '*Formica* spp.') than between these species and stDNA from *Polyergus* (Table S1). To clarify the taxonomic position of *F. subrufa*, we analysed the 16S mtDNA. The analysis of *rrnL* mtDNA sample revealed three highly supported groups (Fig. 3): *F. subrufa,* '*Formica* spp.', and finally the clade of *Polyergus* species, clearly showing that

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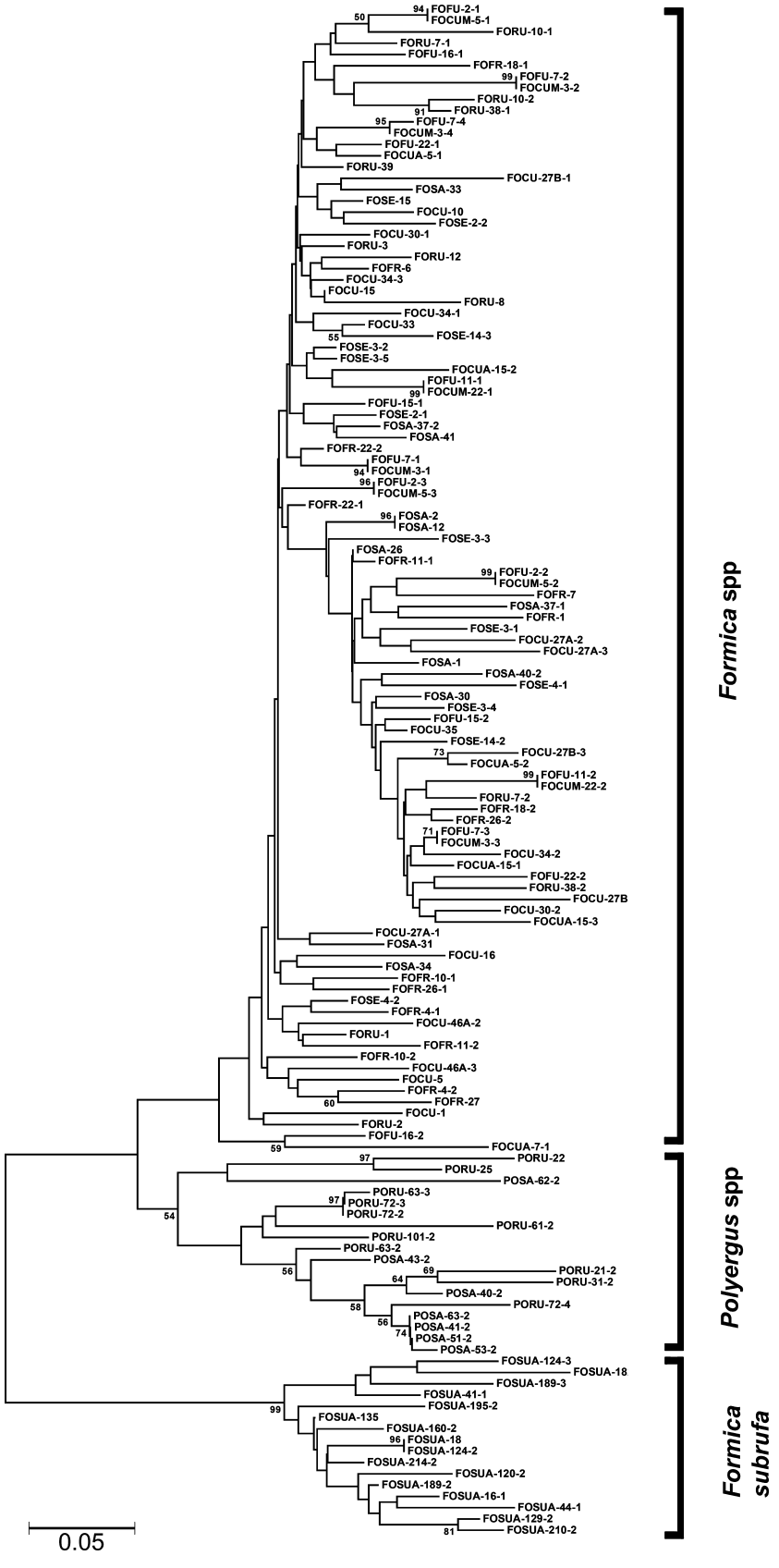

Fig. 2. Neighbour-joining phylogenetic tree of satellite-DNA monomers from species from the genus *Formica*, *Polyergus rufescens* (PORU) and *P. samurai* (POSA). The *Formica* species used were as follows: *F. fusca* (FOFU), *F. cunicularia* (FOCU, FOCUM and FOCUA), *F. rufibarbis* (FORU), *F. frontalis* (FOFR), *F. selysi* (FOSE), *F. sanguinea* (FOSA) and *F. subrufa* (FOSUA). The sequences designated 1, 2 and so on are monomeric units from the same clone. Numbers indicate bootstrap values over 50% in 1000 replications

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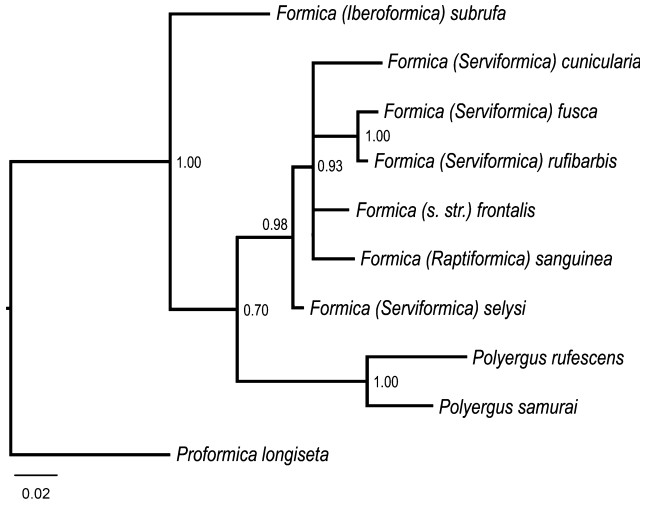

Fig. 3. Bayesian tree obtained using *rrnL* sequences (after recoding gaps as binary characters and removing gappy regions within loops with Gblock). Numbers indicate the *posteriori* probabilities of the Bayesian analysis. Brackets indicate the subgenus name for the Formica species

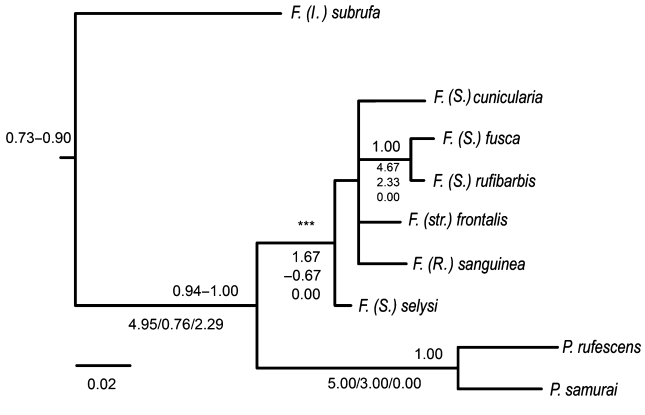

Fig. 4. Bayesian tree obtained with the combined analysis of *rrnL* sequences (after recoding gaps as binary characters and removing gappy regions within loops with Gblock), and consensus satellite-DNA sequences. Numbers above nodes indicate the range of Bayesian credibility values from four different analyses: (1) MAFFT-aligned sequences, (2) MAFFT aligned but removing poorly aligned regions with Gblocks, (3) MAFFT plus gap information also recoded as binary states and (4) the latter but removing poorly aligned regions. Numbers below nodes indicate the partition of Bremer supports from three regions in a parsimony analysis: *rrnL* sequences without poorly aligned positions, recoded information of gaps, stDNA sequences. Brackets indicate the subgenus name for the *Formica* species

parasites (*Polyergus* species) and hosts (*Formica* species) are closely related but not sister species. These results are also in accordance with a loose version of Emery's rule. The *rrnL* DNA does not discriminate between the remaining *Formica* species that are not grouped according to the subgeneric classification. Only *rrnL* DNA indicates that *F. fusca* and *F. rufibarbis* are the most closely related species among the *Formica* species analysed. Identical phylogenetic relationships were found despite the reconstruction model implemented or the molecular marker used (stDNA, consensus-specific stDNA and *rrnL* mtDNA sequences). Moreover, analyses indicate that the phylogenetic signal is fully congruent, even that from loops that are AT biased and varied in length. In summary, the phylogenetic signal was robust.

It is assumed that the mitochondrial DNA has a constant mutation rate and therefore could be used not only to determine the phylogenetic relationships among related species but also the time at which the species diverged (Arbogast et al. 2002). By contrast, the mutation rate of stDNA is more variable. Clearly, the stDNA family described may have been present in a common ancestor of *Polyergus* and *Formica*, whose divergence seems to have occurred more the 44 million years ago (Moreau et al. 2006). According to the *rrnL* DNA results, it is tempting to assume that in the evolutionary line of the analysed species, *F. subrufa* separated first from the common ancestor of other *Formica-Polyergus* species. This early separation could allow the rapid accumulation of differences in the stDNA in relation not only to species of the genus *Formica* but also to the species of *Polyergus*. The stDNA evolution appears to be a partially stochastic process. There are examples in which stDNA has diverged between different populations of one species and others in which the stDNA has been conserved for very long evolutionary periods (reviewed by Ugarković and Plohl 2002; Palomeque and Lorite 2008).

The evolutionary relationships between the different species of the genus *Formica* are currently controversial. The species analysed belong to the subgenera *Serviformica* (*F. cunicularia*, *F. rufibarbis*, *F. fusca*, *F. selysi*), *Formica* s. st. (*F. frontalis*), *Raptiformica* (*F. sanguinea*) and *Iberoformica* (*F. subrufa*). The stDNA and the *rrnL* DNA do not clarify the relationships among the different *Formica* species. However, both molecular markers, one of them nuclear (stDNA) and other mitochondrial (*rrnL* DNA), with different evolutionary dynamics showed the presence of three highly supported groups: genus *Polyergus*, '*Formica* spp', and finally *F. subrufa*, which are sister to all the others. In addition, *F. subrufa* shows morphological features that differentiate it from other species of the genus *Formica*. Differential characteristics are found in all three castes (Tinaut 1990). Among them, workers present the nodiform petiole and concave mesonotum. Both characters are very remarkable as the remaining species of the genus *Formica* present a distinctly scale-like petiole, and the thoracic profile shows no depression along the dorsal region. Mesothorax and metathorax of *F. subrufa* queens are less developed than other species for the genera, with the first gastral segment almost rectangular, giving it a resemblance to the workers. Finally, the males are similar in size to the workers, with the petiolar node low and triangular. Tinaut points out as the main difference the peculiar morphology of the male genitalia, especially the sagittae and the volsellae. In addition, *F. subrufa* has a different chromosome number ($n = 26$) than the found in *Serviformica* ($n = 27$) (Lorite et al. 2002a; Lorite and Palomeque 2010). Thus, the morphological characters (Tinaut 1990), the cytogenetic data (Lorite et al. 2002a; Lorite and Palomeque 2010) and the molecular markers (Lorite et al. 2004, and this paper) all indicate that *F. subrufa* is clearly differentiated not only from the *Serviformica* species (the subgenus to which *F. subrufa* was once ascribed) but also from the species of other *Formica* subgenera.

According to Ward (2011), the goals of Systematics include the discovery and delimitation of clades and species, the estimation of the phylogenetic relationships among taxa, and the establishment of a classification reflecting this information. In this article, we show that both stDNA and *rrnL* DNA

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invariantly suggest that *F. subrufa* represents a separate clade from the two monophyletic clades: *Polyergus* and the clade including the remaining *Formica* species. These results are consistent with the cytogenetic, molecular and morphological results reported elsewhere; therefore, to retain *F. subrufa* in the subgenus *Serviformica* would cause the genus *Formica* to be paraphyletic. Ward (2011) indicates that two requirements are necessary to consider a clade as formal taxon: first, that molecular data strongly support it as a monophyletic group, and second that it presents phenotypic features that allow it to be distinguished from related taxa. Both conditions are given for *F. subrufa*. Therefore, we propose that the taxon *Iberoformica*, formerly described as a subgenus of *Formica* Linnaeus, 1758 and even synonymized under it (Agosti 1994), should now be raised to the status of genus. This genus is currently monotypic, only including *Iberoformica subrufa* (Roger 1859). Our results do not allow conclusions to be drawn about the validity of the other subgenera of *Formica*.

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Resumen

Un nuevo estatus taxonómico para Iberoformica (Hymenoptera, Formicidae) *basado en marcadores moleculares*

La división en subgéneros del género *Formica* es aún controvertida. En este trabajo realizamos un estudio filogenético de varias especies del género *Formica*, así como de especies relacionadas de los géneros *Polyergus* y *Proformica*, usando como marcadores moleculares el DNA satélite nuclear y el *rrnL* mitocondrial. Nuestro objetivo es aclarar las relaciones filogenéticas y la posición sistemática de *F. subrufa*. Esta especie se incluyó inicialmente en el subgénero *Serviformica* pero después se estableció un nuevo subgénero (*Iberoformica*), que incluía solo a esta especie.

Los resultados muestran que la familia de DNA satélite, descrito previamente en las especies de *Formica*, con unidades repetitivas de 129 pb, también está presente en *Polyergus rufescens* y en *P. samurai*, pero no en *Proformica longiseta*. Es el primer caso de presencia de una misma familia de DNA satélite en dos géneros diferentes de hormigas. Las secuencias de este DNA satélite en *F. subrufa* son claramente diferentes de las aisladas en las otras especies de *Formica* y de *Polyergus*. El análisis Bayesiano, usando las secuencias de DNA mitocondrial *rrnL,* muestra tres grupos altamente soportados: *F. subrufa*, las demás especies estudiadas de *Formica*, y las del género *Polyergus*, sugiriendo que las especies parásitas (del género *Polyergus*) y sus hospedadores (del género *Formica*) son especies relacionadas pero no especies hermanas. Los análisis conjuntos del DNA satélite nuclear y el DNA mitocondrial *rrnL* muestran que hay concordancia filogenética, a pesar de que ambos marcadores tienen una dinámica evolutiva distinta. Este análisis no es capaz de discriminar entre las restantes especies de *Formica*, que no se agrupan de acuerdo a la clasificación de subgéneros.

A la vista de estos resultados no se puede seguir asumiendo que *F. subrufa* pertenezca al subgénero *Serviformica* o al denominado 'grupo *fusca*'. Anteriores estudios morfológicos, citogenéticos y moleculares apoyan también esta diferenciación. Por tanto, y teniendo en cuenta estos argumentos, y otros que se explican en detalle en este trabajo, se propone elevar el taxón *Iberoformica*, un subgénero que ha sido sinonimizado, a la categoría de género. Este género sería monotípico y compuesto, por el momento, por *Iberoformica subrufa* (= *F. subrufa* Roger, 1859).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Multiple-sequence alignments of all satellite-DNA sequenced clones from *Polyergus rufescens* (PORU) and *P. samurai* (POSA). Monomer consensus sequence is shown. The primers used are also shown (underlined). The sequence primers have been eliminated from the sequence alignments.

Table S1. Satellite DNA. (a) Nucleotide diversity, Pi (JC), in each species (diagonal) and pairwise divergence, Dxy (JC), values between species. (b) Pi (JC) in all species of *Formica* analysed except *F. subrufa* (*Formica* spp.), in *Polyergus rufescens* and *P. samurai* (*Polyergus* sp.) and in *F. subrufa* and the corresponding Dxy (JC) between them. The number of fixed differences is shown in brackets.

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