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Nest composition and worker relatedness in three slave-making ants of the genus *Rossomyrmex* Arnoldi and their *Proformica* Ruzsky hosts (Hymenoptera, Formicidae)

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Abstract In this paper, we analyze and compare nest composition and architecture as well as worker relatedness in three related species of slave-making ants: *Rossomyrmex anatolicus*, *R. minuchae*, and *R. quandratinodum*. Colony structure within nests is an important trait in ants, especially in the case of mixed societies, when host and parasite coexist in the same nest. Data for their respective free-living hosts, *Proformica korbi*, *P. longiseta* and *P.* sp., are also provided. For our study, we integrated a meticulous excavation procedure with a genetic method. We conclude that the average number of parasites, as well as of slaves, is species-specific, whereas nest depth depends on the nest architecture of the host. The genus *Rossomyrmex* seems to be monogynous and monandrous, whereas *Proformica* shows differences in the number of queens and mating frequency. *R. quandratinodum* shows different traits in nest composition (host/parasite ratio: P/R) and architecture. The difference in traits may account for some differences in parasitism: raid process or avoidance of parasitism.

Key words nest architecture, nest composition, *Proformica*, relatedness, *Rossomyrmex*, slave-making ants

Introduction

A basic aspect in the study of social insects is to know their colony structure, especially the number of queens. Wilson (1993) describes the number of queens as "like some anatomical features…shaped by multiple influences during evolution…". Many ant species are monogynous (single-queen colonies) while others are polygynous (several queens). Polygyny is considered to arise from monogyny through the adoption of new queens in different ecological and physiological conditions (Goropashnaya *et al.*, 2001), such as nest site limitation or high risks associated with independent nest founding. Polygyny is also associated with weaker nestmate recognition as compared to that of monogyny (Bourke & Franks, 1995). Thus, estimating worker–worker relatedness may approximate the average effective number of reproducing queens in colonies as well as mating frequency (Savolainen & Sëppa, 1996). This method is helpful given that queens are often missing during excavation of nests and this can mislead data. Moreover, other traits are fundamental to the knowledge of social structuring, such as the number of workers and the architecture of the nest, both being modulated by multiple influences during

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evolution. The number of workers may be directly related to the number of queens (Elmes & Keller, 1993) while the architecture of the nest is determined by environmental variables. Both traits involve a major phylogenetic component common to a genus or related genera, as these usually share similar nest architecture (Ruano & Tinaut, 1993; Mikheyev & Tschinkel, 2004) and composition (Baroni-Urbani *et al.*, 1978; Baroni-Urbani & Pisarski, 1978). In parasite–host societies, two species generally from different genera, live together in the same nest. Cohabitation can engender conflicts of interest since obligated parasite workers do not perform colony maintenance tasks (Hölldobler & Wilson, 1990). They may also present different preferences for specific nest architecture because of a different phylogenetic constraint. Therefore, parasitized and free-living nests could differ in composition and architecture.

The ants of the genus *Rossomyrmex* Arnoldi are slave-makers that invade nests of the genus *Proformica* Ruzsky to steal brood. Lacking their own queen, the *Proformica* brood then grow as slaves (Arnoldi, 1932; Ruano & Tinaut, 1999). There are four species known in the genus *Rossomyrmex*: *R. proformicarum* Arnoldi from the Caspian steppes (Russia), *R. minuchae* Tinaut in Sierra Nevada (Spain), *R. quandratinodum* Xia and Zheng in the region of Urumchi (China), and *R. anatolicus* Tinaut from the Anatolian steppes (Turkey). The parasitic life, the short activity period, and low number of raids per nest are assumed to occur in the whole genus (Marikovsky, 1974; Ruano & Tinaut, 1999, 2004). These traits together with the isolated and distant distribution areas of the genus make them very difficult to find and work with; thus, their biology and distribution are not fully understood (Arnoldi, 1932; Marikovsky, 1974). Marikovsky (1974) referred to *R. proformicarum* but we consider the data to be applied to *R. quandratinodum* (Tinaut *et al.*, 2008). More recently, a considerable number of articles have focused on the different aspects of the biology and behavior of *R. minuchae* (see Ruano & Tinaut, 1999 references), presently the best-known species of the genus. For *R. anatolicus*, only the aspects related to its taxonomy are known to-date (Tinaut, 2007).

In the present paper, we provide new data and analyze differences in nest composition (number of slaves and slave-makers), worker relatedness and inner nest architecture in *R. anatolicus*, *R. quandratinodum* and *R. minuchae* and also in the non-parasitized nests of their respective *Proformica* host species. In terms of relatedness estimates, we aimed to test the reliability of our excavating method in finding queens and obtaining information on colony structure that may have gone unnoticed by random excavation of nests.

Material and methods

Field methods

Five nests of *Rossomyrmex anatolicus* were excavated in May 2008 from two different populations in Anatolia (Turkey). The first corresponds to the type location: Belembaçi Beli (37°53′N, 32°22′E), north-west of Konya, where we collected three complete nests. The second comes from Ziyaret Tepesi (38°49′N, 36°54′E), in the province of Sivas where Schulz and Sanetra (2002) had mentioned the presence of *R. minuchae*. In the second site, we only found *R. anatolicus* after excavating two complete nests and another one partially (not included in this paper). Moreover, two free-living nests of *Proformica korbi* (Emery), host of *R. anatolicus*, were excavated in Belembaçi Beli. For *R. quandratinodum*, three nests were excavated in June 2007 located in the steppes between the Ily and the Charyn rivers (43°22′N, 49°01′E), about 200 km north-east from Almaty (Kazakhstan), near the Charyn canyon. Also, the data of Marikovsky on four nests were included in this analysis (nests 1–4 in Table 1). In this region, another two free-living nests of the host species called *Proformica* sp. were excavated. Seven nests of *R. minuchae* and another seven nests of free-living *P. longiseta* Collingwood studied here were located in Sierra Nevada (Granada, Spain; 37°07′N, 32°54′W) and excavated between 1993 and 1997.

The excavation procedure of the nest, similar to that described in Plaza and Tinaut (1989), consisted in making a deep rectangular trench of about 20 cm far from the entrance of the nest. From this position, we moved forward through the nest with uniform vertical cuts, in layers, so that we can easily detect its length, the main gallery and the chambers. This method allowed us to follow the main gallery to the end and therefore its chambers, giving us an expected high certainty that the nests have been excavated completely, including the queen(s). The whole population, including existing broods, was collected with an aspirator. The entire procedure under standard conditions required approximately 1.5–2.0 h. Collected ants were counted and installed in the laboratory or stored in 99% ethanol.

Genetic analyses

Five to eight workers per colony were genetically analyzed. Total DNA was extracted from workers using the Puregene DNA Isolation Kit (Gentra Systems Minneapolis, MN, USA). *Rossomyrmex* workers were genotyped at 11 microsatellite loci: Ccur11, Ccur46, Ccur63b, Ccur76, Ccur89, Ccur99, FE11, FE19, FE21,

Table 1 Colony structure, nest depth and worker relatedness of mixed colonies. Data of nests 1–4 of *R. quadratinodum* come from Marikovsky 1974, and the rest are the authors'.

	Locality	Queens	*Rossomyrmex* workers	*Proformica* workers	P/R ratio	Nest depth (cm)	*Rossomyrmex* relatedness
R. minuchae	Spain						
Nest 1		–	116	537	4.63	–	–
Nest 2		1	142	414	2.92	40	–
Nest 3		1	64	387	6.05	–	–
Nest 4		1	122	803	6.58	42	–
Nest 5		1	235	2 252	9.58	36	–
Nest 6		1	81	414	5.11	32	–
Nest 7		1	128	710	5.55	33	–
Mean ± s.e.		1	126.86 ± 20.77	788.14 ± 251.34	5.77 ± 0.77	36.60 ± 1.64	0.72 ± 0.10
R. quandratinodum	Kazakhstan						
Nest 1 (Marikovsky)		–	200	650	3.25	132	–
Nest 2 (Marikovsky)		–	23	65	2.83	120	–
Nest 3 (Marikovsky)		1	76	594	7.82	88	–
Nest 4 (Marikovsky)		1	53	84	1.58	48	–
K1N1		1	23	43	1.87	85	0.83
K1N2		–	15	21	1.4	50	0.40
K1N3		1	21	30	1.43	38	0.36
Mean ± s.e.		1	58.71 ± 24.97	212.43 ± 106.23	2.88 ± 0.87	80.14 ± 13.87	0.53 ± 0.13
R. anatolicus	Turkey						
T1N2		1	95	389	4.09	30	0.78
T1N3		1	133	893	6.71	43	0.75
T1N4		1	66	621	9.41	22	0.73
T2N1		1	50	362	7.24	29	0.84
T2N2		1	49	177	3.61	30	0.48
Mean ± s.e.		1	78.60 ± 15.94	488.40 ± 123.31	6.21 ± 1.07	30.80 ± 3.40	0.72 ± 0.03

– indicates the absence of queen. Relatedness for *R. minuchae* was calculated from 26 nests that were not included in the table. P/R, *Proformica/Rossomyrmex*.

FE37 and FE51 (Gyllenstrand *et al.*, 2002; Pearcy *et al.*, 2004). *Proformica* workers were genotyped at seven loci: Ccur11, Ccur26, Ccur63, Ccur76, FE19, FE37 and FL12 (Chapuisat, 1996). Microsatellites were amplified by polymerase chain reaction (PCR) in a 10 μL reaction containing 2.5 ng of DNA, 0.2 nmol/L of each primer, 0.25 nmol/L of each deoxynucleotide triphosphate (dNTP), 1 × MBL buffer (MBL, Cordoba, Spain) and 2.5 mmol/L of $MgCl_2$ (overall), 0.5 units/μL of MBL *Taq* DNA polymerase and 7 μL of distilled water. PCR conditions were 94°C for 3 min to denature the samples, followed by 11 touchdown temperature cycles of denaturing at 94°C for 15 s, annealing at 55–45°C for 30 s and extension at 72°C for 30 s, followed by 30 cycles at 94°C for 15 s, 50°C for 15 s and 72°C for 30 s. PCR products were genotyped at Unidad de genómica. Scai (University of Córdoba) using an ABI310 sequencer.

We scored genotypes using GENESCAN software v3.7 (Applied Biosystems, Foster City, CA, USA). The genetic data of *R. minuchae* and *P. longiseta* do not correspond to excavated nests but to more recent samplings in the same population (summers 2004–2007). Relatedness for *R. minuchae* was calculated over 26 nests and 10 nests were used for *P. longiseta*.

Statistical analyses

Most of the variables studied followed a normal distribution. However, because of the relative low number of samples (19 *Rossomyrmex* nests and 12 of *Proformica*), (Tables 1 and 2), we applied the Kruskal-Wallis test to detect differences in means. The Mann-Whitney *U*-test was used to test the significance of differences between

Table 2 Colony structure, nest depth and worker relatedness in free-living nests of *Proformica*.

	Locality	Queens	Workers	Nest depth (cm)	Relatedness
Proformica longiseta	Spain				
Nest 1		1	478	30	–
Nest 2		3	565	16	–
Nest 3		4	316	45	–
Nest 4		1	470	42	–
Nest 5		4	453	48	–
Nest 6		1	622	55	–
Nest 7		–	69	55	–
Mean ± s.e.		2.33 ± 0.57	424.71 ± 69.46	41.57 ± 5.35	0.27 ± 0.02
Proformica sp.	Kazakhstan				
PK1N4		1	359	130	0.66
PK1N2		–	218	110	0.69
Mean ± s.e.		1	288.5 ± 70.50	120 ± 10	0.67 ± 0.02
Proformica korbi	Turkey				
PT1N1		1	178	12	0.20
PT1N2		1	88	15	0.09
Mean ± s.e.		1	133 ± 45.00	13.50 ± 1.50	0.14 ± 0.05

– indicates the absence of queen. Relatedness for *P. longiseta* was calculated from 10 nests that were not included in the table.

groups. Spearman's rank correlation was applied to test correlation among variables (STATVIEW 5.0). All measures are expressed in the text as mean ± s.e. Worker nestmate relatedness was calculated using the software RELATEDNESS 5.0 (Queller & Goodnight, 1989) weighting nests equally. Standard errors were obtained by jack-knifing over loci. Expected and observed values were compared with *t*-tests.

Results

Nest architecture

Nests of *Rossomyrmex* consisted of one entrance with several shallow galleries (1–5 cm deep), leading to a single vertical gallery with small lateral chambers. This vertical gallery ended in a final chamber, slightly larger than the others, in which the queen is usually found. In relation to nest depth, Marikovsky provided no specific value for *R. quandratinodum* (= *R. proformicarum sensu* Marikovsky), but his data on chamber distribution implied a depth of approximately 70–100 cm, consistent with the nests that we excavated in the same study area (Table 1). There were significant differences for nest depth among the examined *Rossomyrmex* species (Kruskal-Wallis test, $P = 0.005$), with *R. quandratinodum* being the species with significantly the deepest nests (Mann-Whitney *U*-test *vs. R. minuchae* $P = 0.012$;

vs. R. anatolicus $P < 0.01$). No significant differences for this variable were found between *R. minuchae* and *R. anatolicus* (Fig. 1).

Nest architecture of free-living *Proformica* was similar to those nests parasitized by their corresponding *Rossomyrmex*. Overall, we found no significant differences in depth between parasitized and non-parasitized nests (Kruskal-Wallis test, $P > 0.5$). We found significant differences in nest depth among *Proformica* species (Kruskal-Wallis test; $P = 0.02$), with *P.* sp. having the deepest nests (120 ± 10 cm) and *P. korbi* (host of
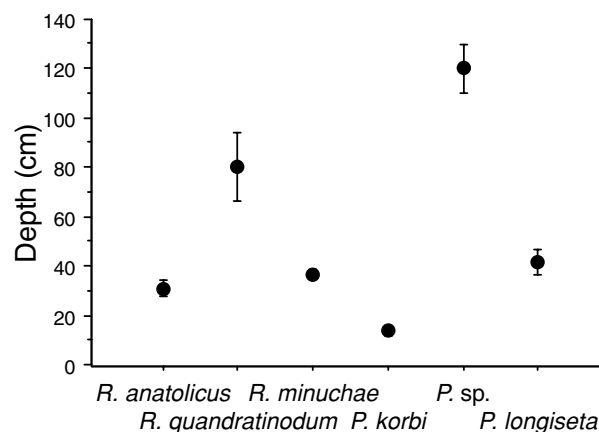
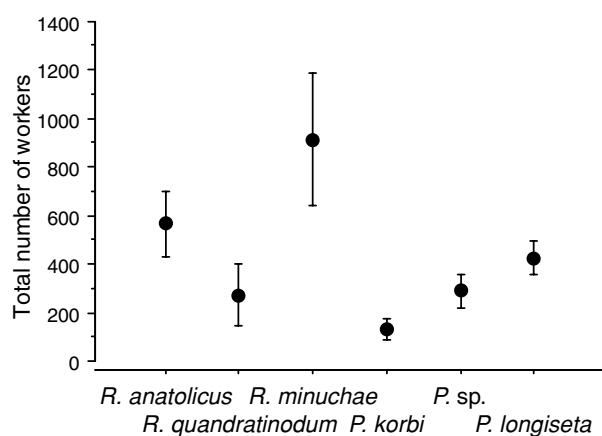**Fig. 1** Depth of mixed and free-living nests (bars mean ± s.e.).

Fig. 2 Number of workers in mixed and free-living nest (bars mean ± s.e.).

Fig. 3 Mean ratio of *Proformica* and *Rossomyrmex* workers per nest (bars mean ± s.e.).

R. anatolicus) having the shallowest nests (13.5 ± 1.5 cm). *P. longiseta*, host of *R. minuchae*, had intermediate nest depths (41.6 ± 5.3 cm) (Mann-Whitney *U*-test *P. longiseta vs. P. korbi* and *P.* sp., $P = 0.04$) (Table 2, Fig. 1).

Nest composition

In 15 *Rossomyrmex* nests, one queen was found, whereas in the others (4) there was none (Table 1). The mean overall number of workers found in parasitized nests was 89.30 for *Rossomyrmex* and 497.15 for *Proformica*. Nests of *R. minuchae* were the most populated for parasites and slaves, followed by *R. anatolicus*. In *R. quandratinodum*, excavated nests generally consisted of a very low number of workers of both species, similar to the lowest data from Marikovsky (1974) (Table 1). We found significant differences in the numbers of workers in the nests of each *Rossomyrmex* species (Kruskal-Wallis test, $P = 0.04$). Significance is due principally to differences between *R. minuchae* and *R. quandratinodum* (Mann-Whitney *U*-test, $P = 0.02$), where *R. anatolicus* is the intermediate (Fig. 2). Nevertheless, the relationship between the number of *Proformica* slave and *Rossomyrmex* slave-maker workers (P/R ratio) significantly varied among species (Kruskal-Wallis test, $P = 0.03$). Again, the means were similar between *R. anatolicus* and *R. minuchae* and both were significantly higher than in *R. quandratinodum* (Fig. 3) (Mann-Whitney *U*-test, $P = 0.03$).

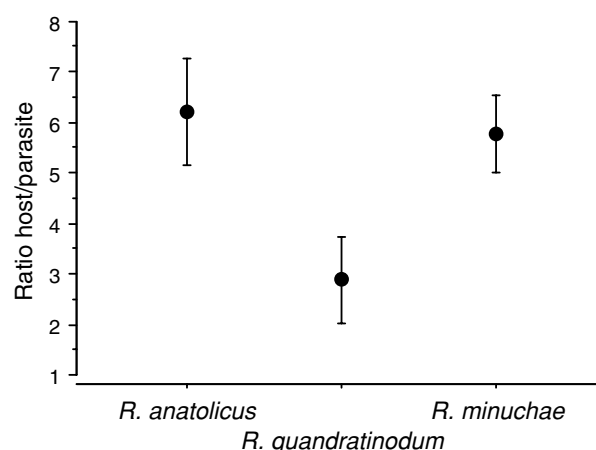From the host species, and as a result of the excavation procedure, all but *P. longiseta* seemed to be monogy-nous (Table 2). *Proformica korbi* had the lowest number of workers, while the highest number corresponded to *P. longiseta* (Fig. 2). However, these differences were not significant (Kruskal-Wallis test, $P = 0.13$).

Overall, there was no correlation between the number of workers in a nest and its depth (Spearman's rank correlation Rho $= -0.120$, $P = 0.53$).

Worker relatedness

Intracolony worker relatedness was quite low in *R. quandratinodum* (Table 1) although this did not differ significantly from the other *Rossomyrmex* values (Kruskal Wallis test, $P > 0.05$), meaning they were close to that expected under single mating by queens (0.75). Conversely, we found significant differences among the three host species (Kruskal Wallis test, $P = 0.03$). Obtained values of relatedness for *P. longiseta* (Table 2) are in accordance with polygyny (0.27 ± 0.02) as well as the observed number of queens in the nests (2.33 ± 0.56). *P. korbi* and *P.* sp. nests had only one queen, which was reflected in a high value of relatedness not different from 0.75 just for *P.* sp. (0.67 ± 0.02, *t*-test, $P = 0.19$). In the case of *P. korbi*, the low values (0.14 ± 0.05) were similar to *P. longiseta* (Mann-Whitney *U*-test, $P = 0.08$) meaning that in fact, nests must be polyandrous and even polygynous. *P. longiseta* had significantly lower levels of worker nestmate relatedness than monogynous and monoandrous *P.* sp. (Mann-Whitney *U*-test, $P = 0.03$), although we found no significant differences between *P. korbi* and *P.* sp. (Mann-Whitney *U*-test, $P = 0.12$).

Discussion

Nest architecture

By comparing the general nest architecture and depth of mixed societies of *Rossomyrmex* and *Proformica* with monospecific nests of *Proformica*, we found that they do not differ significantly in each pair host–parasite. Thus, after the usurpation by *Rossomyrmex*, there are no changes made in the nest architecture of the *Proformica*. The zero effect of the parasite on the original nest architecture of the host differs from other slavemakers like *Polyergus samurai* Yano, which presents larger nests than its unparasitized host (Tsuneoka, 2008). Nest architecture in all cases fits the limited information known on *Rossomyrmex*, *Proformica* (Marikovsky, 1974; Tinaut & Fernández Escudero, 1993) and even for *Cataglyphis* (Plaza & Tinaut, 1989; Ruano & Tinaut, 1993). The nest architecture may be attributed to their phylogenetic relationship (Hasegawa *et al.*, 2002). In relation to nest depth, *R. anatolicus–P. korbi* have the shallowest nests whereas the deepest are clearly that of *R. quandratinodum–P.* sp. When this data is compared with the number of workers living in the nests of each species, it appears that nest depth is not directly related to colony size (except for the minimum space requirement of a whole colony). Thus, other factors apart from the number of workers must influence nest depth in these species (climate, soil structure, etc.).

Nest composition

In a mixed society formed by a slave-maker species and its host, the number of parasite workers usually grows gradually, depending on the age of the nest, to reach a maximum determined not only by environmental factors but also by a phylogenetic component (see data on Baroni-Urbani *et al.*, 1978; Baroni-Urbani & Pisarski, 1978). However, in the case of enslaved workers that lack their own queen, their number will depend on intrinsic traits of the parasite species such as the number of raids per year and their magnitude. The number of slave workers could depend also on stochastic factors like the number of workers in the raided nests, their accessibility, and/or aggressiveness (Zamora *et al.*, 2003). In addition, the number of ants in a sampled nest can vary depending on the moment of the excavation; by chance, this could take place before or after an assault, so the worker numbers would vary markedly from one sampling date to another. In the case of the genus *Rossomyrmex*, although the activity period is more or less known (Arnoldi, 1932; Marikovsky,

1974; Ruano & Tinaut, 1999) the number of raids per nest or the exact moment in the activity period is unpredictable (Ruano & Tinaut, 1999). According to our results, the number of host and parasite workers is variable within a species. However, we found significant differences at the species level for the number of parasite workers in each nest and the proportion of slave and parasite workers. The host–parasite ratio (P/R) is not a fixed parameter, but quite variable (Table 1) and independent of total numbers. In the three species studied, the ratio is similar to *R. minuchae* and *R. anatolicus* (Fig. 3) but significantly lower in *R. quandratinodum*. However, there are no differences in the number of workers in free-living nests for the three host species. Obtained values for the number of workers are similar to other slavemakers and free-living hosts, such as *Polyergus lucidus* and its *Formica* hosts (King & Trager, 2007) but much lower than others like *Polyergus samurai* and its host *Formica japonica* (Tsuneoka, 2008). The proportion of *Rossomyrmex* workers for the three species is similar to the slavemakers mentioned above, which is related to their inability to perform worker tasks.

Our observations on *R. minuchae* indicate that raids are long (2–3 days) and what is stolen depends basically on the population of the assaulted nest. In general, the whole brood is harvested. Thus, our results suggest that on average, *R. quandratinodum* should show lower levels of raid efficiency given that its nests present significantly fewer enslaved workers than the other parasites but there are no differences when comparing free-living nests for the number of workers. This can be due to two reasons: *R. quandratinodum* performs fewer raids per season or *P.* sp. can avoid raids more efficiently than the other *Proformica* species (escaping before the assault starts, pulling down the galleries, repelling the parasites, etc.). Unfortunately, the exact circumstances of raiding behavior remain unknown and further studies would be needed to shed light on this. Therefore, our results would show different co-evolutive outcomes from the action of the arms race as a result of the selective pressure of the slave-making ants on different host species.

Worker relatedness

According to relatedness estimates, *R. minuchae* and *R. anatolicus* were, as expected, monogynous and generally monoandrous (Ruano & Tinaut, 2005). The relatively low values of nestmate relatedness among workers in *R. quandratinodum* may be due to the occurrence of intraspecific raids (as we observed in the field while excavating one of the nests), or multiple mating by the queen because only

one or no queen was found in all its nests (polygyny should therefore be excluded). In cases where the queen was not found (4 nests), the reason may be due to accidental death or age of the nest. Intraspecific raids were also observed in *R. minuchae*, and we can assume this to be a common trait to the genus and to other ants like *Pogonomyrmex* (Gadau *et al.*, 2003) or *Myrmecocystus* (Kronauer *et al.*, 2002).

Proformica longiseta was consistently polygynous and according to Fernandez-Escudero *et al.* (2002), polyandrous. On the contrary, *P.* sp. seems to be monogynous and apparently monoandrous. Data on *P. korbi* are misleading with one queen per nest. While there were similar values of worker nestmates' relatedness to *P. longiseta*, they were not significantly lower than *P.* sp.; nevertheless, polygyny and polyandry are presumed to occur in this species. Our results on *P.* sp. are not surprising given that other species of *Proformica* are in fact monogynous, such as *P. ferreri* (A. Tinaut, unpublished data) or *P. nasuta* (Stumper, 1957). In general, our sampling method has proven to be quite reliable in accounting for the number of workers as well as for the queens, at the moment of the excavation. In this sense, worker nestmate relatedness estimated from genetic data confirms our sampling results in monogynous species.

Acknowledgments

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