Coevolution in the Slave-Parasite System *Proformica longiseta-Rossomyrmex minuchae* (Hymenoptera: Formicidae)

by

Carmen Zamora-Muñoz[1], Francisca Ruano[2], Christine Errard[3], Alain Lenoir[3], Abraham Hefetz[4] & Alberto Tinaut[1]

ABSTRACT

Previous articles on slavemaker ants have identified different traits in slave species as a result of a potential coevolution with the slavemaker, demonstrating that species or populations used as slaves show an overall aggressive reaction against the slavemaker. The pairing of slave-parasite ants *Proformica longiseta-Rossomyrmex minuchae* (Hymenoptera: Formicidae) constitutes a good model system to study the coevolution between the two species, because the parasite lives only in a very restricted area of the host's distribution and the strategies shown by the parasitized *P. longiseta* may differ from the rest of the non-parasitized population. We investigated the aggressive behavior of the potential host *P. longiseta* from different locations (allopatric and sympatric with respect to the parasite) against *R. minuchae* and in-between locations. The phylogenetically related *Proformica ferreri*, a neighboring non-host species, was used as a reference outgroup. Aggressiveness level in confrontations was related to differences in cuticular hydrocarbon profiles and to geographical distances between the tested nests. Hydrocarbon profiles were correlated with geographical distances but not with aggression level. The slavemaker invariably won in all the confrontations but fights were less intensive against sympatric *P. longiseta*, also the location in which the host is less aggressive.

We found comparative evidence suggesting that this low level of aggression might be an apomorphic trait, evolved by the parasite-selection pressure. For the first time in slavemaker ants, we demonstrate that the decreasing of a trait (aggressiveness) could be considered a counter-defense consequence of an arms race, a coevolutionary process in progress that matches with a Mafia system.

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[1]Departamento de Biología Animal y Ecología, Universidad de Granada, SPAIN.
[2]Estación Experimental del Zaidín, CSIC, Granada, SPAIN.
[3]IRBI, CNRS, Université de Tours, FRANCE.
[4]Department of Zoology, Tel Aviv University, ISRAEL.
Correspondence: A. Tinaut. E-mail: hormiga@ugr.es

INTRODUCTION

Coevolution between hosts and parasites is a proven fact (Dawkins & Krebs 1979, Brooks & McLennan 1993). The general laws applied to host-parasite systems could be applied to slave-slavemaker ant systems, because they live at the expense of other individuals or societies (the slaves) throughout their life causing the host a real damage and interfering with host-nest reproduction (Brooks & McLennan 1993, Tinaut & Ruano 1999).

Avian brood parasitism has been suggested as an ideal system to study coevolutionary process because (i) parasites impose sever costs on their host, (ii) a high degree of specificity appears between brood parasite and hosts, (iii) there is central focus (nests) where adaptations and counter-adaptations should be detected, and also because most of those parasite and host adaptations would be based on visual or sonorous stimuli easily detectable by human senses (Rothstein 1990). Moreover, both host and parasites have similar generation times and thus parasites do not have faster mutation potential than their hosts (Price 1980).

Slave-slavemaker ant systems in ants resemble those described for avian brood parasites and therefore provide good system for exploring coevolutionary patterns.

Davies *et al.* (1989) suggested that, as in hosts of avian brood parasites, several lines of defenses should evolve in slave ants to prevent slavemaker ants, facilitation recognition of non-nestmate and non-conspecific ants, better immunity to appeasement substances, and better fighting ability than among non-host ant species. In accordance with this coevolutionary scenario, several studies have shown that species or populations selected as hosts by slave-making ants show an overall aggressive reactions against the parasites, as a consequence of a long host-parasite coevolutionary arms race (Alloway 1990, Stuart 1993, Foitzik *et al.* 2001).

In general, host defenses against parasites are costly, but the net benefits of defensive parasitized phenotypes are much greater than in non-defensive parasitized ones (Brooks & McLennan 1993, Ridley 1993). However, sometimes it would not benefit the host to increase or display its defense, depending of the level of parasite counter-defenses. This would be the case of a parasite who is able to counteract host defense and facultatively inflict extra costs on the defensive host phenotypes but not on the non-defensive ones (Soler *et al.* 1998). This facultative virulence by the parasite has been detected in birds (Soler *et al.* 1995) and has been proved in other host-parasite systems (Li &

Graur 1991, Michalakis *et al.* 1992, Ewald 1994) and has never been reported in slave makers ants.

Social insects vary extensively in their relative aggressiveness. Moreover, there is evidence showing that aggressiveness in ants is linked to differences in cuticular profiles (Nowbahari *et al.* 1990), both increasing with geographical distance (Beye *et al.* 1997, 1998). Differences in cuticular hydrocarbons are considered genetically derived with important environmental components (Vander Meer & Morel 1998, Lenoir *et al.* 1999 for reviews).

In this context, aggressiveness in the slave-slavemaker system could be influenced by the coevolutionary process in two different ways. We hypothesize that, as in other slave-slavemaker systems, a coevolutionary process between *Rossomyrmex minuchae* and *Proformica longiseta* would select for hosts that recognize their parasitic species and show a higher level of aggressive-defensive behavior against the parasite. Hence, we predict that the sympatric host nests should be more aggressive against the slavemakers than allopatric host nests.

The alternative hypothesis is that the sympatric host species would be less aggressive because of slavemaker selection pressure that favors non-aggressive host ants. In this case we predict less aggressive behavior in encounters of sympatric host ants with the slave-making species, than in those with allopatric host ants.

However, this alternative scenario can also be explained by the possibility of slave-making ants selecting host-ant nests that show low aggressiveness and are parasitized for a short period (short coevolutionary time). In this case the low level of aggressiveness would not result from coevolution but from the parasite selecting non-defensive host nests. A phylogenetic approach would potentially distinguish between these two possibilities. By using the parsimony rules (Maddison & Maddison 1992) and an outgroup (aggressiveness against the parasite shown by an ant species which has never been used as host for a parasitic species), we might be able to statistically determine whether the highly aggressive behavior is the ancestral or the apomorphic trait in the host species.

To test these hypotheses, we investigated the aggressive behavior of two groups of nests of *P. longiseta* (the host ant), located in two different places, one allopatric and the other sympatric with respect to the distribution of *R. minuchae* (the slave-making ant). We also studied the aggressive behavior of a closely related species of the host (*Proformica ferreri*). Specifically, we determined cuticular hydrocarbons (HCs), measured geographical distances between the nests collected from each ant species or location (sympatric or allopatric) and estimated aggressiveness in experimental encounters between potential slaves and the slavemaker.

MATERIAL AND METHODS

The ant species

The parasite genus *Rossomyrmex* is comprised of two obligate slavemaker species, *R. proformicarum* Arnoldi, 1928 from Russia and Kazakstan and *R. minuchae* Tinaut, 1981 endemic to Sierra Nevada Mountains (South Spain) (Tinaut 1981). The host species of *R. minuchae* is *Proformica longiseta* Collingwood, 1979, an endemic species from the high-mountain peaks of the southeastern Iberian Peninsula, which nest underground at an average depth of 60 cm, with a number of workers of around 500 individuals/nest (mean ± SE = 497.7 ± 48.8 individuals/nest; $n = 31$ nests). In the Sierra Nevada Mountains, *P. longiseta* is found between 2000 and 2700 m a.s.l. in the most arid biotopes in a continuous distribution (Tinaut 1979), but the known slavemaker distribution is limited to a very particular area (of about 50 km^2) in the lowest parts of the host distribution (Ruano & Tinaut 1999, Ruano 2000). *P. longiseta* is a polygyne species that reproduces by budding (Fernández-Escudero & Tinaut 1999) and this makes the genetic flow between distant nests and the breeding contact between nearby locations difficult.

Nests density of *R. minuchae* is very low (mean ± SE = 0.002 ± 0.002 nests/m^2; Ruano & Tinaut, unpublished data) this being an extremely scarce species (Ruano & Tinaut 1999) included in the red list of protected animals of the I.U.C.N.

Proformica ferreri Bondroit, 1918 is an endemic species from the Iberian Peninsula. In the Sierra Nevada this species inhabits a more or less continuous area below 900 m a.s.l., nesting underground (65 cm in depth) (Tinaut, unpublished data).

Nests sampled

We sampled non-parasitized *P. longiseta* nests from two different locations: (1) from the sympatric zone with *R. minuchae* (hereafter Pls), from Dornajo (2000-2200 m a.s.l.); and (2) from the allopatric zone with *R. minuchae* (hereafter Pla), where years of study of the potential host have never revealed a *R. minuchae* nest (Fernández-Escudero *et al.* 1993;1997, Fernández-Escudero & Tinaut 1999, Tinaut *et al.* 1999). The zone where *R. minuchae* has never been found is located at 2400 m a.s.l., in Borreguiles of the San Juan River, 6 km apart from the place where *R. minuchae* and *P. longiseta* live in sympatry. From the sympatric zone, we also sampled *R. minuchae* nests (hereafter Rm) containing enslaved *P. longiseta* (hereafter Ple). Finally, nests of *Proformica ferreri* (hereafter Pf) were sampled in Dilar (800 m a.s.l.), 15 km apart from the habitat of Pls and 22.5 km from the locality of Pla.

Chemical analyses

For cuticular HCs analyses, we obtained the chemical profiles of 42 Ple extracts and 20 Rm extracts from two Rm nests, 18 extracts from two Pls nests, 7 extracts from two Pla nests and 15 extracts from two Pf nests. Cuticular HCs were extracted by immersing individual worker ants, previously killed by freezing, in 1 ml of pentane. Each pentane extract was evaporated and subsequently redissolved in 50 µl of pentane of which 1 µl was injected into an on-column Varian 3300 GC equipped with a CPSil 5 capillary WCOT Chrompack column (25 m, 0.25 mm Internal Diameter) that was temperature programmed from 80°C to 150°C at 10°C per minute and from 150°C to 280°C at 5°C per minute. Quantification was done by peak integration using an Enica integrator. Only peaks with a relative intensity above 1% were used for analysis. For the statistical analysis the relative percentage of each peak was recalculated from the total peaks considered. We used a discriminant analysis for the hypothetical differences between the chemical profiles of the different nests.

Aggression tests

For the behavioral tests, we used three Rm nests, two Pls nests, two Pla nests and two Pf nests. These nests were maintained in the laboratory in artificial nests with an 8D: 16L photoperiod and 25°C, and honey and water were provided regularly.

Using a protocol based on Retana & Cerdá (1995) especially designed for interspecific interactions, we performed two types of aggression tests (dyadic and group tests) by the same experimenter (CZM).

Dyadic tests (10 min in duration) were performed in a circular plastic container (8.8 cm of diameter) by putting together two individuals, each belonging to a different species or location (sympatric or allopatric). Analyzing tape-recorded behavior of the ants, we distinguished the following behaviors: indifference, inspection (antennae inspection), escape and aggressive behaviors (threats with open mandibles, jumps towards the opponent, bites). Also, we determined the frequencies, duration of inspections and aggressive behavior and latency to attack (when no attack occurred, a 10-min latency was determined). Frequency values (number of items per minute) were given as means ± SE.

Group tests consisted of simultaneously placing five workers from each nest in a similar arena for 3 h. At the end of the test the activity and the number of living, dead, and injured ants were recorded. Scores were calculated according to the following aggression index: (1) No aggression (all individuals were alive and calm at the end of 3 hours); (2) Low aggression (attacks were observed at the end of the test, but there were no injuries); (3) Moderate aggression (a few individuals were injured but none dead); (4) High aggression (≤ 50% of the individuals of one nest were dead) and (5) Very high aggression (>50% of the individuals of one nest were dead).

For control, we observed five individuals of each nest, isolated under similar conditions.

In the above-mentioned tests (dyadic and group) we performed two types of confrontations: (1) All species and locations against Rm (Rm/Pls, Rm/Pla, Rm/Pf, Rm/Rm) and (2) Confrontations between the two *Proformica* species and their locations (Pls/Pla, Pf/Pla, Pla/Pla, Pls/Pf, Pls/Pls, Pf/Pf). Confrontations among different species and locations will give us a reference of how they behave against non slave-making ants.

Three replicates were made per test, and the number of Rm nests available was the limit for the total number of test carried out (102 tests each per dyadic and group tests, see Appendix 1 for confrontation design). Each individual worker ant was used once to avoid possible learning effects.

The protocol of the aggression tests used for the experiments, with three replicates per test, and the capacity of the social insects to display strongly agonistic reactions towards non-nestmates (Wilson 1971, Hölldobler & Michener 1980) led us to consider the results of behavioral tests strong enough despite the low number of nests used.

Statistical analyses of behavioral data

To analyze the levels of aggression of the different species and locations (independent variable), we used the following variables (dependent variables): duration of inspections, duration of aggressive behavior, and latency to attack in the dyadic tests; and aggression index in the group tests. For approximately normally distributed variables, the duration of inspections and the latency to attack were transformed by log (X + 0.001), while duration of aggression were transformed by log (X + 0.01). The aggression index could not be normalized.

To analyze effects of species or location confronted in our experimental tests of aggressiveness, avoiding pseudoreplication problems, we used a three-factor nested ANOVA (MANOVA), where replicates were nested within nest, and nest was nested within species or locations confronted. This model enabled us to examine the effect of species (or location), while controlling for the possible differences among replicates, or among nests, of the same species or location. Moreover, we also used that nested MANOVA model when analyzing the effect of sympatry and allopatry in our experiment, but changing species confronted by sympatry or allopatry (i.e. Rm and Pls are sympatric, Pf and Pla are allopatric).

In the absence of knowledge concerning the genetic relatedness among confronted nests, the respective roles of genetic affinity and geographical distance in nest recognition generally cannot be dissociated (Thomas *et al.* 1999). In an effort to estimate the influence of cuticular cues and geographical distances on aggressive behavior, a multiple autocorrelation analysis was performed (Foster & Cameron 1996), which also controls the phylogenetic effects (Gittleman & Luh 1992, Edwards & Kot 1995, and references therein). We constructed a matrix of geographic distances between all species or locations (measured in meters between the different nests, and after calculating the average distance between locations or species), another one of cuticular-cues differences between species or locations (Mahalanobis distances based on the discriminant analyses) and a third one using mean values of each type of behavioral data (duration of inspections, duration of aggressive behavior, latency to attack and aggression index). In this way, we have three matrices with the same number of cells, with each cell reflecting geographic and cuticular differences as well as the result of the confrontations from two target species or locations. We used the computer program "Progiciel R" (Legendre & Vaudor 1991) with the methodology of Smouse *et al.* (1986), which allows the calculation of partial correlation coefficients. The results are presented as the Mantel statistic r, which is the correlation coefficient between two matrices, both being controlled for the effect of a third matrix, as well as r_{std}, which is the standardization of r proposed by Hubert (1985) and varies between +1 and -1. This consists of a real value of r produced by the extreme values (maximum and minimum) obtained from the permutations (Legendre & Vaudor 1991). Probabilities of the correlation coefficients were calculated using 1000 permutations.

RESULTS

Chemical data

The three species are so similar in chemical profiles, however a discriminant analysis performed for all the nests (Fig. 1) distinguished two well-separated groups (100% well clustered, $F_{23,48} = 6.48$, $p < 0.0001$). The first discriminant variable (78.9% of the variance) separated Pf nests from all others (respectively, $F = 28.97$; $F = 25.81$; $F = 23.13$; $F = 21.58$, $p < 0.0001$). The second discriminant variable (13.6

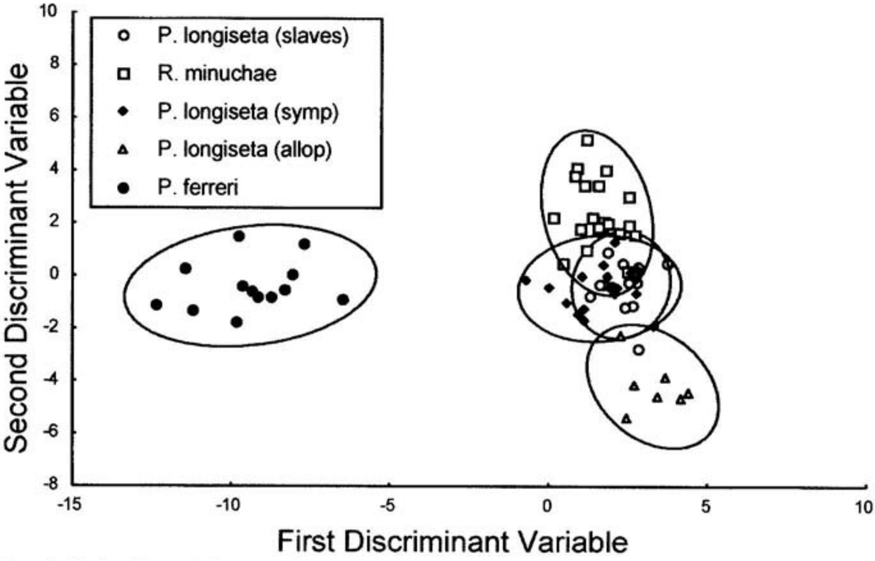

Fig. 1. Projection of the two first axes of discriminant analysis of cuticular substances for *Rossomyrmex minuchae*, *Proformica longiseta* (symp = sympatric; allop = allopatric) and *P. ferreri*.

% of the variance) separated Ple, Pls and Rm from Pla (respectively, $F = 4.51$, $p < 0.0001$; $F = 6.85$, $p < 0.0001$; $F = 3.01$, $p < 0.001$). Within the Pls workers and Ple group, it appeared that the chemical profiles of Ple were closer to those of Pls workers ($F = 1.54$, $p = 0.099$, NS) than to Rm profiles ($F = 5.09$, $p < 0.0001$).

Aggression tests

In dyadic encounters against Rm, the confrontations with conspecific Rm (Rm/Rm) and sympatric host location (Rm/Pls) showed the least aggressive behavior (see number of threats and bites in Table 1). In comparison, the encounters between Rm/Pla or Rm/Pf proved the most aggressive. The frequencies of inspections were greater for Rm/Pls and Rm/Rm encounters, as compared to Rm/Pla and Rm/Pf (Table 1). Likewise indifference in Rm/Pls and Rm/Rm encounters occurred more frequently than in those for Rm/Pla and Rm/Pf (Table 1). The latency to attack in the Rm/Pls encounters was longer than either in the Rm/Pla or Rm/Pf encounters (Table 2).

We also observed a behavior called pseudotrophallaxis, which occurred only in Rm/Pls encounters (Table 1), in which both ants undergo an antennae inspection face to face, touching each other with forelegs and with mandibles open but without food transmission, this being related to the low level of aggression of the hosts.

There were no attacks at all in the encounters between two alien nests of Rm (Rm/Rm encounters).

A nested MANOVA between groups of encounters of all the species (or locations) against Rm (Rm/Pf, Rm/Pls, Rm/Pla, Rm/Rm) revealed significant effects of both sympatry/allopatry and species (or locations) for variables measured during dyadic tests (duration of inspections, duration of aggressive behavior, latency to attack), and during groups tests (aggression index) (Table 3). These variables summarize the different recorded types of behavior during the aggression tests. The duration of aggressive behavior and the aggression index for the above encounters are shown in Fig. 2 (A and B).

The duration of aggressive behavior in dyadic tests (Fig. 2A) were significantly less in encounters of Rm against the sympatric locations (Pls or alien nests of Rm) than in encounters of Rm against the allopatric locations (Pla or Pf) (Post-Hoc comparisons, LSD test Rm/Pls vs. Rm/Pf, $p = 0.0074$; Rm/Pls vs. Rm/Pla, $p = 0.0073$; Rm/Rm vs. Rm/Pf, $p = 0.0298$; Rm/Rm vs. Rm/Pla, $p = 0.0295$). There were no significant differences in the duration of aggressive behavior between dyadic Rm/Pla vs. Rm/Pf encounters (Figure 2A; Post-Hoc comparisons, LSD test, $p = 0.9944$).

The variable measured in group tests (the aggression index) shows a bimodal distribution, that is, in most of the encounters there was no aggression (57 %, $n = 57$) or this aggres-

Table 1. Mean (± SE) values of different ant behaviors per 10 min in dyadic tests. *N*, number of confrontations. Abbreviations of species are as follows: Rm, *R. minuchae*; Pls, sympatric location of *P. longiseta*; Pla, allopatric location of *P. longiseta*; Pf, *P. ferreri*.

	N	Threats	Jumps	Bites	Inspection	Indifference	Escapes	Pseudotrophallaxis
Rm/Pls	18	1.06 ± 0.38	0.56 ± 0.33	0.06 ± 0.06	17.28 ± 2.95	5.00 ± 1.07	9.78 ± 1.90	0.89 ± 0.43
Rm/Pla	18	7.78 ± 2.35	0.39 ± 0.20	1.94 ± 0.78	7.17 ± 1.24	1.83 ± 0.58	11.33 ± 2.57	0.00 ± 0.00
Rm/Pf	18	3.50 ± 0.84	0.11 ± 0.08	0.83 ± 0.37	4.06 ± 1.42	1.39 ± 0.46	2.17 ± 0.58	0.00 ± 0.00
Rm/Rm	3	0.33 ± 0.33	0.00 ± 0.00	0.00 ± 0.00	16.67 ± 10.14	3.33 ± 1.86	11.00 ± 7.77	0.00 ± 0.00
Pls/Pf	12	1.25 ± 0.57	0.75 ± 0.51	0.42 ± 0.23	2.50 ± 0.67	0.42 ± 0.23	4.25 ± 0.98	0.00 ± 0.00
Pls/Pla	12	9.33 ± 3.34	0.42 ± 0.23	4.83 ± 2.26	14.92 ± 5.23	0.42 ± 0.19	15.75 ± 5.54	0.00 ± 0.00
Pf/Pla	12	4.75 ± 0.84	1.33 ± 0.43	3.50 ± 0.93	6.17 ± 1.63	0.83 ± 0.37	8.67 ± 2.35	0.00 ± 0.00
Pls/Pls	3	1.33 ± 0.88	0.00 ± 0.00	0.00 ± 0.00	19.33 ± 3.18	1.00 ± 0.58	4.67 ± 1.67	0.00 ± 0.00
Pf/Pf	3	2.67 ± 1.45	0.33 ± 0.33	0.00 ± 0.00	10.33 ± 5.17	6.00 ± 2.08	7.33 ± 4.33	0.00 ± 0.00
Pla/Pla	3	7.00 ± 2.00	0.00 ± 0.00	0.00 ± 0.00	20.67 ± 6.36	8.33 ± 8.33	14.33 ± 5.04	0.00 ± 0.00

Table 2. Duration of inspections, aggressive behavior and latency to attack (mean values per minute ± SE) for confrontations in dyadic tests and aggression index (means ± SE) in group tests.

	N	Duration of inspections	Duration of aggressive behaviors	Latency to attack	Aggression index
Rm/Pls	18	1.59 ± 0.25	0.08 ± 0.03	4.94 ± 1.13	1.33 ± 0.23
Rm/Pla	18	0.15 ± 0.08	0.98 ± 0.41	0.73 ± 0.55	4.39 ± 0.30
Rm/Pf	18	0.61 ± 0.48	0.79 ± 0.31	1.98 ± 0.91	2.33 ± 0.39
Rm/Rm	3	4.13 ± 1.76	0.01 ± 0.01	10.00 ± 0.00	1.00 ± 0.00
Pls/Pf	12	0.03 ± 0.01	0.62 ± 0.55	6.08 ± 1.41	3.17 ± 0.32
Pls/Pla	12	0.19 ± 0.10	3.23 ± 1.12	1.84 ± 1.10	2.83 ± 0.32
Pf/Pla	12	0.07 ± 0.02	3.74 ± 1.21	0.79 ± 0.33	3.83 ± 0.11
Pls/Pls	3	2.06 ± 0.57	0.16 ± 0.10	3.45 ± 3.27	1.00 ± 0.00
Pf/Pf.	3	1.46 ± 0.93	0.37 ± 0.21	3.96 ± 3.03	2.67 ± 0.88
Pla/Pla	3	0.90 ± 0.47	0.61 ± 0.43	0.21 ± 0.04	1.00 ± 0.00

Table 3. MANOVA between-groups nested design; results of the confrontations of *P. ferreri* (Pf) and the two locations of *P. longiseta*, allopatric (Pla) and sympatric (Pls) against *R. minuchae* (Rm).

	Effect of species			Effect of sympatry		
	F	df	*p*	*F*	df	*p*
Dyadic tests:						
Duration of inspections	8.74	3, 27	0.0003	26.33	1, 27	0.0000
Latency to attack	7.34	3, 27	0.0010	20.21	1, 27	0.0001
Duration of aggressive behavior	4.90	3, 27	0.0076	14.68	1, 27	0.0007
Group tests:						
Aggression index	16.06	3, 27	0.0000	28.16	1, 27	0.0000

sion was high or very high (37 %, $n = 57$), indicating that when ants are in groups can behave very aggressively or not aggressively at all. Again, the least aggressive encounters in the groups tests were between Rm/Pls, in Rm/Rm encounters no aggression was evident, and the highest aggression was shown in Rm/Pla encounters (Table 2; Fig. 2B). There were significant differences between the aggression index of Rm/Pls vs. Rm/Pf encounters (Post-Hoc comparisons, LSD test, $p = 0.0423$), Rm/Pls vs. Rm/Pla (Post-Hoc comparisons, LSD test, $p < 0.0001$) and Rm/Rm vs. Rm/Pla (Post-Hoc comparisons, LSD test, $p = 0.0006$).

The results of the aggressions in the group tests did not exactly match with those of the dyadic tests. In dyadic tests, the encounters between Rm/Pla and Rm/Pf showed a similar levels of aggressive behavior but when these species are in group, the encounters between Rm against Pla were significantly more aggressive than against Pf (Rm/

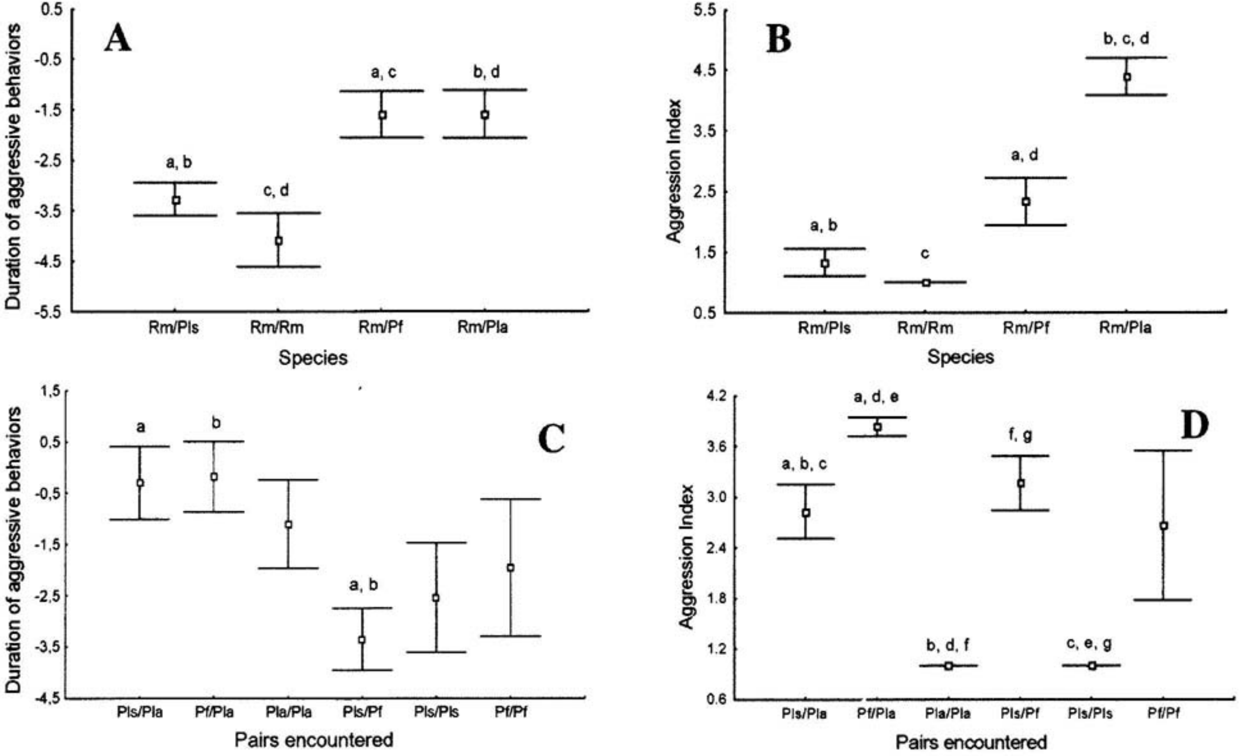

Fig. 2. A) Duration of aggressive behavior (log (X + 0.01)) for dyadic encounters against *Rossomyrmex minuchae*. B) Aggression index for group encounters against *Rossomyrmex minuchae*. C) Duration of aggressive behavior (log (X + 0.01)) for dyadic encounters between *Proformica* species and locations. D) Aggression index for group encounters between *Proformica* species and locations. Pls = sympatric location of *Proformica longiseta*; Rm = *Rossomyrmex minuchae*; Pf = *Proformica ferreri*; Pla = allopatric location of *Proformica longiseta*. Values are mean ± SE. Pairs of confrontations significantly different ($p < 0.005$) after Post-Hoc comparisons (LSD test) are marked with the same small letter.

Pf vs. Rm/Pla Post-Hoc comparisons, LSD test, $p = 0.0002$).

In all the aggressive encounters, Rm won the struggle, and injured or dead ants invariably pertained to *Proformica*. Often Rm killed all *Proformica* individuals, varying that frequency in relation to the species or location encountered. The highest frequency of experiments finished with all *Proformica* individuals being killed by Rm corresponded to the encounters with the most aggressive species/location (Rm/Pla, 55.56 %, $n = 18$). The smallest frequencies corresponded to encounters with the least aggressive locations (Rm/Pls, 0.00 %, $n = 18$ and Rm/Rm, 0.00 %, $n = 3$), and the encounters Rm/Pf had intermediate frequencies of experiments with all the potential host being dead at the end of the experiment (16.67 %, $n = 18$). Thus, Rm killing behavior correlates with the aggressive behavior of their potential slave in group tests (Spearman rank correlation: $r_s = 0.68$, $n = 57$, $p < 0.0001$).

In the dyadic tests between *Proformica* species and locations (Pla, Pls and Pf), Pla was consistently the most aggressive. In these encounters, threats and bites were the most frequent acts (Table 1). The lowest duration of aggression corresponded to encounters of conspecifics or between Pls/Pf (Table 1).

The nested MANOVA between groups of encounters of *Proformica* species and locations (Pf, Pla, Pls) showed a significant effect of species and locations in the dyadic tests for the duration of inspections and the duration of aggressive behavior, but not for the latency to attack (Table 4; Fig. 2C). However, the duration of aggressive behavior proved significant only between Pls/Pf vs. Pf/Pla encounters (Fig. 2C; Post-Hoc comparisons, LSD test, $p = 0.0022$) and Pls/Pf vs. Pls/Pla (Post-Hoc comparisons, LSD test, $p = 0.0030$).

The result of MANOVA for the aggression index in group tests was also significant (Table 4; Fig. 2D). In these encounters, Pf behaved as aggressively as Pla, and encounters Pf/Pla and Pls/Pf registered the

Table 4. MANOVA between-groups nested design; results for the confrontations between species and locations of *Proformica* (Pla, Pls and Pf).

	F	df	p
Dyadic tests:			
Duration of inspections	5.95	5, 18	0.0020
Latency to attack	0.72	5, 18	0.6186
Duration of aggressive behavior	3.50	5, 18	0.0222
Group tests:			
Aggression index	5.19	5, 18	0.0040

highest aggression index, and their values did not significantly differ (Post-Hoc comparisons, LSD test, $p = 0.1657$). Pls/Pla encounters followed in aggression, its aggressive index showing significant differences with respect to Pf/Pla encounters (Post-Hoc comparisons, LSD test, $p = 0.0439$). On the other hand, the aggression index in the encounters between alien nests of the same locations of *P. longiseta* (Pla and Pls) showed the lowest values and did not significantly differ (Fig. 2D).

Relationships between cuticular HCs, geographic distances and aggressive behavior.

Table 5 presents the differences in cuticular profiles (expressed as Mahalanobis distances) and the geographic distances (in meters) between the tested nests. The autocorrelation analysis showed that aggressiveness was significantly related both to differences in cuticular profiles and to differences in geographic distances between encounters (Table 6). Either duration of inspections or latency to attack were negatively related to differences in cuticular profiles and geographic distances. Moreover, HC profiles or geographic distance were positively related to duration of aggressive behavior and aggression index –that is, when aggression was high, inspections and latency to attack were reduced. As these variables are related, we present for simplification only the data pertaining to duration of aggressive behavior and the aggression index.

Differences in cuticular HCs were positively related to geographic

Table 5. Mean values of Mahalanobis distances between hydrocarbon profiles, geographic distances (meters) between species and locations used to calculate the matrices of differences for the autocorrelation analysis.

Encounters	Mahalanobis distances for CHs	Geographic distances (meters)
Rm/Rm	1.77	350
Rm/Pls	15.68	592
Rm/Pf	50.82	15,834
Rm/Pla	46.93	6,391
Pls/Pf	54.49	15,526
Pls/Pla	38.87	6,008
Pf/Pla	70.06	22,584
Pls/Pls	9.05	25
Pf/Pf	4.40	25
Pla/Pla	0.00	25

Pls: sympatric location of *P. longiseta*. Pla: allopatric location of *P. longiseta*. Pf: *P. ferreri*. Rm: *R. minuchae*.

Table 6. Values of r, r_{std} and associated p calculated with Mantel test.

Behavior	Cuticular HC distances (Mahalanobis distances)			Geographic distances (meters)		
	r	r_{std}	p	r	r_{std}	p
Duration of inspections	- 0.82	- 1.00	0.001	- 0.67	- 1.00	0.001
Latency to attack	- 0.50	- 0.82	0.013	- 0.37	- 0.65	0.03 *
Duration of aggressive behavior	0.71	1.0	0.001	0.64	1.00	0.001
Aggression index	0.81	1.00	0.001	0.65	0.996	0.002

* Non-significant after Bonferroni correction.

distances (Mantel test: $r = 0.94$; $r_{std} = 1.00$; $p = 0.001$). When controlling for the effect of geographic distances, we found differences in cuticular profiles to be positively related to aggressive behavior (Mantel tests, partial correlation coefficient for aggressive duration, $r = 0.39$, $r_{std} = 0.78$, $p = 0.005$; partial correlation coefficient for aggression index, $r = 0.76$, $r_{std} = 1.00$, $p = 0.001$). However, when controlling for differences in cuticular profiles, we found geographic distances to be negatively related to differences in aggressive behavior (Mantel tests, partial correlation coefficient for the aggression duration, $r = -0.07$, $r_{std} = -0.14$, $p = 0.294$; partial correlation coefficient for the aggression index, $r = -0.53$, $r_{std} = -1.00$, $p = 0.001$). However, this negative relationship disappeared and became positive on excluding the non-host species Pf (Mantel tests, partial correlation coefficient for the aggressive duration, $r = 0.66$, $r_{std} = 1.00$, $p = 0.002$; partial correlation coefficient for the aggression index, $r = 0.37$, $r_{std} = 0.48$, $p = 0.105$). Thus aggressiveness of *P. longiseta* increased with the distance between nests, independently of the differences in the cuticular profiles.

DISCUSSION

The results from the analyses of the aggression tests support our alternative hypothesis and prediction. The sympatric host species is less aggressive than the allopatric host and the outgroup species, not only in encounters against the slavemaker ant, but also in encounters against the other *Proformica* locations. The most of published commentaries on coevolution in slave-slave maker systems reports an overall aggressive reaction of the host to the slave-maker ant (Alloway 1990, Stuart 1993, Foitzik *et al.* 2001). Nevertheless, a number of other authors have registered a lower level of aggression against the slaves in obligatory than in facultative slave-making ants (Grasso *et al.* 1992). Differences in the virulence of slave-maker attacks even of the same species (Sanetra & Güsten 2001), and advantages in fitness of the less aggressive slave-making ants (Hare & Alloway 2001) have been also reported. These last references support the idea that other kind of coevolution, different than arms race, is possible in slave-parasite systems in ants.

Using the most parsimonious solution, we assume that the aggression level showed by *P. ferreri* or by the allopatric *P. longiseta* matches the ancestral trait because in all the encounters they behave more similarly than the sympatric location of *P. longiseta*. Moreover, these locations are not under the parasite-selection pressure. Thus, the low level of aggressiveness showed by sympatric *P. longiseta* against the slave-maker ant, must be considered an apomorphic trait. This result does not agree with the possibility that the parasite searches for mild host locations which have not evolved defenses (high aggressiveness) against the parasite due to the short coevolutionary time in the parasite-host relationship, because low aggressiveness is showed only for the location suffering parasite pressure.

Furthermore the parasite presents facultative virulence, which was demonstrated in the group tests. In encounters against the aggressive allopatric location of *P. longiseta*, all the host individuals were usually killed by the parasite, whereas in encounters between *R. minuchae* and the non-aggressive sympatric location of *P. longiseta*, no host ants were killed by the parasite. In group encounters against *P. ferreri*, a species showing an intermediate level of aggression in group confrontations against *R. minuchae*, the virulence level of the slavemaker also proved intermediate. These results suggest that the parasite is able to raise the cost of host resistance through increasing its negative effects on their hosts by killing the aggressive ants. In this way, only mild nests of *P. longiseta* have any chance of reproducing in the distribution zone of the slavemaker *R. minuchae*. This scenario closely resembles a Mafia system as described by Soler *et al.* (1998).

On the other hand, although all species and locations studied generally proved to have similar chemical profiles, cuticular hydrocarbons correlated with geographical distances probably because of environmental similarity (Vander Meer & Morel 1998, Lenoir *et al.* 1999), but not with aggression level because of a different selection pressure –that is, the parasite-selection pressure, which favors the non-aggressive host phenotypes.

In relation to the aggressiveness between species or locations of *Proformica*, the lowest aggression index occurred in conspecific encounters between the allopatric and sympatric locations of *P. longiseta*, meanwhile *P. ferreri* registered a higher and more variable aggression index.

In conclusion, we have demonstrated for the first time a slave-slavemaker system in which the low aggressiveness of the slaves is the result of a Mafia System, as a consequence of a coevolutionary process in progress. Nevertheless, this evolutionary scenario could be more frequent than expected among slave-making ants, as suggests the data and observations of other authors.

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Appendix 1. Confrontation design in dyadic and group tests.

APPENDIX I

- 3 Rm nests x 2 Pls nests x 3 replicate = 18 confrontations
- 3 Rm nests x 2 Pla nests x 3 replicate = 18 confrontations
- 3 Rm nests x 2 Pf nests x 3 replicate = 18 confrontations
- 2 Pls nests x 2 Pla nests x 3 replicat = 12 confrontations
- 2 Pls nests x 2 Pf nests x 3 replicate = 12 confrontations
- 2 Pla nests x 2 Pf nests x 3 replicate = 12 confrontations
- 1 Rm nest x 1 Rm nest x 3 replicate = 3 confrontations
- 1 Pls nest x 1 Pls nest x 3 replicate = 3 confrontations
- 1 Pla nest x 1 Pla nest x 3 replicate = 3 confrontations
- 1 Pf nest x 1 Pf nest x 3 replicate = 3 confrontations

Total = 102 confrontations