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doi: 10.1111/j.1755-0998.2009.02553.x



# Isolation and characterization of microsatellite loci in the plant-ant *Pseudomyrmex ferrugineus* (Formicidae: Pseudomyrmecinae) and cross-testing for two congeneric species

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**Abstract**

**To investigate the population structure of the obligate plant-ant *Pseudomyrmex ferrugineus*, we developed primers for 12 microsatellite loci. We tested the variability of the markers on 11 individuals from each of two populations (totalling 22 individuals) and found two to 12 alleles per locus and population. No deviations from Hardy–Weinberg equilibrium were detected. Observed and expected heterozygosities at each locus ranged from 0.00 to 0.50 and from 0.08 to 0.46, respectively. We also investigated suitability of these primers in two congeneric species.**

*Keywords*: Hymenoptera, mutualism, obligate *Acacia*-ant, *Pseudomyrmex mixtecus*, *Pseudomyrmex peperi*, social insects

*Received 19 November 2008; revision accepted 4 December 2008*

Among ant–plant mutualisms, the association of ants from the *Pseudomyrmex ferrugineus* group with swollen-thorn acacias of Central America has been thoroughly studied in ecological and evolutionary terms (e.g. Janzen 1966; Heil *et al*. 2004; Ward & Downie 2005). The mutualism is strictly obligate for the ants: *P. ferrugineus* group ants have never been found nesting outside an *Acacia* host plant. The geographical distribution of these obligate *Acacia*-ants coincides, therefore, strictly with the sites where its host plants grow, a situation that likely affects the ants' genetic population structure. Moreover, ant colonies monopolize host plants and founding queens have to search for uncolonized plants (Clement *et al*. 2008), which should have significant consequences for the ants' mating system and dispersal strategies. Microsatellites are ideal means to investigate such aspects and we thus have developed 12 microsatellite markers for *P. ferrugineus*, which we also tested in *Pseudomyrmex mixtecus* and *Pseudomyrmex peperi*.

Specimens were collected from swollen-thorn acacias in Southern Mexico (states of Oaxaca and Veracruz) in

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September 2007, and stored immediately in 96% ethanol. DNA was extracted from 36 workers belonging to six colonies following a modified cetyltrimethyl ammonium bromide protocol (Sambrook & Russell 2001) and pooled. Genomic DNA was restricted with Tsp 509 I (New England Biolabs) and ligated using two adaptors (MWG Biotech; Tsp AD short and Tsp AD long, Tenzer *et al*. 1999). After purification (Ultrafree-4 spinning columns, Millipore), fragments were amplified in a total of 32 polymerase chain reactions (PCR) consisting of 25 μL each, containing 0.5 μL restricted and ligated product, 1.25 U *Taq* DNA polymerase (MBI Fermentas), 1 μM Tsp AD short, 1× *Taq* buffer (containing 100 mM Tris-HCl pH 8.8, 500 mM KCl, 0.8% Noidet P40; MBI Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 250 μM of each dNTP (Fermentas). Thermal cycling was performed in a T-Gradient Thermocylcler (Whatman-Biometra) as follows: 20 cycles of 93 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, preceded by 72 °C for 5 min to synthesize the nick between the linker and the genomic DNA and a final elongation at 72 °C for 10 min.

To enrich repeat motifs, $(GA)_{13}$ biotinylated probes were linked to streptavidin-coated magnetic beads (Dynabeads M-280 m Streptavidin; Dynal) and probes were subsequently hybridized to the DNA. Hybridization and washing was carried out following Tenzer *et al*. (1999). Enriched DNA was recovered from the beads and amplified again using the same settings as before but without the initial extension step. The PCR was directly performed with 1 μL of bead solution as template.

Subsequently, PCR fragments were cloned using the TOPO TA Cloning kit (Invitrogen) following the manufacturer's protocol. White colonies ($N = 355$) were identified and dot blotted on nylon membranes (Hybond N+ Amersham). These clones then were probed with $(GA)_{13}$ oligonucleotide labelled with fluorescein (MWG Biotech) and detected by Gene Images CDP-Star detection module (Amersham Life Science). We identified 247 positive clones, of which 123 were sequenced using the BigDye Cycle Sequencing version 1.1 Ready Reaction kit (PE Biosystems) and T7 or M13 reverse primers (MWG Biotech). Samples were run on an ABI PRISM 310 Genetic Analyser used with a 310 Genetic Analyser Capillary 47 cm and POP4-Polymer (PE Biosystems). Sequences were assembled and edited in Sequencing Analysis 3.4.1 (PE Biosystems) and visually checked for microsatellites. Repeat motifs were identified in 87 clones, and primers flanking the core microsatellite repeats were designed and tested for 44 loci. Primers were designed manually in cases where repeat motifs were homogenous and where the flanking regions allowed primer design.

For all primers, PCR amplification was performed in 20-μL reactions containing 1–50 ng DNA template, 0.5 U *Taq* polymerase (Fermentas), 0.5 μM of each forward and reverse primer (MWG Biotech), 1× *Taq* buffer (Fermentas, content see above), 1× Enhancer (PEQLAB), 2 mM $MgCl_2$, 250 μM of each dNTP (Fermentas) using a T-Gradient Thermocylcler (Whatman-Biometra). Cycling conditions were 4 min at 94 °C, 35 cycles of 1 min 15 s at 95 °C, 1 min at 55 °C, 45 s at 72 °C and a final extension of 10 min at 72 °C. In cases of successful PCR with four individuals, the PCR was repeated with the forward primer 5′-labelled with 6-FAM, TET or HEX (MWG Biotech). The labelled products were diluted with water, mixed with GeneScan-500 (Tamra) size standard and scored on an ABI PRISM 310 Genetic Analyser used with a 310 Genetic Analyser Capillary 47 cm and POP4-Polymer. Loci were genotyped using GeneScan 3.1 (PE Biosystems). To assess variability of the microsatellites, DNA was extracted from individual ants from two different populations, located close to Puerto Escondido at the Pacific coast in South Mexico (15°55′N, 097°09′W), and close to Matias Romero in the Isthmus of Tehuantepec (17°06′N, 094°55′W). Each primer pair was tested on 22 individuals of *P. ferrugineus* with each 11 individuals derived from 11 colonies per population (i.e. one individual per colony) and on 20 individuals (three colonies) of *P. mixtecus* and 24 (five colonies) of *P. peperi*, respectively. For the latter two species, we tested only samples from Puerto Escondido. In cases of failure of PCR amplification, doubling DNA content always led to successful PCR. In one case (population Puerto Escondido, locus Psfe19), we ran out of DNA.

Twelve primer pairs were flanking polymorphic loci that comprised two to 10 alleles per population of *P. ferrugineus* (Table 1), suggesting that they are sufficiently variable for population genetic analyses. Observed and expected heterozygosities, and exact Hardy–Weinberg probability test using the Markov chain method with default parameters were calculated using the GenePop software (Raymond & Rousset 1995) (Tables 1 and 2). No significant deviation between expected and observed heterozygosities were detected. We tested for null alleles using Micro-Checker (Shipley 2003) and found no evidence for null alleles. Linkage disequilibrium between the loci Psfe06, Psfe07 and Psfe13 in the Matias Romero population was detected based on Fisher's exact test as implemented in the online version of the GenePop software (Raymond & Rousset 1995; $P < 0.05$). Thus, we developed at least nine primer pairs for *P. ferrugineus* that show no deviations from Hardy–Weinberg equilibrium or linkage disequilibrium and amplify reliably. PCR primers and characteristics for 10 additional microsatellite loci can be found in Table S1, Supporting information (accession nos EU864155–EU864159, EU864163–EU864164, EU919670–EU919671 and EU919681). For *P. mixtecus* and *P. peperi*, 10 of 12 primers amplified successfully (Table 2). Deviations from Hardy–Weinberg equilibrium at four loci in *P. mixtecus* and at seven loci in *P. peperi* might be due to the sampling strategy. We sampled several individuals from one colony. However, in the

**Table 1** Primer sequences and characteristics of 12 microsatellite loci for *Pseudomyrmex ferrugineus*. The repeat motif is given for the cloned allele

| Locus | GB accession | Primer sequence | Repeat motif | $T_a$ (°C) | Size range (bp) | Puerto Escondido | | | | Matias Romero | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | *N* | *A* | $H_E$ | $H_O$ | *N* | *A* | $H_E$ | $H_O$ |
| Psfe06 | EU864160 | 5′ TET-ACGAAAAGGTTTTTAATAAGC<br>GCTGACAGATTAATAGTATGC | $(TC)_{20}$ | 50 | 76–103 | 11 | 3 | 0.31 | 0.32 | 11 | 7 | 0.43 | 0.41 |
| Psfe07 | EU864161 | 5′ 6-FAM-AAGGCTTGAAATATCGTTCGTTGC<br>AAAGTAGATGTTTCAGTCCATCGC | $(GA)_{11}$ | 55 | 108–123 | 11 | 3 | 0.30 | 0.27 | 11 | 6 | 0.42 | 0.50 |
| Psfe08 | EU864162 | 5′ 6-FAM-ATTAATGCTCGAAGGCAAA<br>ATCGAACACGTTGAATGATAC | $(CT)_{25}$ | 55 | 124–150 | 10 | 2 | 0.25 | 0.30 | 11 | 7 | 0.44 | 0.41 |
| Psfe13 | EU919672 | 5′ HEX-TATTGAGAAGTCAGACGGTTTCGC<br>GAGTCATTACTATCTATTAACAGG | $(AG)_{20}AA(AG)_9$ | 55 | 161–205 | 11 | 4 | 0.35 | 0.41 | 11 | 10 | 0.46 | 0.50 |
| Psfe14 | EU919673 | 5′ TET-AATAGTAATTACCGAGATAATAAC<br>ACAAGACAAGCTCGAGGATTTAAA | $(CT)_{28}$ | 50 | 112–164 | 11 | 4 | 0.35 | 0.41 | 11 | 10 | 0.44 | 0.50 |
| Psfe15 | EU919674 | 5′ TET-TTGCGTTTCCGAGAAACAAC<br>CCGATGCGTTCATTAAAA | $(CT)_{22}$ | 55 | 106–134 | 11 | 4 | 0.25 | 0.14 | 11 | 7 | 0.38 | 0.27 |
| Psfe16 | EU919675 | 5′ TET-TTCGCTAAAAGATTCTTCCGTATT<br>AAACCTTTCATGTGCGTTACATCG | $(GAA)_9(AG)_3AA(AG)_{13}AAAGAA$<br>$(AG)_{14}AA(AG)AA(AG)_{17}$ | 55 | 180–207 | 11 | 6 | 0.39 | 0.36 | 11 | 7 | 0.44 | 0.50 |
| Psfe17 | EU919676 | 5′ HEX-AGTGCCAATTCTAAACATTATCGC<br>ATCGTGCTGTTAGAATGATGGACC | $(TC)_{26}$ | 55 | 111–154 | 11 | 5 | 0.39 | 0.28 | 11 | 9 | 0.43 | 0.41 |
| Psfe18 | EU919677 | 5′ HEX-TTTTGATAATGACAGGTTTTGGTA<br>ATAATGCATTCCGATTGACTGTGC | $(TC)_{19}$ | 55 | 128–150 | 11 | 4 | 0.08 | 0.09 | 11 | 8 | 0.46 | 0.50 |
| Psfe19 | EU919678 | 5′ TET-TCACAAAACGTCTTGAAACTTTCC<br>TAAAGCAAAAGAGATTCTACCCTA | $(AG)_{32}$ | 55 | 113–174 | 10 | 8 | 0.41 | 0.35 | 11 | 5 | 0.29 | 0.32 |
| Psfe20 | EU919679 | 5′ HEX-ACTCTGAATTGTTGCATTGTTTGC<br>CCATTACGAATATTCAAATACGTG | $(TC)_{10}GTTCTTTCCG(TCTG)_2$<br>$(TC)_3TT(TC)_3TT(TC)_{10}$ | 55 | 159–169 | 11 | 3 | 0.08 | 0.00 | 11 | 5 | 0.34 | 0.19 |
| Psfe21 | EU919680 | 5′ 6-FAM-TCGCCGGAGATAGGGAGGAAC<br>TAAGGAGCGTGGAAGTTAGC | $(GA)_3AA(GA)_{20}$ | 55 | 100–132 | 11 | 5 | 0.37 | 0.45 | 11 | 3 | 0.24 | 0.32 |

GB, GenBank; $T_a$, annealing temperature; *N*, number of genotypes that amplified from eleven individuals of each population screened; *A*, observed number of alleles; $H_E$, expected heterozygosity; $H_O$, observed heterozygosity.

**Table 2** Results of cross-species testing of microsatellite primers developed for *Pseudomyrmex ferrugineus* on *Pseudomyrmex mixtecus* and *Pseudomyrmex peperi*. Shown are only those loci that amplified in at least one of the two species

| Locus | *P. mixtecus* | | | | | *P. peperi* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Size range (bp) | *N* | *A* | $H_E$ | $H_O$ | Size range (bp) | *N* | *A* | $H_E$ | $H_O$ |
| Psfe06 | 93 | 20/3 | 1 | — | — | 85–93 | 24/5 | 4 | 0.59 | 0.54 |
| Psfe13 | 164–166 | 20/3 | 2 | 0.33 | 0.30 | 179–187 | 24/5 | 2 | 0.51 | 0.17* |
| Psfe14 | 126–146 | 20/3 | 8 | 0.77 | 0.55* | 136–154 | 24/5 | 7 | 0.84 | 0.75* |
| Psfe15 | 109–127 | 20/3 | 4 | 0.62 | 0.35* | 105–143 | 24/5 | 7 | 0.78 | 0.58* |
| Psfe16 | 160–210 | 20/3 | 6 | 0.83 | 0.95* | 152–170 | 24/5 | 6 | 0.84 | 0.54* |
| Psfe17 | 110–150 | 20/3 | 5 | 0.73 | 0.80 | 112–146 | 24/5 | 6 | 0.80 | 0.42* |
| Psfe18 | 126–130 | 20/3 | 4 | 0.63 | 0.50 | 132–154 | 24/5 | 5 | 0.56 | 0.46 |
| Psfe19 | 120–166 | 20/3 | 7 | 0.64 | 0.55* | 101–103 | 24/5 | 2 | 0.08 | 0.08† |
| Psfe20 | 163–171 | 20/3 | 6 | 0.60 | 0.70 | 163–191 | 24/5 | 7 | 0.81 | 0.54* |
| Psfe21 | 103–109 | 20/3 | 5 | 0.57 | 0.45 | 124–130 | 24/5 | 4 | 0.64 | 0.13* |

*N*, number of genotypes obtained from 20 individuals screened of *Pseudomyrmex mixtecus* and 24 of *Pseudomyrmex peperi* (for both species genotypes were obtained for all individuals screened)/number of colonies screened; *A*, observed number of alleles; $H_E$, expected heterozygosity; $H_O$, observed heterozygosity; *significant deviation ($P < 0.001$) between expected and observed heterozygosities based on Hardy–Weinberg probability tests; †Hardy–Weinberg probability tests not possible.

polygynous species *P. peperi* inbreeding might occur. Since cross-tests were positive for two species, our primers might be broadly applicable for genetic studies of *Pseudomyrmex* ants, which is a species-rich genus with amazing interactions with myrmecophytic plants.

## Acknowledgements

This work was financially supported by the DFG (grants He 3169/4-2 to M.H. and He 1623/22 to J.H.). We would like to thank Alison Surridge (Cambridge) and Steffen Pauls (St. Paul) for valuable discussion and comments on earlier versions of this manuscript.

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## Supporting information

Additional supporting information may be found in the online version of this article:

**Table S1** Primer sequences and characteristics of 10 additional microsatellite loci for *Pseudomyrmex ferrugineus*. The repeat motif is given for the cloned allele. GB, GenBank; $T_a$, annealing temperature; *N*, number of genotypes that amplified from 12 individuals of each population screened/number of colonies screened; *A*, observed number of alleles; $H_E$, expected heterozygosity; $H_O$, observed heterozygosity; *significant deviation ($P < 0.001$) between expected and observed heterozygosities based on Hardy–Weinberg probability tests; †Hardy–Weinberg probability tests not possible.

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doi: 10.1111/j.1755-0998.2009.02569.x



# Microsatellite markers for the silver arowana (*Osteoglossum bicirrhosum*, Osteoglossidae, Osteoglossiformes)

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### Abstract

***Osteoglossum bicirrhosum* (silver arowana) is an important fish for the economy of the Amazon region, both as an ornamental fish and as a food fish. To provide tools for addressing ecological and genetic questions, we developed 19 polymorphic microsatellite markers that had between 2 and 7 alleles per locus in the 24 tested individuals. The transferability of many of the loci was confirmed for *Osteoglossum ferreirai* (black arowana) and *Arapaima gigas*, and for three African osteoglossiform species.**

*Keywords*: aruanã, microsatellites, Osteoglossiformes, *Osteoglossum bicirrhosum*

*Received 25 July 2008; revision accepted 9 December 2008*

## Introduction

The two species of arowana have an allopatric distribution: *Osteoglossum ferreirai* (black arowana) is restricted to the Negro River basin, while *Osteoglossum bicirrhosum* (silver arowana) occurs in rest of the Amazon basin, in the Orinoco basin and in many of the drainages of the Guyanas (Cala 1973). According to Saint-Paul *et al*. (2000), the difference in distribution is associated with water type where *O. ferreirai* occurs in highly acidic black waters, while *O. bicirrhosum* is found in neutral/slightly alkaline waters. Arowanas

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