

PRIMER NOTE

Species-diagnostic microsatellite loci for the fig wasp genus *Pegoscapus*

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Abstract

To obtain tools for the estimation of inbreeding and assignment of offspring to matrilines, we developed 13 microsatellite loci from the fig wasps that pollinate *Ficus obtusifolia*. Based on morphological studies, it was thought that a single species (*Pegoscapus hoffmeyer*) pollinated this fig. However, our data revealed the presence of two coexisting cryptic species. Several diagnostic microsatellite markers may be used to distinguish these two cryptic species. The new microsatellites can be used across a wide range of fig-pollinating wasp species for both evolutionary and population genetic studies.

Keywords: cross-species amplification, cryptic species, fig wasp, microsatellites, *Pegoscapus*, primers

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Fig-pollinating wasps have been used as model organisms to study evolution of mutualism (Machado *et al.* 2001) and sex ratio theory (Hamilton 1967; Frank 1985; Herre 1985). Among these, the theory of local mate competition (LMC) has been considered 'the section of evolutionary theory that best proves the power and accuracy of the Neodarwinian paradigm as a whole' (Hamilton 1996). In spite of their importance, most of the assumptions about mating structure on which previous tests of LMC were based have never been tested (Herre *et al.* 2001). Genetic markers would allow the testing of these by enabling estimation of inbreeding coefficients and observation of the reproductive behaviour (sex ratios and brood size) of individual foundresses (Molbo & Parker 1996). Here we report the first microsatellite markers developed for fig wasps.

Primers were developed using modifications of protocols described by Rassman *et al.* (1991). Genomic DNA was extracted from approximately 800 *Pegoscapus hoffmeyer* wasps (Wiebes 1995) collected from several Panamanian *Ficus obtusifolia* trees. Wasps were used only from figs without wasp-parasitizing nematodes or mites. Total genomic DNA was extracted with phenol-chloroform and digested with the restriction enzymes *AluI*, *HaeIII* and *RsaI* according to the manufacturer's protocols. Ten µg of 200–

600 bp DNA fragments were ligated into pBluescript® II KS (+) vectors (Stratagene) to transform Epicurean coli® XL1-Blue MRF' supercompetent bacteria (Stratagene). Approximately 6000 clones were screened by hybridization with three oligonucleotides (TC₁₀, TG₁₀, AAT₁₀) labelled with the DIG system (Boehringer Mannheim) using the manufacturer's directions. To verify microsatellite presence and locate them within the insertion, 112 recombinant clones were polymerase chain reaction (PCR) screened with all combinations of oligonucleotides and flanking primers (T3 and T7). Twenty-five positive clones were sequenced on an ABI377 automated sequencer by Microsynth GmbH, Switzerland. Primers flanking repeat sequences were designed for 20 loci using Primer 1.0 (Lincoln *et al.* 1993). Thirteen of these amplified satisfactorily (Table 1).

DNA was extracted from single wasps using the Puregene® DNA isolation kit (Gentra) modifying the manufacturer's single *Drosophila melanogaster* protocol as follows: individual wasps were homogenized using liquid nitrogen, then 100 µL cell lysis solution (Gentra) and 0.01 µg Proteinase K were added and samples were incubated at 55 °C overnight. Protein and DNA were precipitated according to Gentra's protocols, and the DNA was redissolved in 50 µL DNA hydration solution and stored at –20 °C. One µL extraction was used per PCR reaction.

PCR reactions were set up in 10-µL volumes containing 1 × PCR buffer (Quiagen), 0.5 µM of each primer, 0.1 mM of

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Table 1 Characteristics of microsatellite loci for *Pegoscapus* fig wasps

Locus	Primer sequence F = forward primer R = reverse primer	T_a (°C)	MgCl ₂ mM	Repeat motif	GenBank accession no.	Product length ranges bp (numbers of observed alleles)		Cross-species amplification
						<i>P. hoffmeyer</i> sp. A <i>n</i> = 40	<i>P. hoffmeyer</i> sp. B <i>n</i> = 25	
Pe 3	F: GCTAATTCCTCCCAACTCACA R: GCCTTGGCCAATGATCAC	55	1.88	(GA) ₅ AA(GA) ₃	AF491966	143 (1)	141 (1)	4A, 4B, 5, 14, 15A, 15C, 17B
Pe 9	F: AGATCCGAAAGCGACACG R: CCGCGCTAGAAACATCTCTC	55	1.88	(GA) ₁₉	AF491967	100–132 (10)	108 (1)	3, 5*, 7, 14, 17A*
Pe 28	F: ACGGGTGAACCTCGATTTC R: CCCTGCATCCATTCTCTCTC	55	1.88	(GA) ₉	AF491968	143–149 (4)	145 (1)	3*, 4A, 4B, 5, 15A, 15C, 17B
Pe 45	F: CGAGACAATTTCTTCTCGTG R: ACCTGCGCGACTTACTTTTC	55	1.88	(GA) ₆	AF491969	145 (1)	141 (1)	3*, 4A, 4B, 5*, 7, 14, 15A, 15C, 17A
Pe 51	F: ACGACGGAGAGAGAGAAAGAG R: CCGATTACACCCCACTC	55	1.88	(GA) ₅ AA(GA) ₂ AA(GA) ₈	AF491970	114 (1)	—	4A*, 14*, 15A*, 15C, 17A*, 17B*
Pe 52	F: CAACTCACTTGCAACAAAGAAA R: GTAACGCATCAAGAGGAAAAGG	55	1.88	(TC) ₇ (N) ₄₂ (CGTG) ₄ CG	AF491971	149 (1)	145 (1)	3, 4A, 4B, 6, 14, 15A, 15C, 17A, 17B
Pe 77	F: AAACCTGCTGTGCGCGAG R: ACTCCTTGTCCATCTGTTGACA	55	1.88	(TC) ₈	AF491972	114 (1)	116 (1)	3, 4A, 4B, 5, 6, 7*, 14, 15A, 15C, 17A, 17B
Pe 84	F: GTTACCGTATAACAACCTTCGC R: TCTCCATCTACCCCGTTGAC	55	2.5	(AC) ₁₀ AT(AC) ₁₃ A	AF491973	111–115 (3)	133–137 (3)	4A, 4B, 6, 14*, 15A, 15C*, 17A
Pe 91	F: AATATAGTGCCAATCCAGTGGG R: AATTACCGAGTTGTGCGAGGC	55	1.88	(GA) ₁₂	AF491974	82 (1)	78 (1)	4A, 4B*, 5, 14, 15A, 15C, 17B
Pe 99	F: ACGGAATCGAAAATGCATTTC R: CATAAAATCTTGTGCTACCGTG	54	1.88	(CT) ₅ (N) ₁₂₅ (TC) ₁₄	AF491975	230–242 (6)	220–222 (2)	3, 4A, 4B, 5, 6, 7, 14, 15A, 15C, 17A, 17B
Pe 103	F: ACGCTTAAATTTTACAACGCG R: TTTTACCGCGTTTGAATGTG	55	2.5	(TAA) ₁₀	AF491976	131–152 (4)	122	3*, 5, 6, 7*, 14, 15C*, 17A
Pe 107	F: CCTTACCGTAAAAATCTCGACG R: CTTTATAAAGTAGCGCGTTGG	55	1.88	(GA) ₄ GC(GA) ₄ GC(GA) ₃	AF491977	212 (1)	202 (1)	3*, 4A, 4B, 5*, 7, 14, 15A, 15C, 17A, 17B
Pe 109	F: TATCGGAAATGGAAGGCAAC R: AGCATGAAAATGTGATCGAGG	54	1.88	(TG) ₅ T	AF491978	105 (1)	105 (1)	4A, 4B, 5, 6*, 14*, 15A, 15C, 17A, 17B

T_a , annealing temperature; MgCl₂, the concentration of MgCl₂ in PCR reactions; *n*, numbers of individuals that had allele length ranges characterized in two cryptic *Pegoscapus* species from *F. obtusifolia*. Cross-species amplification was recorded for the *Pegoscapus* species *P. tonduzi* (3), *P. gemellus* sp. A collected from *Ficus popenoi* (4A), *P. gemellus* sp. B (4B), *P. picipes* (5), *P. grandii* (6), *P. lopesi* (7), *P. silvestrii* (14), *P. gemellus* sp. A collected from *F. bullenii* (15A) *P. gemellus* sp. C (15C), *P. insularis* sp. A (17A), *P. insularis* sp. B (17B); * following a species number indicates the PCR products were faint but could be scored.

Table 2 Expected heterozygosity, H_O (gene diversity, Nei 1987; equation 7.39, p. 164) and observed heterozygosity, H_E , for selected loci in two *Pegoscapus* species

Locus	<i>P. hoffmeyerii</i> sp. A			<i>P. hoffmeyerii</i> sp. B		
	<i>n</i>	H_E	H_O	<i>n</i>	H_E	H_O
Pe 9	339	0.853	0.125			
Pe 28	58	0.149	0.048			
Pe 84	338	0.204	0.041	115	0.507	0.051
Pe 99	39	0.521	0.103	60	0.066	0
Pe 103	40	0.316	0.055			

each of dCTP, dGTP and dTTP and 0.02 mM dATP, 1.88–2.5 mM MgCl₂ (see Table 1) and 0.02 µL ³³P-dATP and 0.31 U *Taq* polymerase (Quiagen). The cycling program was 4 min at 94 °C, then 30 cycles of (92 °C, 30 s; annealing temperature (see Table 1), 30 s; and 72 °C, 1 min). Thermal cyclers used were PTC-100 Programmable Thermal Controller (MJ Research, Inc.) and GeneAmp® PCR System 9700 (Applied Biosystems).

An analysis of the genotypes of female (diploid) fig wasps collected from *F. obtusifolia* revealed the presence of two cryptic species (*P. hoffmeyerii* sp. A and *P. hoffmeyerii* sp. B). Ten of the 13 loci were species diagnostic (Table 1). Nine of the diagnostic loci had nonoverlapping allele ranges and one locus amplified DNA only from *P. hoffmeyerii* sp. A (Table 1). Two of the three nondiagnostic loci (Pe 9 and Pe 28) had a single fixed allele in *P. hoffmeyerii* sp. B that was included in the allele length range in *P. hoffmeyerii* sp. A (Table 1). The last nondiagnostic locus (Pe 109) was fixed for the same allele in both species (Table 1). We include information on this locus because cross-species amplifications (Table 1) showed that it was polymorphic in *P. piceipes* (three alleles 116–120 bp), diagnostic for two cryptic *P. gemellus* spp. (107 vs. 108 bp) and two *P. insularis* spp. (105 vs. 109 bp).

Two lines of evidence suggest that gene flow is absent or very restricted between *P. hoffmeyerii* sp. A and *P. hoffmeyerii* sp. B. First, we observed only four hybrids among the 431 females (one per fruit) genotyped. Second, the four hybrids were F₁ and no back-cross (F₂) genotypes were observed, suggesting that hybrid fitness is severely reduced.

The observed heterozygosity (Table 2) was extraordinarily low, in accordance with the known breeding structure of fig wasps. Interestingly, we found that inbreeding was higher than previously estimated by nonmolecular methods (Molbo *et al.* in prep). This is not surprising, given that earlier estimates of inbreeding were based erroneously

on the assumption that there was only one fig wasp species.

Screening across species revealed that other fig trees may commonly be pollinated by several cryptic species. The 13 loci were variable and/or diagnostic for cryptic species among pollinators from seven other *Ficus* species studied (Table 1, Molbo *et al.* in prep). These microsatellite markers are currently the only way to distinguish the cryptic *Pegoscapus* species. They provide a useful diagnostic tool for a wide range of both evolutionary and population genetic studies in fig wasps of the genus *Pegoscapus* (Molbo *et al.* submitted).

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