Molecular Ecology Notes (2002) **2**, 440–442 doi: 10.1046/j.1471-8278 .2002.00268.x

PRIMER NOTE

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

D. MOLBO,* M. J. D. KRUGER,† L. A. HERRE‡ and L. KELLER*
*Institute of Ecology, University of Lausanne, 1015 Lausanne, Switzerland, †Department of Entomology, University of Georgia, Athens, GA 30602–2603, USA, ‡Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Republic of Panama

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 hoffmeyeri*) 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

Received 26 April 2002; revision received 29 May 2002; accepted 29 May 2002

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 hoffmeyeri* 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–chlorophorm and digested with the restriction enzymes *Alu*I, *Hae*III and *Rsa*I 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 (TC_{10}, TG_{10}, AAT_{10}) 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

Correspondence: Drude Molbo, IE-ZEA, Batiment de Biologie, Université de Lausanne, 1015 Lausanne, Switzerland. Fax: (+ 41) 216924165; E-mail: drude.molbo@ie-zea.unil.ch

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) *P. hoffmeyeri* sp. *A* *n* = 40	Product length ranges bp (numbers of observed alleles) *P. hoffmeyeri* sp. *B* *n* = 25	Cross-species amplification
Pe 3	F: GCTAATTCCCCCAACTCACA R: GCCTTGGCCAATGATCAC	55	1.88	$(GA)_5AA(GA)_3$	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: ACGGGTGAAACCTCGATTC R: CCCTGCATCCATTCTCTCTC	55	1.88	$(GA)_9$	AF491968	143–149 (4)	145 (1)	3*, 4A, 4B, 5, 15A, 15C, 17B
Pe 45	F: CGAGACAATTTCCTTCTCGTG 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: CCGATTCACACCCACACTC	55	1.88	$(GA)_5AA(GA)_2AA(GA)_8$	AF491970	114 (1)	—	4A*, 14*, 15A*, 15C, 17A*, 17B*
Pe 52	F: CAACTCACTTGCAACAAAGAAA R: GTAACGCATCAAGAGGAAAAGG	55	1.88	$(TC)_7(N)_{42}(CGTG)_4CG$	AF491971	149 (1)	145 (1)	3, 4A, 4B, 6, 14, 15A, 15C, 17A, 17B
Pe 77	F: AAACCTGCTGTTGCCGAG 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: GTTACCGTATAACAACCCTCGC 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: AATTACCGAGTTGTGCAGGC	55	1.88	$(GA)_{12}$	AF491974	82 (1)	78 (1)	4A, 4B*, 5, 14, 15A, 15C, 17B
Pe 99	F: ACGGAATCGAAAATGCATTC 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: CTTCATAAAGTAGCGCGTTGG	55	1.88	$(GA)_4GC(GA)_4GC(GA)_3$	AF491977	212 (1)	202 (1)	3*, 4A, 4B, 5*, 7, 14, 15A, 15C, 17A, 17B
Pe 109	F: TATCGGAAATGGAAGGCAAC R: AGCATGAAAATGTGATCGAGG	54	1.88	$(TG)_5T$	AF491978	105 (1)	105 (1)	4A, 4B, 5, 6*, 14*, 15A, 15C, 17A, 17B

T_a, annealing temperature; $MgCl_2$, the concentration of $MgCl_2$ 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. gememllus* sp. A collected from *Ficus popenoei* (4A), *P. gemellus* sp. B (4B), *P. piceipes* (5), *P. grandii* (6), *P. lopesi* (7), *P. silvestrii* (14), *P. gemellus* sp. A collected from *F. bullenei* (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

	P. hoffmeyeri sp. *A*			*P. hoffmeyeri* sp. *B*		
Locus	*n*	H_E	H_O	*n*	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 $MgCl_2$ (see Table 1) and 0.02 μL ^{33}P-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. hoffmeyeri* sp. *A* and *P. hoffmeyeri* 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. hoffmeyeri* sp. *A* (Table 1). Two of the three nondiagnostic loci (Pe 9 and Pe 28) had a single fixed allele in *P. hoffmeyeri* sp. *B* that was included in the allele length range in *P. hoffmeyeri* 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. hoffmeyeri* sp. *A* and P. *hoffmeyeri* sp. *B*. First, we observed only four hybrids among the 431 females (one per fruit) genotyped. Second, the four hybrids were F_1 and no back-cross (F_2) 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).

Acknowledgements

Funding was provided by the Smithsonian Tropical Research Institute and Augustinus fondet to DM, and several grants from the Swiss NSF to LK.

References

Frank SA (1985) Hierarchical selection theory and sex ratios II. On applying the theory, and a test with fig wasps. *Evolution*, **39**, 949–964.

Hamilton WD (1967) Extraordinary sex ratios. *Science*, **156**, 477–488.

Hamilton WD (1996) *Narrow Roads of Geneland I, Evolution of Social Behaviour*. Freeman, Oxford, UK.

Herre EA (1985) Sex Ratio Adjustment in Fig Wasps. *Science*, **228**, 896–898.

Herre EA, Machado CA, West SA (2001) Selective regime and fig wasp sex ratios: towards sorting rigor from pseudo-rigor in tests of adaptation. In: *Adaptationism and Optimality* (eds Orzack SH, Sober E). Cambridge University Press, Cambridge, UK.

Lincoln SE, Daly MJ, Lander ES (1993) *Primer 1.0: A Computer Program for Automatically Selecting PCR Primers*. MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, MA.

Machado CA, Jousselin E, Kjellberg F, Compton SG, Herre EA (2001) Phylogenetic relationships, historical biogeography and character evolution of fig-pollinating wasps. *Proceedings of the Royal Society of London — Series B: Biological Sciences*, **268**, 685–694.

Molbo D, Parker ED Jr (1996) Mating structure and sex ratio variation in a natural population of *Nasonia vitripennis*. *Proceedings of the Royal Society of London — Series B: Biological Sciences*, **263**, 1703–1709.

Nei M (1987) *Molecular Evolutionary Genetics*. Columbia University Press, New York.

Rassman K, Schlötterer C, Tautz D (1991) Isolation of simple sequence loci for use in polymerase chain reaction-based DNA fingerprinting. *Electrophoresis*, **12**, 113–118.

Wiebes JT (1995) Agaonidae (Hymenoptera, chalcidoidea) and Ficus (Moracea): fig wasps and their figs, XV (Mesoamerican *Pegoscapus*). *Proceedings of the Koninklijke Nederlandse Akademie Van Wetenschappen*, **98**, 167–183.