

## PRIMER NOTE

# Development and characterization of microsatellite markers for *Liporrhopalum tentacularis* Grandi, the pollinator fig wasp of *Ficus montana* Blume

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## Abstract

Microsatellite markers for the pollinator fig wasp *Liporrhopalum tentacularis* were developed using genomic libraries enriched for di-, tri- and tetranucleotide repeats. A subset of 31 positive clones was sequenced and primers were designed. Eleven primer pairs produced polymorphic amplification products in *L. tentacularis*. Eight markers gave unambiguously scorable patterns and were further characterized on 29 individuals collected from different fruits of the dioecious host fig *Ficus montana* in Indonesia. Three to 19 alleles per locus were detected in this set of samples. The observed heterozygosity ranged between 0.10 and 0.55.

**Keywords:** Agaonidae, breeding system, *Ficus*, habitat fragmentation, Krakatau islands, simple sequence repeat (SSR)

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The family Agaonidae (Hymenoptera: Chalcidoidea) includes several hundred wasp species closely associated (as pollinators or nonpollinators) with the plant genus *Ficus* (Moraceae), which is recognizable by its characteristic enclosed inflorescence called a syconium. Generally, different fig species have their own specific pollinating wasp species. This relationship is mutualistic, as fig seed production is completely dependent on pollination by minute wasps and the wasps depend on the fig syconia for completion of their life cycle. Since wasps mate within a syconium immediately after hatching, sib-mating is a general rule for fig wasps, and so local mate competition (LMC) is likely to occur. The extent of LMC depends on the number of females ovipositing in a given syconium. Many studies have used fig pollinator wasps as model species for testing sex allocation and sex ratio theories (Frank 1985; Herre 1985; Greeff & Compton 1996; West & Herre 1998; Kathuria *et al.* 1999; Kinoshita *et al.* 2002). However, differentiation of the progenies of individual foundresses

(ovipositing females) with respect to the sex ratio has not yet been investigated, as appropriate molecular markers were not available.

The species-specific mutualistic relationship between fig species and their pollinating wasps is expected to have important consequences for the maintenance and establishment of viable fig populations in disturbed and fragmented habitats. Moreover, *Ficus* species provide keystone food resources in many tropical ecosystems and hence the long-term preservation of these species is essential for tropical forest conservation. To investigate these mutualistic effects, codominant highly polymorphic molecular markers, like microsatellite markers, are indispensable.

In this primer note, we report on the development and characterization of eight microsatellite markers for *Liporrhopalum tentacularis*, the pollinator wasp of the dioecious *Ficus montana*. Di-, tri- and tetranucleotide repeat enriched libraries of *L. tentacularis* genomic DNA were constructed by a selective hybridization procedure (Karagyozyov *et al.* 1993), using the method described by Van der Schoot *et al.* (2000), with the following modifications: genomic DNA of *L. tentacularis* (153 individuals from the glasshouse experimental *F. montana*/*L. tentacularis* population at the University of Leeds, UK) was digested with *Rsa*I. Fragments in the

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**Table 1** Characterization of 8 microsatellite markers in *Liporhopalum tentacularis*

Microsatellite marker	Primer sequence Forward, Reverse, 5'-3'	Amplification conditions‡	Repeat†	Expected product length (observed range)	Number of alleles among 29 individuals	$H_O/H_E$	EMBL Nucleotide Sequence Database Accession no
LT3-08	GTGGGGATGCCGTTCAAACAG TCGCGTGGAGCCTTCAAGTG	LT60/27	(GCA) <sub>13-3</sub> (ACA) <sub>13</sub>	231 (222-231)	4	0.35/0.49	AJ494983
LT3-11	GCCCTGCTGTTGTGGATGTGGT GGCGTCCCCGGCTTTGTGTC	LT55/32	(GCT) <sub>9-1</sub> (GTT) <sub>58-34</sub>	282 (278-293)	6	0.52/0.73	AJ494984
LT4-15	GCCATTGATGCAGAGCTGTTTACA TTCGCCCTCCTTACAATTTTCACTTT	LT60/27	(GAT) <sub>17</sub>	289 (270-309)	10	0.55/0.77	AJ494985
LT4-16	GCGATGGCCTGATTGGTG CGCCTTTGTTTCTTTTCCTTTTATG	LT60/27	(ACG) <sub>10</sub>	265 (263-302)	12*	0.48/0.75*	AJ494986
LT4-19	CTCAACGCCAACTGCCACGAC TCAAGCGATCAACCGAGAACG	LT60/27	(GAC) <sub>14</sub>	129 (101-137)	7	0.48/0.73	AJ494987
LT4-21A§	AGATCCCCTCCTTTGCCCTTGAA GCCGTGTGGATCGATCGTAGTAAT	LT60/27	(CGT) <sub>9</sub>	203 (193-205)	3	0.10/0.46	AJ494988
LT4-21B§	CGGCAAGCTAAAACCTCGTC CTCGTAGGCTCGCACAGTAG	LT55/32	(GAT) <sub>22</sub>	246 (215-254)	10	0.52/0.81	AJ494988
LT4-27	GAATAGACGCCAATCGCATCCATA GCGGCAATTAATCACCTGTCCCTT	LT55/32	(TCT) <sub>32-2</sub>	223 (201-297)	19*	0.52/0.95*	AJ494989

\*among 21 individuals.

†'-'' denotes an imperfect repeat with the first number being a total number of repeats and the second being a number of mismatches within.

‡LT55/32: 1 cycle 3 min at 94 °C, 32 cycles (15 s at 94 °C, 45 s at 55 °C, 2 min at 72 °C), 20 min at 72 °C.

LT60/27: 1 cycle 3 min at 94 °C, 27 cycles (15 s at 94 °C, 45 s at 60 °C, 2 min at 72 °C), 20 min at 72 °C.

§two markers for the same locus.

size range of 300–1000 bp were isolated from an agarose gel and ligated to a blunt adapter, consisting of a 32-mer (5'-ACTCGATTCTCAACCCGAAAGTATAGATCCCA-3') and 28-mer (5'-TGGGATCTATACTTTCGGGTTGA-GAATC-3'). After the enrichment procedure polymerase chain reaction (PCR) fragments were cloned according to the TOPO TA protocol (Invitrogen), using the pCR2.1-TOPO vector and TOP10F' *Escherichia coli* competent cells. Insert-containing colonies were transferred onto Hybond N + membranes and screened by hybridization to a mixture of the synthetic oligonucleotides at 65 °C. Out of 280 positive clones, 31 were sequenced using PRISM dGTP Big Dye Terminator Ready Reaction Kit (Applied Biosystems) and analysed on an ABI 3700 sequencer (Applied Biosystems). Primer pairs for 15 microsatellite repeats were designed using the software package LASERGENE (DNA star). The markers were tested using different PCR protocols (Table 1) on a PTC-200 thermocycler (MJ). The amplification reaction was performed in 20 µL volume containing 75 mM Tris-HCl pH 8.8, 1.5 mM MgCl<sub>2</sub>, 20 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.01% (v/v) Tween 20, 100 µM of each dNTP, 200 nM of each primer, 1 ng template DNA and 0.3 unit Goldstar™ DNA Polymerase (Eurogentec). Amplification products were mixed with an equal volume of 8 M urea containing 10 mM NaOH and 0.05% bromophenol blue, denaturated for 5 min at 80 °C, separated on 6% denaturation polyacrylamide gels (National Diagnostics), and visualized by silver staining (Promega Silver Sequence DNA Sequencing System).

Out of 15 tested markers, 11 primer pairs were polymorphic in *L. tentacularis*. The eight best scorable markers were further characterized on 29 individuals collected from the fruits of dioecious host fig *Ficus montana* from Sumatra (05°44' S 105°35' E), Krakatau (05°00' S 105°00' E) and Java (06°10' S 105°50' E and 06°34' S 106°45' E) islands (Indonesia). DNA was extracted from single wasps from different fig plants (6–8 plants per location and one wasp per syconium) using the PUREGENE® DNA extraction kit (Gentra). Loci showed 3–19 alleles per locus and expected heterozygosities ranged between 0.46 and 0.95. For all markers the observed heterozygosities were lower than the expected values (Table 1), suggesting either the presence of null alleles or inbreeding. As all markers were affected

equally, the latter possibility is more likely and may result from the pollinator's mating system. The obtained microsatellite markers will allow us to differentiate between progeny of the individual female wasps in multi-foundress syconia and therefore, they will provide a sensitive tool for the investigation of the wasp mating system and for testing sex ratio theories using *Liporhopalum tentacularis*. In addition, the markers will be used to investigate how colonization affected the level of genetic variation in fig wasp populations on the Krakatau Islands after the sterilizing volcanic eruption of 1883.

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