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PRIMER NOTE

Isolation of 10 polymorphic microsatellite loci from *Antirhea borbonica* (Rubiaceae)

I. LITRICO, J. GILLES, P. SOURROUILLE and H. VIGNES
Centre d'Ecologie Fonctionnelle et Evolutive, CNRS, 1919 route de Mende, 34293 Montpellier cedex 5, France

Abstract

We report here the characteristics of 10 microsatellite markers isolated from a microsatellite-enriched DNA library from *Antirhea borbonica*, Gmel (Rubiaceae). *Antirhea borbonica* is an endemic tree on the islands of La Réunion and Mauritius (Indian Ocean) where it occurs on young lava flows (fragmented and perturbed habitat) and in old primary forest. Ten polymorphic loci were characterized, with two to 15 alleles per locus, based on samples from six populations. These loci will be useful for analysing population structure in a metapopulation context where populations frequently go extinct.

Keywords: *Antirhea borbonica* (Rubiaceae), fragmented habitat, microsatellites

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Antirhea borbonica (Rubiaceae) is endemic to the islands of La Réunion and Mauritius in the Indian Ocean. This species is an abundant pioneer shrub on early lava flows and persists through different successional stages and climax rain forest (Strasberg 1995). It often occurs in disturbed or fragmented habitats; indeed on La Réunion *A. borbonica* is abundant on the slopes of the 'Piton de la Fournaise' volcano. Populations experience recurrent colonization and extinction which might be indicative of metapopulation functioning. This species thus constitutes an interesting model for the study of colonization dynamics in fragmented habitats. Moreover, the reproductive system is variable among populations of this species along a successional gradient (Litrico 2001). However, genetic markers are not available for this species, which prevents further work on these issues. Consequently, we have developed microsatellite markers to analyse the genetic diversity and population structure in relation to reproductive system in the fragmented habitats where this species occurs.

Plant DNA was extracted from leaves using the QIAmp plant kit (QIAGEN, IMP). The isolation of microsatellite loci from an enriched (GAA_n and TAA_n) library was performed following Dutech *et al*. (2000), using biotin-labelled microsatellite oligoprobes and streptavidin-coated magnetic beads, with the following modifications: (i) there was no size selection after digestion with the restriction enzyme; (ii) XL10 Gold ultracompetent cells (Stratagene) were used; (iii) recombinant clones were screened with GAA_8 and TAA_8 and AGE1 (AAACAGCTATGACCATGATTAC) or AGE2 (TTGTAAAACGACGGCCAGTG) oligonucleotides using a modified polymerase chain reaction (PCR) method (Waldbieser 1995); (iv) the PCR reagents were 75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20, 1 μM of each primer, 1.875 mM $MgCl_2$, 1 mM dNTP and 0.75 U of *Taq* DNA polymerase (Red Gold Star; Eurogentec) and (v) the PCR program was 2 min of denaturation at 94 °C, 27 cycles at 94 °C for 30 s, 60 °C for 1 min and 72 °C for 2 min and 5 min of elongation at 72 °C. One hundred and twenty-two clones were screened, 61 of which gave a positive signal. Thirty-five positive clones were sequenced using an ABI PRISM® 310 genetic analyser (Applied Biosystem). It was possible to define the PCR primer in 16 sequences. Amplification reactions were carried out separately for each locus in 20 μL including 0.1 μL of each 10 μM fluorescent-labelled primer and unlabelled primer (Table 1), 2 μL of 10× manufactured reaction buffer, 2 μL of 25 mM $MgCl_2$, 0.3 μL of 20 mM dNTP mix, 0.08 μL of 5 U/μL *Taq* DNA polymerase (Red Gold Star; Eurogentec) and 1 μL of genomic DNA. The PCRs were performed using a PTC100 thermocycler (MJ Research) in the following conditions: 2 min of denaturation at 94 °C and 35 cycles of 30 s of initial denaturation at 94 °C, 30 s of annealing at a suitable temperature (Table 1), 35 s of extension at 72 °C and 5 min of elongation at 72 °C. Diluted (1:20) PCR products (1 μL) were pooled in 15 μL of deionized formamide and 0.2 μL of GeneScan-500XLROX size standard and analysed on an ABI PRISM® 310 genetic analyser.

Correspondence: Isabelle Litrico. Fax: (+ 33) 467 412138; E-mail: litrico@cefe.cnrs-mop.fr

Table 1 Characteristics of 10 microsatellite loci isolated from *Antirhea borbonica*

Locus	Repeated motif	Forward and reverse primer sequences	Annealing temp. (°C)	Size range (bp)	No. of alleles	GenBank Accession no.
AntbT6F	$(GAA)_{25}$	HEX F: TGCAACAGCTAATGATCTTAACC R: GGCAGCAAAATCTGACGAG	59	135–158 Discontinuous	15	AY583767
AntbT10B	$(GAA)_8G_3(GAG)_3$	HEX F: GTCTCAACGGACGGGTCTAG R: TTGGACTTCTGGACGATTTG	59	235–238	2	AY583768
AntbT2D	$(GAA)_{10}$	NED F: TTCCAGAGATAAGGGCTGTG R: ATGGCGTATGGTTTGTGC	59	175–199 Discontinuous	7	AY583770
AntbT7H	$(TCT)_8$	6-FAM F: CAGATCGCAGCACCACTAG R: GATTCCATCTCTCGATGACG	59	113–125 Continuous	5	AY583772
AntbT7C	$(TTC)_7$	6-FAM F: AAGCTCATTTTGGGTGATTTAC R: TGGCATTGGAAACATAATACTG	59	212–221 Continuous	4	AY583769
AntbT6Ea	$(CTT)_8$ CC$(CT)_4$	NED F: CCTATCTCTATCTAGGGCTTGC R: GTGAGAGTGTTGCCTCGAC	59	133–148 Continuous	2	AY583771
AntbP367	$(CTT)_{10}$	6-FAM F: GCCTTGGGCTAGAAGAATTAG R: AGAAGAATTATGGTGTCGAAGC	58	280–305 Discontinuous	7	AY583765
AntbP5A9	$(CTT)_8$	6-FAM F: TCTGAGTTGCCTCTTACTAGCC R: CAGTGAACCCAACCTTACTTG	58	212–224 Discontinuous	5	AY583766
AntbP5A4	$(CTT)_{10}$	HEX F: GTTACATCGCAATCCAAAGC R: ATTTCTGTCATGGTCTTGCAC	58	131–146 Discontinuous	4	AY583774
AntbT6A	$(AAG)_{10}$	NED F: GTAAGTGCGAGCCGACTG R: CTCAACGCCTGTTAGCTCTC	59	224–239 Discontinuous	4	AY583773

Table 2 Mean number of alleles per locus (N_{all}), observed heterozygosity (H_O), Nei's diversity (H_E) and F_{IS} (Weir & Cockerham 1984)

Site	N_{all}	H_O	H_E	F_{IS}
C1976	3.3	0.27	0.41	0.35
C1961	3.2	0.30	0.39	0.24
Kipuka	3	0.22	0.37	0.40
Mare Longue	3.1	0.21	0.40	0.48
Cilaos	2.8	0.27	0.37	0.27
Petrin	3.3	0.22	0.37	0.39

The variability was screened at 16 loci in six populations (Table 2) with 15 individuals per population. Five populations of *A. borbonica* were sampled on La Réunion: three populations on the lowland slopes of the volcano where lava flows occur (C1976, C1961 and Kipuka) and two in old successional stages outside the main area of lava flows (Mare Longue and Cilaos). A population from Mauritius (Petrin) was also analysed. Ten of the 16 loci were found to be polymorphic (Table 1). A mean of 5.5 alleles was detected per locus over populations with a mean per locus and population of 3.11. Linkage disequilibrium was tested in 123 cases using 1000 permutations implemented by GENETIX version 4.04 (Belkhir *et al*. 2001). Only three tests were significant at 5%, a level less than that expected by chance. Nei's gene diversity values ranged from 0.37 to 0.41 and the observed heterozygosity ranged between 0.21 and 0.30 (Table 2). In all cases we observed a heterozygosity deficit which was variable among loci. The large F_{IS} value at locus T6F (0.73) may result from null alleles which may thus contribute to the deficit of heterozygotes. The F_{ST} estimates are in accordance with those of Weir & Cockerham (1984) (locus T6F removed), ranging between 0.007 and 0.386. All population pairs were significantly different except for comparisons between Kipuka and C1961 and C1976 and C1961 (1000 permutations conducted using GENETIX version 4.04).

The loci characterized will thus be particularly useful for the analysis of diversity and population structure in this species. A study based on these markers is now under way to analyse the process of colonization of *A. borbonica* in relation to reproductive trait variations in perturbed and fragmented habitats. Moreover, these microsatellite markers may be useful for other species in the genus *Antirhea* which occur in central and South America (Jansen *et al*. 1984; Castillo-Campos & Lorence 1985).

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