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TECHNICAL NOTE

Development of micro satellite markers for a critically endangered species, *Ceropegia fantastica* from the Western Ghats, India

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Abstract *Ceropegia fantastica* L. (Asclepiadaceae) is a highly endemic and endangered species in the Western Ghats of India. Fourteen microsatellite markers were developed for *C. fantastica.* Eight microsatellite primers screened had 2–5 alleles per locus and the observed and expected heterozygosity ranged from 0.48 to 0.83 and 0.48 to 0.62, respectively. The primers were also evaluated for their cross amplification against two related species *Ceropegia hirsuta* and *Ceropegia oculata*. The microsatellites developed for this species could be used for addressing population genetics of this endemic and critically endangered species.

Keywords Microsatellites · *Ceropegia fantastica* · Western Ghats · Asclepiadaceae · Cross amplification · Endangered species

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Ceropegia L. (Apocynaceae) is an old world tropical genus containing about 200 species (Bruyns 1985). There are about 48 species of *Ceropegia* in India, with many of them being endemic (Huber 1957). *Ceropegia fantastica* is an endangered species mostly confined to the northern regions of Western Ghats, a mega diversity hotspot in south India. The species is sparsely distributed with no more than 50 individuals at any given population (Yadav et al. 2006). In this paper, we report the development of microsatellite markers and discuss the utility of these markers in addressing questions related to the population genetics of this species.

The plant material of *C. fantastica* and its closely related species, *Ceropegia hirsuta* and *Ceropegia oculata* were collected from north Western Ghats (N 16°06′041″ E074°08′ 8″) in south India. Genomic DNA was extracted from all the individuals of each species using CTAB method (Doyle and Doyle 1987).

The extracted DNA was purified and digested with *Rsa*I and ligated to linker oligonucleotides SNX-F and SNX-R (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′; 5′-GATTC TGCTAGCTAGGCCTTAAACAAAA-3′) using the rapid DNA ligation kit (Fermentas International). These DNA fragments were hybridized to biotinylated oligos (microsatellite probes) and captured by using dynabeads. Beads and attached probes were separated magnetically from the supernatant. Following stringent washes, the bound DNA (pure gold) was recovered using Fermentas TA cloning kit. The pure gold DNA was incorporated into a pTZ57R plasmid (NEB) vector. Ligation and transformation was performed as per Fermentas TA cloning kit. The positive clones were picked and subjected for colony PCR using the universal M13 forward and M13 reverse primers. The purified PCR products were sequenced using Cycle sequencer (ABI PRISM 3100 Genetic Analyzer, Applied

Table 1 Locus, repeat motif, primer sequence, expected product size range, annealing temperature (T), allele number (A), observed heterozygosity (H_O) and expected heterozygosity (H_E) for eight microsatellites in *Ceropegia fantastica*

Loci and (GenBank accession number)	Repeat unit	Left sequence (5′–3′)	Right sequence (5′–3′)	Size range (bp)	Annealing temperature	A	H_O	H_E
CF1 (FJ175184)	(TG)11	GTGTGACTATCGACCCAGTC	GTGATGAGGCTGTAGGAAAG	180–240	58	5	0.8333	0.5966
CF2 (FJ175185)	(CT)13	GAGAAGCAGTCGAGTTGTCT	CAGAATCACCCAGACATAGG	210–240	57	4	0.4762	0.6167[a]
CF4 (FJ175187)	(GT)12	GTTCTTTGACCAACAGCG	CTACCTATGGACACTTCCGA	360–400	55	5	0.7500	0.5789
CF7 (FJ175190)	(CT)16	GTCTGGTGGGATGATGAGT	GGACGGTGGTTGTCGGCT	105–140	58	4	0.6667	0.5151
CF8 (FJ175191)	(TG)16	CTAGATCTGAGAAGGATGCC	AGACGGGACCCAAATCAT	280–350	56	5	0.5217	0.5681[a]
CF10 (FJ175193)	(ATG)6	TGAAATTGTCGTCGTGGTTG	TCGACATCGACATCATCCTC	150–245	52	5	0.7619	0.5447
CF12 (FJ473407)	(ACA)7	TCAGTGCCCGTATTGTCAAA	GCAGCACTCGATGGGATAGT	180–220	51	5	0.7728	0.5899
CF13 (FJ473408)	(TG)12	CAGGACGAGGGTGCTGAG	TCACCAGCTTCAACTTGCAC	210–220	51	2	0.7500	0.4787[a]

[a] Indicate significant deviations from Hardy–Weinberg equilibrium at $P < 0.05$

Biosystems). Sequences that contained micro satellites were short-listed. Primers were designed for the flanking sequences of the microsatellites by using web-based software Primer3 (Rozen and Skaletsky 2000).

A total of 14 primers were designed from the sequencing results and of these only eight successfully amplified at the expected size. The details of the primer have been provided in Table 1. The amplification was carried out in 20 µl reactions containing 20–30 ng of DNA, 1 mM dNTPs, each primer of 5 pmole, 1× PCR reaction buffer, containing 1.5 mM $MgCl_2$) and 1 unit Taq polymerase. PCR condition for all loci were carried out at 94°C for 2 min, 35 cycles of denaturation for 50 s, 94°C, annealing temperature at 1 min, and elongation for 1.30 min at 72°C followed by final elongation for 72°C at 10 min. The aliquots of 5 µl PCR reaction products were fractionated along with a 20 bp ladder on 12% polyacrylamide gel consisting of 12 ml of 40% acrylamide solution, 8 ml of 5× TBE buffer and sterile water added to the final volume 22 ml. The gel was run at 150 V for about 12–13 h. After electrophoresis, the gels were silver stained as described by Creste et al. (2001).

Twenty-one individuals of *C. fantastica* were used for the estimation of the genetic variability using these eight loci. The number of alleles, size range and expected and observed heterozygosities (H_E and H_O) at each microsatellite loci are presented in Table 1. All the loci screened had a minimum of two and a maximum of five alleles per locus. The observed and expected heterozygosity ranged from 0.48 to 0.83 and 0.48 to 0.62, respectively (Table 1). Exact tests for departure from Hardy–Weinberg equilibrium (HWE) were performed using Markov-chain random walk algorithm (Guo and Thompson 1992) using the programme Arlequin 3.1 (Excoffier et al. 2005). Three loci, namely CF 2, CF 8 and CF 13 showed significant deviations from HWE ($P < 0.05$), indicating perhaps the presence of null alleles at these loci (Table 1). We also performed an exact test for genotypic linkage disequilibrium between loci based on 560 permutations using the FSTAT ver 2.9.3 (Raymond and Rousset 1995). None of the loci showed significant linkage disequilibrium after multiple tests correction of the probability value ($P > 0.0017$).

Table 2 Cross amplification of the eight micro-satellite markers in *Ceropegia hirsuta* and *Ceropegia oculata*

Locus	*C. hirsuta*	*C. oculata*
CF1	+	+
CF2	+	+
CF4	+	+
CF7	+	+
CF8	+	+
CF10	+	+
CF12	+	+
CF13	+	+

The microsatellite markers thus obtained were cross amplified in two related species namely *C. hirsuta* and *C. oculata.* The cross amplification of the primers have been provided in Table 2. All the primers cross amplified with both the species. These primers could be used for studying the population genetic structure of other closely related species of *Ceropegia.*

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