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TECHNICAL NOTE

Development of polymorphic microsatellite markers of the Seychelles endemic tree *Glionnetia sericea* (Rubiaceae)

Aline Finger · Christopher N. Kaiser-Bunbury · Chris J. Kettle

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Abstract *Glionnetia sericea* (Rubiaceae) is an endemic and rare tree species of the Seychelles, restricted to altitudes between 500 and 900 m with less than 1,000 remaining individuals. It survives in mist forests but also in smaller populations on granitic outcrops (inselbergs) and is pollinated by hawk moths which might ensure long-distance pollen flow. Understanding the reproductive ecology of this species will allow a better understanding on how such species survive in naturally fragmented habitats and will provide scientifically informed management recommendations. Here we report on ten species specific polymorphic microsatellite loci developed for a study of historic and contemporary gene flow. Based upon a sample of 81 adults, the number of alleles per locus ranged from 3 to 12 (mean of 6.1 per locus) with an average polymorphic information content of 0.52 across loci. Expected heterozygosity ranged from 0.27 to 0.82 with two of ten primers showing some deviation from Hardy–Weinberg expectation.

Keywords Microsatellites · *Glionnetia sericea* · Population genetics · Seychelles · Gene flow

The rare tree *Glionnetia sericea* (Rubiaceae) is endemic to the Seychelles archipelago. It is found on two Islands, Mahé and Silhouette, where probably less than 1,000 individuals survive at ten known discrete sites. The species is classified as vulnerable in the IUCN red list (2011). *G. sericea* is distributed in mist forests at higher altitudes between 500 m and 900 m and is representative of a plant community occurring in remnants of virgin forest. The current distribution includes some relatively large and continuous populations, consisting of more than 100 individuals, and some smaller more isolated populations found on granitic outcrops (inselbergs). Increase in habitat degradation and invasive species may threaten the long-term survival of this species (Daehler et al. 2004) and the inselbergs provide important refugia for *Glionnetia sericea*. The species' naturally patchy population structure on inselbergs provides a useful study system for investigations of the genetic consequences of habitat fragmentation. Seeds are dispersed by wind and it is pollinated by hawk moths (*Agrius convolvuli* and *Cenophodes tamsi*) (Kaiser-Bunbury et al. 2011), traits that might render the species less vulnerable to habitat fragmentation.

Our work aims to investigate the ability of this species to survive in fragmented populations through a better understanding of its reproductive ecology and specifically, historic genetic differentiation, contemporary gene flow by pollen dispersal, and genetic diversity among remaining large continuous and small isolated populations.

Here we describe the characterization of 10 microsatellite markers for *G. sericea.* Enriched libraries were established from size selected (300–750 bp) genomic DNA ligated into the *Hin*d III site of a pUC19 plasmid and enriched using magnetic bead capture (CPG, Inc., Lincoln Park, New Jersey) with biotin-labelled CA(15), Biotin-GA(15), Biotin-AAC(12) and Biotin-TAGA(8). Microsatellite-containing clones were identified from two di-nucleotide and two tri-nucleotide repeat libraries. Plasmids from 96 positive clones were sequenced and primers designed for microsatellite-containing clones using DesignerPCR version 1.03 (Research Genetics,

A. Finger (✉) · C. N. Kaiser-Bunbury · C. J. Kettle
ETH Zürich, ITES—Ecosystem Management,
Universitätsstrasse 16, 8092 Zurich, Switzerland
e-mail: aline.finger@env.ethz.ch

C. N. Kaiser-Bunbury
Department of Bioscience, Genetic and Ecology Group,
Aarhus University, 8000 Aarhus C, Denmark

Table 1 Characteristics of ten polymorphic microsatellite loci in *Glionnetia sericea*

Locus	GenBank accession. No.	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	Ta (°C)	*A*	H_o	H_e	*PIC*
B6	JN377940	F: CCACCCTGGAAAGAAAGTG R: GGGACTGTTGCTACTGAAGG	$(AG)_{18}$	239–264	56	11	0.76	0.79	0.76
C104	JN377941	F: CAGCCATCAGAACCTTACA R: ATAGCCGACCCCACATAG	$(CAA)_8$	131–143	56	3	0.44	0.46	0.38
A104	JN377942	F: GCGATTTTGTTCAGGGTC R: ATTTTAGCAGACACAGGATGAC	$(CA)_{18}$	161–183	56	6	0.49	0.66	0.61
B110	JN377943	F: TCCCTCCTATGAAAATTACTG R: TATCCCTTTAGCATTGGAACA	$(AG)_{13}$	219–227	56	5	0.70	0.73	0.68
B102	JN377944	F: ATTAGCATCTAACGCACGATA R: AGCAAAGCCATTACTTGTAGTC	$(TC)_5(CT)_{14}$	295–315	56	4	0.33	0.39	0.35
C10	JN377945	F: ATAGCCGACCCTACATAGTGG R: TGATTGCTGGAGTACCTTCTG	$(GTT)_7$	211–217	56	3	0.24	0.28	0.26
C12	JN377946	F: TGGTGGAACTACTTGAGCA R: TTGCGTTAGGTTGACAGC	$(ACA)_7$	138–148	56	3	0.30	0.27	0.24
A106	JN377947	F: CACCAACCAAATTAACAAGAT R: GTCAGCACAAATCAATCTATCC	$(CA)_{13}$	194v222	56	12	0.78	0.82	0.80
B109	JN377948	F: GCAAAATCAAATCAGGTGAC R: GCTCCAAAGAGAGAAGAAAAG	$(CT)_{18}$	108–130	56	11	0.58	0.82	0.79
C105	JN377949	F: CTGTCCTTTTCACATTGTTCTG R: GCGAAACATCATCCATATAGC	$(CAA)_6$	128–134	56	3	0.28	0.48	0.37

F forward primer, *R* reverse primer, T_a annealing temperature, *A* mean number of alleles, H_o observed heterozygosity, H_e expected heterozygosity, *PIC* Polymorphism information content, 81 individuals were analysed for each locus

Inc), of which a subset were tested for polymorphism. The ten most promising loci were optimized. All primers were labeled using an M13-tag at the 5′-end following Schuelke (2000) (Table 1). Polymorphism of the ten selected primers were evaluated using 81 adult trees of *G. sericea* samples collected from seven different sites on Mahé.

Leaf genomic DNA was extracted from *G. sericea* ($n = 211$) using the QIAGEN DNeasy Plant Maxi Kit, following the manufacturer's protocol. PCRs for the M13 primers were carried out in 10 µl reactions with 2 µl of 1× PCR buffer (Promega colorless Flexi GoTaq PCR buffer), 15 mM $MgCl_2$, 0.2 µM dNTPs, 0.2 µl of the 0.04 µM M13 forward primer, 0.8 µl of the 0.16 µM reward primer and 0.8 µl of the 0.16 µM M13 primer, 0.025 U *Taq* polymerase (Promega), and 2 µl DNA template (*c.* 10 ng). Cycling conditions were as follows: 1 × (95°C for 15 min), 30 × (95°C for 30 s, primer-specific temperature (56°C) for 45 s, 72°C for 45 s), 8 × (95°C for 30 s, primer-specific temperature (53°C) for 45 s, 72°C for 45 s), 1 × (72°C for 30 min) (Table 1) carried out in a Bio-Rad Dyad Cycler. We used an ABI3730 for genotyping and Genemapper 3.5 software (Applied Biosystems) for fragment analysis.

The number of alleles and deviations from Hardy–Weinberg equilibrium (HWE) were generated using GenAlEx 6.2 (Peakall and Smouse 2006). The polymorphism information content (PIC), observed and expected heterozygosities was calculated in Cervus 3.0 (Kalinowski et al. 2007). Linkage disequilibrium was implemented in GENEPOP (Raymond and Rousset 1995). All ten loci were polymorphic with 3–12 alleles and a total number of 61 alleles detected over all populations for *G. sericea*. Observed heterozygosity values ranged from 0.24 to 0.78 and expected heterozygosity from 0.27 to 0.82. There was no evidence for scoring error due to stuttering, no evidence for large allele dropout and no evidence for null alleles according to microchecker 2.2.3 (Van Oosterhout et al. 2004). Significant deviations from Hardy–Weinberg equilibrium (HWE, $P < 0.05$) were detected in loci B109 and C105. No significant linkage disequilibrium was detected after Bonferroni correction suggesting that all 10 loci segregate independently of each other. The results indicate that these 10 primers will provide a valuable tool for evaluating genetic diversity and the reproductive ecology of this rare tree species.

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