Conserv Genet (2009) 10:1911–1914
DOI 10.1007/s10592-009-9852-x

TECHNICAL NOTE

Isolation and characterization of eleven polymorphic microsatellite loci from an endemic species, *Piper polysyphonum* (Piperaceae)

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Received: 7 January 2009 / Accepted: 4 February 2009 / Published online: 17 February 2009

Abstract *Piper polysyphonum* is an endemic species in southeast Asia, in the narrow habitat located in the Chinese provinces of Guizhou and Yunnan, and the country of Laos. Recently, loss of forests due to agricultural activity has dramatically reduced the habitat and population size of *P. polysyphonum*. In this study, eleven primer sets of polymorphic microsatellite DNA loci were developed for *P. polysyphonum*. Allele numbers ranged from two to ten, with observed heterozygosities ranging from 0.222 to 0.889. Four loci exhibited a departure from Hardy–Weinberg equilibrium, possibly due to population admixture. No loci pairs revealed significant linkage disequilibrium. Among the eleven loci, two with extremely high numbers of TCG repeats were obtained. The polymorphic microsatellite DNA markers reported here should provide a helpful means to address questions concerning population structure and demographic history of *P. polysyphonum* for conservation efforts.

Keywords *Piper polysyphonum* · Endemic species · Microsatellite DNA

Piper polysyphonum C. de Candolle (Piperaceae) is an endemic species distributed in the wet forests of Laos and southern China (SW Guizhou and S Yunnan provinces) and grows at altitudes of 800–1,400 m (Shu 1999). *Piper* is a large aromatic genus with nearly 2,000 species (Quijano-Abril et al. 2006; Wanke et al. 2007) of shrubs or climbers and rarely herbs or small trees. It also includes several economically and medicinally important plants such as *P. nigrum* (black pepper), which is used as a spice (Jaramillo and Manos 2001), and *P. betle* (betel leaf), which is used as a traditional medicine in India (Prabhu et al. 1995). Several new phytochemicals have been isolated from *P. polysyphonum*, including polysyphoside A, B and C (Ma and Han 1993); isonectandrin B (Zhang et al. 1997); and polysyphorins (Ma et al. 1991). With in vitro testing, polysyphorins have been shown to have inhibitory activity against platelet aggregation (Ma et al. 1991) as a potential medical use.

With regard to phylogeny, *Piper* belongs to the basal lineages of angiosperms (Jaramillo and Callejas 2004) and is one of the largest and most diverse genera. Based on the ITS of nuclear ribosomal DNA, the *Piper* genus divides to three major clades, including clades in Asia, the South Pacific and the Neotropics (Jaramillo and Manos 2001). New evidence has shown that the Asia and the South Pacific clades combine to form a Paleotropical clade and differ from their congeners in the Neotropics (Wanke et al. 2007). Three hundreds species are recognized in the Asian clade and are distributed in the tropical region (Jaramillo

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Table 1 Characteristics of eleven microsatellite loci isolated from *Piper polysyphonum* (Piperaceae)

Locus	Primer sequence(5′→3′)	Repeat motif	Allelic size (bp)	T_a(°C)	N_A	H_O	H_E	*P* value
Pc-b1	F: CCATCACCTTTTACGGTTCC	$(CGG)_7$	81–87	47	2	0.3333	0.2941	1.0000
	R: TAATTGAGGAGGGGCTCACC							
Pc-b2	F: GAGGTAACTTTCAACACTCCC	$(TCG)_9(ACG)_5$	101–158	47	7	0.4444	0.7451	0.0273
	R: ACTATAGGGCACGCGTG							
Pc-b3	F: ACAATGGATGTCCTTCTGAC	$(TCG)_{81}(ACG)_4$	296–383	47	8	0.8889	0.8039	0.8363
	R: AGTGATTACTATAGGGCACGC							
Pc-b4	F: TCATAGCTGTATCGTGGGCG	$(AG)_5(AGG)_3(AT)_4(GT)_4$	114–152	47	7	0.6667	0.7451	0.3623
	R: CGCACATAGCGTACACACTC							
Pc-b6	F: TGTTATTGTGATCCGGGCAC	$(TG)_{16}$	117–171	47	8	0.3333	0.8562	<0.0001
	R: TTGCTGATGCAATCCGACAC							
Pc-b7	F: TCGGATTGCATCAGCAAGTG	$(TGC)_4(ATG)_8(CA)_6(TCA)_{13}(AC)_{12}(CAT)_{11}(AC)_{11}$	208–283	50	10	0.7778	0.9412	0.1837
	R: TACGGTCATGCGTATCTGCT							
Pc-b8	F: AGCAGATACGCATGACCGTA	$(GC)_4(AC)_8$	95–137	50	5	0.8889	0.6797	0.0339
	R: CTTGCTAGTCTCGATTGCAC							
Pc-b11	F: TCAGGAAACGTATGACACGC	$(TGA)_{18}$	113–194	45	10	0.6667	0.9020	0.1291
	R: CGATGTCGCAACGTATGATG							
Pc-b12	F: GCTGGTGCTGGTGCCCCTTA	$(TCG)_{72}$	260–275	48	3	0.3333	0.3072	1.0000
	R: TGCTGCCGCTGCTTTTGACG							
Pc-b13	F: AACGCGTTGAACGATGCATC	$(CA)_7(TCA)_8$	123–156	48	7	0.3333	0.8497	0.0002
	R: CATATAGTCGTGCTGAGAGG							
Pc-b14	F: CCATCACCTTTTACGGTTCC	$(CGG)_8$	104–116	45	2	0.2222	0.2092	1.0000
	R: GGCTCACCTTGGACAAGTTT							

T_a, optimized annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity

P values indicate a test for a significant deficit of heterozygotes from that expected under Hardy–Weinberg Equilibrium (HWE)

and Manos 2001). Sixty species are distributed in China, and thirty-four are endemic to China (Shu 1999). *P. polysyphonum* has a limited distribution between 18–24°N and 98–106°E. Recently, deforestation within Laos and southern China has destroyed the habitat of *P. polysyphonum* and other *Piper* species. Accordingly, the population structure and the genetic diversity have been affected by colonization and extinction dynamics (Pannell and Dorken 2006) and gene flow among populations (Sork and Smouse 2006). *P. polysyphonum* would be a good model species to develop microsatellite markers to study population genetics for a species with rapid habitat loss.

Piper polysyphonum samples were collected from Xishuangbanna, Simao, and Lincang, southern Yunnan province, China. Total genomic DNA was extracted from adult leaf powder according to the manufacturer's protocol with the DNeasy® Plant Mini Kit (QIAGEN, Germany). Extracted DNA was dissolved in 200 µl of TE buffer (pH 8.0) and stored at −20°C for further experiments. To isolate microsatellite loci, the protocol was modified from the AFLP of sequences containing repeats (FIASCO) proposed by Zane et al. (2002). DNA (10 µg) was digested to completion with *MseI* (Promega, USA), and genomic size fractions of 200–800 bp were recovered from a 1% agarose gel using the MinElute Gel Extraction Kit (QIAGEN). Linkers (complementary oligo A: 5′-TACTCAGGACTCAT-3′ and 5′ phosphorylated oligo B: 5′-GACGATGAGTCCTGAG-3′) were ligated onto the DNA fragments using T4 DNA ligase (Promega, USA). Fifteen cycles of prehybridization polymerase chain reaction (PCR) were performed using adaptor specific primers (5′-GATGAGTCCTGAGTAAN-3′, hereafter refereed to as MseI-N). The DNA was denatured and hybridized to different biotinylated probes [B-$(TG)_{15}$, B-$(TCG)_{10}$, B-$(AC)_{15}$, B-$(CGG)_{10}$, B-$(TCA)_{10}$] at 68°C for 1 h for enrichment. The DNA-probe hybrids were then incubated and captured with 1 mg of Streptavidin MagneSphere Paramagnetic Particles (Promega, USA) at 42°C for 2 h, followed by washing with high- and low-salt solutions. DNA was amplified for 25 cycles by PCR, again using MseI-N. PCR products were then cloned with the pGEM®-T Easy Vector System (Promega, USA) according to the manufacturer's protocol. Plasmids containing the PCR product were further screened using colony PCR and purified with a QiaGen kit (Qiagen, Germany). Subsequently, the selected clones were sequenced in both directions using an ABI BigDye3.1 Terminator Cycle Sequencing Kit (Applied Biosystems, USA) with the ABI PRISM® 3700 DNA Sequencer. For sequencing, T7 and SP6 primers were used for amplification.

To screen polymorphisms in *P. polysyphonum*, 18 samples (six individuals per population) were examined using the designed primers. PCR was performed using the Gene Amp® PCR System 9700 (Applied Biosystems, Singapore). The thermocycling profiles were: initial denaturation at 94°C for 5 min, followed by 30 cycles of 40 s denaturation at 94°C, 1 min annealing at the optimized annealing temperature (T_a) (Table 1), 1 min at 72°C, and a subsequent final extension for 10 min at 72°C. PCR products were all directly sequence in order to confirm the polymorphic patterns of tandem repeats. In total, 11 polymorphic microsatellite loci were obtained. The degree of polymorphism, including the number of alleles (N_A) and the observed and expected heterozygosity (H_O and H_E), were examined for these loci using GENEPOP version 3.4 (Raymond and Rousset 1995). Results for the polymorphisms are shown in Table 1. The number of alleles per locus ranged from two to ten, and the observed and expected heterozygosities ranged from 0.2222 to 0.8889, and 0.2092 to 0.9412, respectively. Exact tests between H_O and H_E for departure from Hardy–Weinberg equilibrium (HWE) were also calculated, and four of eleven loci (Pc-b2, Pc-b6, Pc-b8, and Pc-b13) showed significant departures from HWE ($P < 0.05$). Among these four loci, three resulted from heterozygote deficiencies, which could be due to population admixture. It is worth noting that both Pc-b3 and Pc-b12 had an extremely high number of TCG repeats that were confirmed by sequencing. Despite the high repeat number of these two loci, they showed a different pattern of N_A distribution with relatively high and relatively low numbers (eight and three, respectively). Still, they were not departures from the HWE in our examined populations. Linkage disequilibrium was tested using Arlequin 3.11 (Excoffier et al. 2005) and no loci pairs revealed significant linkage disequilibrium.

For conservation efforts, the heterogeneity between populations and the effective population size of rare species, e.g., *P. polysyphonum*, are necessary in order to give suggestions for restoration. The microsatellite loci reported in this study may have sufficient reliability for parental analysis of *P. polysyphonum*. In spite of the low genetic diversity of rare species, rapidly evolving microsatellites lead to higher variation that is sufficient to evaluate population genetics (Hearne et al. 1992). These microsatellites become effective genetic markers to estimate gene flow, population structure, and historical demography. The highly polymorphic microsatellite loci reported herein could be helpful in genetic studies of *P. polysyphonum* for conservation efforts.

Acknowledgments This study was supported by grants from the National Science Council of the ROC on Taiwan awarded to Yu-Chung Chiang.

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