

Isolation and characterization of 11 microsatellite loci from *Camellia sinensis* in Taiwan using PCR-based isolation of microsatellite arrays (PIMA)

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Abstract We report 11 novel microsatellite primer pairs for the wild tea, *Camellia sinensis* (L.) O. Kuntze forma *formosensis* Kitamura. These simple sequence repeat markers were tested in 24 samples collected from wild tea populations, and in cultivars and *C. japonica*. The number of alleles ranged from 4 to 18. The expected (H_E) and observed (H_O) heterozygosity were 0.687–0.946 and 0.042–0.792, respectively. All loci were significantly deviated from Hardy-Weinberg expectations due to the heterozygote deficiency, indicating a dramatic loss of genetic polymorphisms in the rare species. Significant LD was discovered in most loci. These primers may provide a tool for understanding demography and population structure in wild tea.

Keywords *Camellia sinensis* · Heterozygosity · Microsatellite fingerprinting · PIMA · RAPD-PCR enrichment

Tea, made from *Camellia sinensis* leaves, is one of the popular non-alcohol beverages throughout the world (Mondal 2002). Tea with various aromas, both in oolong and black tea, has been long selected (Mizutani et al. 2002).

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The tea business in Taiwan can be dated back for more than two hundred years and has gradually become flourishing (Lai et al. 2001). Using wild tea to hybridize with cultivars for selecting new breeds has been conducted regularly since 1925 (Cheng et al. 2003). The native wild tea (*C. sinensis* (L.) Ktze. f. *formosensis* Kitam.) is distributed in broadleaf forests at elevations from 900 to 1,800 m in Taiwan (Hsieh et al. 1996). Recent human overexploitation to the original forests inevitably made wild tea suffer habitat degradation and fragmentation, which could reduce the genetic diversity within populations dramatically. Protecting the rare species from extinction is therefore urgent.

In this study, we described the development of 11 microsatellite loci from *C. sinensis* in Taiwan for genetic studies. Genomic DNA was extracted from liquid-nitrogen ground leaf tissue using a CTAB method (Doyle and Doyle 1987). The isolation of microsatellite markers began with a random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) enrichment (Hsu et al. 2004; Huang et al. 2007; Lin et al. 2007). This PIMA (PCR isolation of microsatellite arrays) approach has been proposed by Lunt et al. (1999). It takes advantage of the fact that the RAPD fragments contain microsatellite repeats more frequently than random genomic clones (Cifarelli et al. 1995).

The RAPD-PCR amplifications were performed in a thermal cycler (Bio-Rad) with a reaction mixture (50 µl) containing 20–100 ng DNA, 0.2 mM of each dNTP, 2 mM MgCl₂, 0.5 U *Taq* polymerase (Promega), and 5 pmols of one RAPD primer. The PCR programs were as follows: initial denaturing 3 min at 94°C for 1 cycle, 40 cycles of 1 min at 94°C, 1 min at 42°C, 2 min at 72°C, followed by 10 min at 72°C for additional extension. Several RAPD primers were used to amplify DNA fragments from the target species' genome in separate reactions. PCR products

were size-selected to obtain small fragments (300–800 bp). DNA fragments were ligated into a pGEM T-Easy Vector Systems (Promega); and the plasmids were transformed into *Escherichia coli*. Clones were screened using microsatellite primers and vector primers (Lunt et al. 1999). In positive clones, PCR electrophoresis would show a DNA fragment that contains microsatellite signal, whereas no amplification was found in negative clones. Plasmid DNA from positives was extracted and purified using the High-Speed Plasmid Mini Kit (Geneaid), and then sequenced in an Applied Biosystems Model 377A automated sequencer (Applied Biosystems). Specific primers were designed according to the nucleotide sequences upstream and downstream of the repetitive DNA using Primer 3 (Rozen and Skaletsky 2000). Primers were designed according to the nucleotide sequences upstream and downstream of the repetitive DNA. PCRs were performed in a 20 μ l volume containing 10 ng of genomic DNA, 0.2 mM dNTP, 2 mM MgCl₂, and 5 pmols of each primer. PCR programs took place as follows: 3 min at 94°C; 40 cycles of 30 s at 94°C, 30 s at primer-specific annealing temperature (Table 1), 30 s at 72°C, and a final extension at 5 min at 72°C. Electrophoresis was performed in denaturing 6%

polyacrylamide gels using 10-bp ladder molecular size standard (Invitrogen) to estimate allele sizes via ethidium bromide straining.

The allele number, size range, number of bands per individual, expected (H_E), and observed heterozygosities (H_O) were quantified using the Arlequin version 3.0 (Excoffier et al. 2005). GENEPOP (Raymond and Rousset 1995) online version (<http://genepop.curtin.edu.au/>) and Arlequin version 3.0 were used to assess Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) (Table 1). In total, we present eleven novel microsatellite loci for *C. sinensis* (cf. Ueno et al. 1999; Freeman et al. 2004). The number of alleles per locus ranged from 4 to 18. As shown in Table 1, the H_E and H_O ranged from 0.687 to 0.946 (with an average of 0.784) and 0.042–0.792 (with an average of 0.839), respectively. Significant departures from HWE (Table 1) were detected in all microsatellite loci. These deviations were due to the deficit of heterozygotes within populations, suggesting effects of genetic drift associated with habitat destruction. Significant LD was discovered in most loci, except for loci Ca01, Ca05 and Ca10. The microsatellite primers were also applied to *Camellia japonica* and two cultivars *C. sinensis* var.

Table 1 Primer sequence, repeat motif, size range, number of alleles, expected (H_E) and observed (H_O) heterozygosities, and significance of deviation from Hardy-Weinberg equilibrium for 11 microsatellite primers from *Camellia sinensis*

Locus	F	Primer sequence (5'-3')	Repeat motif	Size range (bp)	Total number of alleles	Tm (°C)	H_O	H_E	HWE P-value
Ca01	F	CCCAGCAAAACCCAGCATG	(CAG)6...(CAG)5	120–218	6	56	0.75103	0.78901	<0.001
	R	CTTCCGAAC TG CAG GTT GTGG							
Ca02	F	TCCAGTCCC AT GG AC AGG AG	(GT)8(TTTTA)5	61–95	18	52	0.04167	0.94592	<0.001
	R	CATAGCACACACCTCCACAGC							
Ca03	F	CAAAGCTGC ACT TA ATT GCGC	(GTTT)7..(AG)8	231–261	9	53	0.33333	0.83599	<0.001
	R	CTCGGCAAAGGTT CACCAGG							
Ca04	F	GAAGGTGGGAAAGGACAACG	(AG)8	131–143	4	56	0.12543	0.68706	<0.001
	R	AGATCCCAGTGA ACC CTCCG							
Ca05	F	CAACAGCTCAGATAGAGCCACG	(GA)18..(GT)16	216–244	5	62	0.79167	0.74645	0.00153
	R	CGACGCCCGGGCTGGTAT							
Ca06	F	CATGTAGAATGCTCAAATGC	(GT)12(GA)10	248–398	11	55	0.29167	0.86436	<0.001
	R	ACCTGAAAACGATCCTGACAT							
Ca07	F	GCACCCCCCATGCTTGGCAG	(CT)7(GT)5	176–191	6	55	0.08333	0.7039	<0.001
	R	GCCTGTCCGATCGACCTGCG							
Ca08	F	TTCAATTACCGCCAATCTC	(CT)10	193–255	11	56	0.54167	0.7234	0.00173
	R	CCAATCTGGATTGAAGAAG							
Ca09	F	CATCTTTTACTGTCTTTC	(AG)20	171–207	6	50	0.08333	0.79433	<0.001
	R	GTTTCAACGACAATGGGCTC							
Ca10	F	TGTTCATCTTTTGCTGT	(AG)35	185–213	4	50	0.04167	0.73138	<0.001
	R	GACAGTAAAAAGAGATGAAC							
Ca11	F	CTCATCACTCTCCCTAGC	(AG)18	107–153	6	50	0.08333	0.8023	<0.001
	R	CAGCAAAAGAGATGAACAA							

sinensis and var. *assamica*. All primer pairs amplified microsatellite fingerprints in cultivars; while cross-species amplification worked with most primer pairs, except for loci Ca02 and Ca03. The application of these microsatellite loci in *C. sinensis* may provide a tool for understanding its demography and population structure.

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