American Journal of Botany

American Journal of Botany: e247–e249. 2011.

AJB Primer Notes & Protocols in the Plant Sciences

Development and characterization of microsatellite markers in the critically endangered species *Acer yangbiense* (Aceraceae)[1]

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- *Premise of the study:* Microsatellite primers were developed to assess genetic diversity and population structure in *Acer yangbiense*, a critically endangered endemic species that occurs in northwestern Yunnan Province, China.
- *Methods and Results:* Using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) protocol, 34 microsatellite loci were isolated and characterized in *A. yangbiense*. Polymorphisms were evaluated in 39 individuals from two distinct populations, one of which was naturally occurring and the other an ex situ grouping. Nine of the markers showed polymorphisms with two to five alleles per locus; observed (H_o) and expected (H_e) heterozygosities ranged from 0.0000 to 0.8000 and from 0.0000 to 0.6889, respectively.
- *Conclusions:* These microsatellite primers will prove useful in the further investigation of population genetics studies in *A. yangbiense* and, in addition, will assist related research of other congeneric species.

Key words: *Acer yangbiense*; genetic diversity; maple; microsatellites; polymorphism; SSR.

Acer yangbiense Y. S. Chen & Q. E. Yang (Aceraceae), a new plant species described in 2003 (Chen et al., 2003), is one of the most endangered Chinese maples. It has a very restricted distribution in the Cangshan Mountains of northwestern Yunnan Province, China. Current field surveys have confirmed that five individuals are scattered within a valley on the western slopes of the mountain and occur within the altitude band of 2300–2400 m. The species is facing a very high risk of extinction because of its small population size, poor reproduction, and habitat degradation. It has been evaluated as a globally critically endangered tree (Gibbs and Chen, 2009). Since 2009, Kunming Botanical Garden has successfully raised more than 1000 seedlings, as part of an ex situ conservation strategy that has succeeded in obtaining viable seed through artificial pollination. The critically endangered status of this species serves to emphasize that an effective conservation strategy is urgently required. The population genetics information obtained from this study will be very useful in designing conservation and management strategies for this species. We aim to assess whether genetic diversity declines within the ex situ saplings and to speculate on the presence of other potential unknown natural individuals through comparison with the natural population. Unfortunately, the majority of microsatellite loci developed for related *Acer* species that have amplified in *A. yangbiense* were not polymorphic (Pandey et al., 2004; Terui et al., 2006; Kikuchi and Shibata, 2008; Segarra-Moragues et al., 2008). Therefore, we developed and characterized 34 microsatellite markers for *A. yangbiense* that will be used for further studies using the Fast Isolation by AFLP of Sequences Containing repeats (FIASCO) protocol (Zane et al., 2002).

[1]Manuscript received 28 March 2011; revision accepted 15 April 2011.

This work was financed by a special fund from the Yunnan Provincial Government of the People's Republic of China, which supports qualified scientists and technicians (grant number 2006PY-48 to W. Sun), and by the generous support of Botanic Gardens Conservation International (BGCI). The study was conducted at the Key Laboratory of the Germplasm Bank of Wild Species in Southwest China, Kunming Institute of Botany, Chinese Academy of Sciences. The authors thank David Paterson of Kunming Botanical Garden for his help in editing the text.

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doi:10.3732/ajb.1100142

METHODS AND RESULTS

Total genomic DNA of *A. yangbiense* was extracted from dry leaf tissue, which was ground in liquid nitrogen using a cetyltrimethylammonium bromide (CTAB) extraction method (Doyle and Doyle, 1987). The genomic DNA (500–800 ng) was completely digested with an *Mse*I restriction enzyme (NEB), then the digested fragments were ligated to an *Mse*I AFLP adaptor. The ligation products were amplified with MseI-N primers (5′-GATGAGTCCTGAGTAAN-3′) (Huang et al., 2009) and were hybridized with 5′-biotinylated $(AC)_{15}$, $(AG)_{15}$, and $(AAG)_{10}$ probes (Zane et al., 2002). The DNA, having been hybridized with probes, was then captured by biotin-streptavidin (Promega, Madison, Wisconsin, USA). After amplifying with MseI-N primers again, the purified PCR products were ligated into pGEM-T vector (Promega) and transformed into *E. coli* strain DH5α (Tiangen, Beijing, China). The positive clones were picked out and tested using $(AAG)_7/(AC)_{10}/(AG)_{10}$ primers and vector primers T7/SP6. A total of 428 clones were chosen for sequencing with an ABI PRISM 3730XL sequencer (Applied Biosystems, Foster City, California, USA). A total of 121 (28%) sequences were found to contain microsatellite repeats, and 79 of them were suitable for designing locus-specific primers, using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, California, USA).

Primer pairs were assessed in 19 *A. yangbiense* samples. The PCR reactions were performed in a 10 μL reaction containing 5 μL 2× Taq PCR MasterMix (Tiangen; 0.1 U *Taq* polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl [pH 8.3], 100 mM KCl, 3 mM $MgCl_2$) and 30–60 ng genomic DNA. The PCR

Table 1. Characteristics of nine primers developed in *Acer yangbiense* showing the forward (F) and reverse (R) primer sequence, repeat motif, number of alleles (*A*), allele size range (bp), optimal annealing temperature (T_a), and GenBank accession number.

Locus	Primer sequence (5′–3′)	Repeat motif	Size range (bp)	*A*	T_a (°C)	GenBank Accession No.
AY10	F: CTTAGAAAGGTAAGCACCCG R: AGAACATGGCAATGGTTAGA	$(CA)_7$	130–137	2	62	JF505497
AY14	F: AAGGCAAGGAAGGAAAAGGG R: TGCTCAGGGGCTAGTCAAAC	$(GT)_8(GA)_9$	163–175	5	65	JF505498
AY29	F: GAATCGCTACCAAAAGAAGA R: AAATACCACAGACAAAATCG	$(GAA)_{13}$	171–179	2	58	JF505499
AY33	F: AATTTATCCCTCTTTACTGC R: AACTGCGTCAAGACATCTAA	$(TC)_{10}(AC)_7$	410–435	3	59	JF505500
AY34	F: ATGAATACAAGGATAATCGG R: TTGAGGGAAAACTACTAAAT	$(GT)_8$	86–90	2	51	JF505501
AY54	F: ATATGCAACATGTGACAGTG R: GAGTGAAGAGCTACAAAGGT	$(GT)_{10}$	148–155	2	61	JF505502
AY64	F: GTCAATTCCATCTAAACCAG R: GGTATGACATCACCAAAGTA	$(TA)_6$	170–180	2	56	JF505503
AY69	F: AAACAAACCCAGAAATCCTA R: CATCATCACCAAACCCTAAT	$(AAG)_{13}(AG)_4$	438–453	2	56	JF505504
AY74	F: TGCGAACATAGAAGACACGA R: GTTTGGCAGAAACCACAACT	$(TC)_{13}$	102–104	3	58	JF505505
AY02	F: TTCAAAGAATAGGGTGGAGA R: TACTCTGGCTACGGAAGGTG	$(GA)_{11}$	251	1	61	JF791788
AY05	F: GCTTGTTGGTCACTCTATTG R: AAGGTCAAAGATTGCATACC	$(CA)_{11}$	269	1	61	JF791789
AY17	F: TTTACCCGAGAAACGAACAC R: ACCATTCTCACCCCTCCATA	$(TG)_5$	245	1	57	JF791790
AY18	F: GAAGCCGGAGAACAAAACCT R: TTTTCCGACACCACAATCAAA	$(GA)_3(GT)_7$	124	1	57	JF791791
AY26	F: TATAATTGACCTCATTCCTC R: GCATAAGCATACTAAAACAT	$(CA)_{15}$	138	1	54	JF791792
AY36	F: CCAAGCCCATAAAGGCACAA R: GAAGGCACGAGCGAACAAAT	$(GA)_6$	201	1	65	JF791793
AY39	F: CAAGGTGGAATTTGTTTTCT R: TTGAAGACATGTAAATCGAA	$(CTT)_{11}$	122	1	52	JF791794
AY43	F: TCGAATCAAGATTTCTACCA R: GAACAGAAGCATCGAAAGAG	$(GT)_8$	221	1	61	JF791795
AY52	F: CAATAGCGAGACAGAAACCC R: TACTTGGCGTCACAGAACAA	$(GAA)_5$	168	1	61	JF791796
AY53	F: AGGTAGCTTGCTTTATTTGA R: AGATGGGACTCCTTAGGATC	$(GT)_6$	100	1	52	JF791797
AY01	F: GAGCAAAAGAAAGAGGGAACG R: GTAAGGTGGGGAATGGCTAG	$(TC)_{10}$	315	1	65	JF802046
AY09	F: AGAAACAGAGGAACGGTAAG R: GAGCGAGTATGTAATGGAAC	$(AT)_8(GT)_{10}$	187	1	61	JF802047
AY11	F: CTATTTAGCCAATGTCGGAT R: GCAAACTTGTCTTGCATACA	$(CA)_{12}$	121	1	58	JF802048
AY19	F: GAAACAAATTACAAGGCACA R: CCAAGCATCTCCGTATCTTC	$(GT)_7$	222	1	60	JF802049
AY24	F: GTTCGCTTTTATTTATGTTATGG R: GCAAGGAAAAGAACGTGATG	$(AC)_8$	132	1	54	JF802050
AY28	F: TGACCTCATTCCTCTTACAA R: CAACTATGCAGGGACACTTA	$(CA)_{11}$	79	1	50	JF802051
AY38	F: GGACCAGCAGCTTCCACTAT R: GAGGGCTTCTTCAACCAAAC	$(CA)_{12}$	262	1	65	JF802052
AY42	F: GAGCACAAATATGCAGGGAC R: GAAGATTGGCAACTCGTCAG	$(TC)_9$	151	1	64	JF802053
AY47	F: CAGGTCTATCATCCGCTTCC R: CTCCGAGTGTCCTTGCTTGT	$(TC)_5$	90	1	62	JF802054
AY49	F: ACGGATATTTCATGGTCATT R: AGTCTTGGCATGGTTATGTT	$(AC)_8$	257	1	61	JF802055
AY55	F: TGCCAAACATTGTGATACAC R: ATGGGACTCCTTAGGAACTG	$(GT)_7$	190	1	62	JF802056
AY59	F: CATACAAGTAAGAGTTATGCAAGT R: GAGAAGAAGCACCAAAGGTA	$(TG)_6$	96	1	60	JF802057
AY60	F: TCGAATCAAGATTTCTACCA R: AGAAGCATCGAAAGAGGATA	$(GT)_8$	217	1	54	JF802058
AY61	F: TACCCTTCGTGCCTGATTTG R: GTGCTTGAAGCTCAGGAATTTGTC	$(CT)_{13}$	118	1	65	JF802059
AY73	F: AAGGAGTTGAGGCAGTTGTT R: AAAATTGAGCTGAGATGGAG	$(AC)_{15}$	145	1	56	JF802060

Table 2. Locus-specific measures of genetic diversity of two populations of *Acer yangbiense*: N = population sample size, A = number of alleles, H_o = observed heterozygosity, H_e = expected heterozygosity, and P = P values of population-level Hardy–Weinberg exact tests (conducted using GENEPOP). P values with an asterisk indicate significant departure from Hardy–Weinberg equilibrium (P < 0.01).

	YB (Yangbi, 25°45′N, 100°0′E)					KBG (Kunming Botanical Garden)				
Locus	N	A	H_o	H_e	P	N	A	H_o	H_e	P
AY10	5	1	0.0000	0.0000	—	34	2	0.2353	0.2107	1.0000
AY14	5	3	0.8000	0.6222	1.0000	34	4	0.5294	0.5909	0.0000*
AY29	5	2	0.4000	0.5333	1.0000	34	1	0.0000	0.0000	—
AY33	5	3	0.6000	0.6889	0.1121	34	2	0.5588	0.5070	0.7309
AY34	5	2	0.2000	0.2000	—	34	3	0.5294	0.6479	0.0000*
AY54	5	2	0.0000	0.3556	0.1122	34	1	0.0000	0.0000	—
AY64	5	2	0.2000	0.2000	—	34	2	0.6176	0.5035	0.2970
AY69	5	2	0.6000	0.4667	1.0000	34	2	0.3824	0.5070	0.1802
AY74	5	1	0.0000	0.0000	—	34	3	0.0000	0.5777	0.0000*

protocols included: initial denaturation of 3 min at 95°C, followed by 35 cycles of 45 s at 94°C, 45 s at 51–65°C, 45 s at 72°C, and final extension at 72°C for 7 min. The amplified products were then separated on 8% denaturing polyacrylamide gels and visualized by silver staining. A 20 bp DNA ladder standard (Fermentas, Shenzhen, China) was used as standard for scoring. Ultimately, 34 microsatellite primers (Table 1) were successfully amplified with expected size and banding patterns (one or double clear bandings), of which nine primers displayed polymorphisms. It is suggested that the polymorphism in these loci should be better evaluated through larger samples.

Five individuals of *A. yangbiense* from the natural population (YB = Yangbi, 25°45′N, 100°0′E) and 34 ex situ individuals (KBG = Kunming Botanical Garden) were used to test the polymorphism of the microsatellite markers. Vouchers were deposited at the Kunming Institute of Botany herbarium (KUN; Appendix 1). Five of the nine primers yielded polymorphic amplification products in both populations. Four markers, AY10 and AY74 in YB and AY29 and AY54 in KBG, were shown to be monomorphic. Population genetic analyses were performed using GENEPOP (Raymond and Rousset, 1995). The polymorphic microsatellite markers amplified alleles ranging from two to five per locus; values for observed (H_o) and expected (H_e) heterozygosities ranged from 0.0000 to 0.8000 and from 0.0000 to 0.6889, respectively (Table 2). Three loci (AY14, AY34, AY74) deviated significantly from Hardy–Weinberg equilibrium (HWE) at KBG (P < 0.01).

CONCLUSIONS

Nine polymorphic microsatellite markers reported here will provide useful tools for the generation of data on population genetics of *A. yangbiense*, including studies on population genetic structure, genetic diversity, and gene flow. These data will help in establishing conservation strategies for this critically endangered maple species. The nine polymorphic loci, together with monomorphic loci, also have potential applicability to other congeneric species.

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Appendix 1. Herbarium vouchers deposited at KUN (Kunming Institute of Botany, Chinese Academy of Sciences). Information presented: taxon, population, herbarium voucher accession code. YB = Yangbi population; KBG = Kunming Botanical Garden.

Acer yangbiense—YB; ZLL-10001

Acer yangbiense—KBG; ZLL-11001