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Isolation and characterization of microsatellite loci in *Ajuga taiwanensis* Nakai ex Murata using PCR-based isolation of microsatellite arrays (PIMA)

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ABSTRACT. *Ajuga taiwanensis* is a valuable herb for traditional Chinese medical treatment. In this study, eight microsatellite loci from *A. taiwanensis* were isolated. The simple sequence repeat (SSR) markers were screened in 15 samples of wild populations of *A. taiwanensis*, and nine samples from its sister *A. bracteosa* and *A. nipponensis*. In *A. taiwanensis*, the number of alleles ranged from 3 to 11, and values of expected (H_E) and observed (H_O) heterozygosity were 0.54253- 0.90575 and 0.0000-1.0000, respectively. All loci deviated significantly from Hardy-Weinberg expectations due to the heterozygote deficiency, indicating a dramatic loss of genetic polymorphisms in the restrictedly distributed species. The markers amplifying well in the three species are useful for examining genetic diversity and population genetic structure, and this can provide information for establishing a conservation strategy for these endangered species.

Keywords: *Ajuga nipponensis*, *Ajuga taiwanensis*, Heterozygosity; Microsatellite; PIMA.

INTRODUCTION

The genus *Ajuga* (Labiatae) comprises about 40 to 50 species all over the world. *Ajuga taiwanensis* Nakai ex Murata, a perennial herb, is restrictedly distributed in the Philippines, Ryukyus, and Taiwan (Hou, 1996). Due to rapid socio-economic development, natural habitats for the plants have been severely disturbed, resulting in population fragmentation. Overexploitation is another reason for the quick population decline. Species belonging to the genus have been used as folk medicinal plants, as anthelmintics against intestinal disorders, and as antifungal, hypoglycaemic, antitumor, and antimicrobial agents (Baytop et al., 1984; Wesnner et al., 1992; Rodriguez-Hann et al., 1994). *Ajuga taiwanensis* is often used for the treatment of hepatitis and hepatoma (Hou, 1996).

Microsatellites, or Simple Sequence Repeats (SSRs), are DNA fragments of nuclear or organellar genomes that consist of repeating units of 1-4 base pairs in length (Turnpenny and Ellard, 2005). SSR fingerprints are co-dominantly inherited and are often used as molecular markers for population studies and conservation genetics (Weising et al., 2005). In this study, *A. taiwanensis* represents some invaluable natural resources. In enforcing the conservation of the rare species, an investigation of genetic diversity and population structure provides vital information. In the study, we isolated eight microsatellites from the Labiatae species and screened its population structure.

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MATERIALS AND METHODS

Genomic DNAs were obtained from ground leaf tissue of *A. taiwanensis* (AJ) using a CTAB methodology (Doyle and Doyle, 1987). Microsatellite markers in AJ were isolated by beginning with a random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) enrichment (Hsu et al., 2004; Huang et al., 2008). This PCR isolation of microsatellite arrays (PIMA) approach was proposed by Lunt et al. (1999). It exploits the fact that the RAPD fragments contain microsatellite repeats more frequently than genomic clones (Cifarelli et al., 1995).

The RAPD-PCR amplification was performed in a thermal cycler (Biometra) with a reaction mixture (50 μL) containing 20 to 100 ng DNA, 0.2 mM of each dNTP, 2

mM $MgCl_2$, 0.5 U *Taq* polymerase (Violet), and 5 pmols of one RAPD primer. The PCR program proceeded as follows: initial denaturing 5 min at 94ºC for 1 cycles, 35 cycles of 30 s at 94ºC, 1 min at 42ºC, 2 min at 72 ºC, followed by 10 min at 72ºC for additional extension step. Several RAPD primers were used to amplify DNA fragments from the genome of target species. PCR products were size-selected to obtain small fragments (ranging from 300 to 800 bp). DNA fragments were ligated into pGEM T-Easy Vector System (Promega), and the plasmids were transformed into DH5α *Escherichia coli* competent cell. Clones were screened using microsatellite-specific primers and two vector primers (Lunt et al., 1999). In positive clones, PCR electrophoresis would show two DNA fragments, of which one PCR product contains microsatellite signal. In contrast, only the whole inserted fragment could be found in negative clones. Extraction of the positive plasmid DNA was conducted and purified using the plasmid purification Mini Kit (Geneaid). Plasmid DNA was sequenced in an Applied Biosystems Model 377A automated sequencer (Applied Biosystems).

Specific-primer pairs were designed according to the nucleotide sequences upstream and downstream of the repetitive DNA using software Primer3 (Rozen and Skaletsky, 2000). PCR amplification was performed in a 25 μL volume containing 10 ng of genomic DNA, 0.2 mM dNTP, 2 mM $MgCl_2$, and 5 pmols of each primer. PCR programs took place as follows: initial denaturing step at 94ºC for 5 min; 35 cycles of 94ºC for 30 s, primer-specific annealing temperature (Table 1) for 30 s, 72ºC for 30 s, and a final extension step at 72ºC for 10 min. PCR products were electrophorezed by ethidium bromide staining in denaturing 8% polyacrylamide gels using 25-bp molecular size ladder (Promega) as a standard to estimate allele sizes. Results of the allele number, size range, number of bands per individual are listed in Table 1. Expected (H_E), and observed (H_O) heterozygosities were calculated using the Arlequin program version 3.1 (Excoffier et al., 2005). Testes of Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted using the GENEPOP program version 3.4 (Raymond and Rousset 1995) (http://genepop.curtin.edu.au/).

RESULTS AND DISCUSSION

Ajuga taiwanensis materials were collected from Nantou, Taichung, and Pingtung County in Taiwan. From each population, samples were collected randomly from four to six subpopulations. In this study, eight novel microsatellite loci in *A. taiwanensis* were isolated (Table 1) and tested in 15 individuals. The number of alleles per locus ranged from 3 to 11. The expected (H_E) and observed (H_O) heterozygosities ranged from 0.54253 to 0.90575 and 0.0000 to 1.0000, respectively. Significant departures from HWE (Table 1) were detected in all microsatellite loci. These deviations were due to the deficit of heterozygotes within populations (loci 3, 6, and 7), suggesting random losses of genetic polymorphisms by genetic drift in

Table 1. Characteristics of eight microsatellite markers isolated from *Ajuga taiwanensis* Nakai ex Murata.

Locus	Primer sequence (5′ to 3′)	Repeat motif	Size range (bp)	Total number of alleles	Ta (°C)	H_O	H_E	HWE p-value
AJ01	F: TCATGCCATCATTAATCAAA	$(GT)_{12}$	135~154	8	62	1	0.85287	<0.001
	R: AACCTCAATCTGTGGCTTCT							
AJ02	F: GGGAGGCGGAAACTGTTTGTGT	$(TC)_4(AC)_7$	229~245	11	53	0.93333	0.90575	<0.001
	R: TCTTTTACTTGCCCTTGCATTTCAGA							
AJ03	F: TCTCACGCATTTTGAATGCAC	$(AC)_9N_{17}(AC)_9$	164~170	4	55	0	0.67126	<0.001
	R: TGAATTAATGTGTGGATGCATGG							
AJ04	F: ATTCGATTTGGTTGCCAGTT	$(AT)_4(AC)_8$	183~235	11	58	0.93333	0.88046	<0.001
	R: GGCGGAGTAGTGAAACACAA							
AJ05	F: AGGCTGCTTGATTTCGCAAG	$(TG)_n$	180~204	4	52	0.93333	0.67126	<0.001
	R: GCGCCTAACAGAGCCTAGT							
AJ06	F: GGCCTCCTTGGTATGTAAGTTG	$(AG)_7TG(AG)_6$	156~170	4	54	0.13333	0.5977	<0.001
	R: GGCATGCCTGCACCAAATTC							
AJ07	F: CCGGGCTGGTGATTCTTCTT	$(AC)_6(GC)_2(AT)_3$	110~114	3	53	0	0.54253	<0.001
	R: GATGATTGCAAAGAGCGGGAAT							
AJ08	F: GCCAAGCACCGTCGTCTAAA	$(CT)_6GAA(C)_{17}(AC)_8$	162~214	6	53	0.73333	0.6919	<0.001
	R: CGTGTGACTGCATTTTCATGG							

AJ01~AJ08, isolated microsatellite locus; F:, forward primer; R:, reverse primer.
Ta, annealing temperature of the primer pair; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy-Weinberg equilibrium.

fragmented populations, or possibly due to the population structuring within specimens (Wahlund effect).

For GENEPOP analysis of linkage disequilibrium (LD), the results revealed no significant LD was discovered in most loci with the exception of loci AJ02 and AJ04 (data not shown). The microsatellite primer pairs were also applied to *A. bracteosa*, and *A. nipponensis*. All of isolated primer pairs from *A. taiwanesis* could cross-species amplify microsatellite fingerprints in these two sister species. The application of these microsatellite loci in *A. taiwanensis* may therefore provide a useful tool for understanding this species demography and population structure during environmental change.

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利用 PIMA 方法分離及分析台灣筋骨草微衛星基因座

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台灣筋骨草（*Ajuga taiwanensis*）為具有價值之傳統中草藥。此研究從台灣筋骨草分別分離出 8 個微衛星基因座，這些微衛星基因座用來篩選台灣筋骨草 15 個野生族群個體以及兩個同屬不同種 *A. bracteosa* 及 *A. nipponensis*，在台灣筋骨草，對偶基因數目範圍為 3 到 11 個，異質度觀測值（H_O）以及期望值（H_E）範圍分別為 0.54253 到 0.90575 以及 0 到 1.0。所有分析之基因座因為異型合子差異性而顯著偏離 Hardy-Weinberg 預估值，顯示侷限分佈物種有明顯遺傳多型性之缺失。這些在三種筋骨草皆能成功增幅之基因座可以用來分析物種遺傳多樣性及族群遺傳結構並且可以對這些瀕危物種建立保育策略及提供有用之資訊。

關鍵詞： 台灣筋骨草 ； 日本筋骨草 ； 遺傳歧異度 ； 微衛星基因座。