Isolation and characterization of polymorphic microsatellite loci for *Pistacia chinensis* Bunge (Anacardiaceae)

Zhaohong Ding and Jianbo Lu

College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou, 310036, China

Corresponding author: Jianbo Lu, E-mail: jianbo.lu@hznu.edu.cn

Abstract

Pistacia chinensis Bunge (Anacardiaceae) is a native, dioecious, perennial arbor, and an important bioenergy plant. Twelve microsatellite markers were developed for *P. chinensis* to evaluate genetic diversity and population genetic structure when this species was subject to landscape fragmentation. Twelve polymorphic microsatellite loci were developed in *P. chinensis* using a microsatellite-enriched genomic library based on magnetic beads. These loci were characterized in 24 individuals from three populations located on Thousand Island Lake, Zhejiang Province, China. The number of alleles per locus varied from three to sixteen. The observed and expected heterozygosity ranged from 0.1250 to 0.8750 and 0.2333 to 0.8917, respectively. These microsatellite loci will be applied in further studies on the population genetic diversity and genetic structure of *P. chinensis*. This study will improve understanding of the effects of landscape fragmentation, and help conserve and manage the species.

Keywords: : *Anacardiaceae, microsatellites, Pistacia chinensis, landscape fragmentation, population genetics*

Introduction

Habitat fragmentation, the reduction of continuous habitat into several smaller spatially isolated remnants, is caused mainly by human activity and includes agricultural development, urbanization, and deforestation etc. (Young et al., 1996). It is a significant threat to the maintenance of biodiversity in many terrestrial ecosystems. Recently, more attention has been paid to the potential genetic consequences of anthropogenic habitat fragmentation on plants (Yuan et al., 2012). Dioecious species, which use a relatively uncommon sexual system with separate female and male individuals, constitute only 6 % of angiosperms (Renner et al., 1995). Some studies have found that dioecious plants are more sensitive to change in population size and structure than self-compatible species because of the restricted number of mating groups within the population. Thus, they are also more sensitive to habitat fragmentation (Stehlik et al., 2008, Yu et al., 2011).

Pistacia chinensis Bunge (Anacardiaceae) is a dioecious, perennial, and deciduous arbor belonging to the *Pistacia* genus, which consists of at least 11 species of dioecious trees and shrubs (Ozden-Tokatli et al., 2010). *P. chinensis* is a widely distributed native tree in China because it has strong adaptability to poor habitats and adverse circumstances. Additionally, it has medicinal functions and its wood is economically useful (Li et al., 2007). Moreover, *P. chinensis* is considered a good raw material for bio-diesel production because of the high content and quality of its oil (Li et al., 2010). In the study of its genus plants, Albaladejo et al. developed eight microsatellite primers from *Pistacia Lentiscus* (Anacardiaceae) by magnetic beads (Albaladejo et al., 2008). Similarly, Chen et al. used magnetic beads to develop microsatellite loci of *Pistacia weinmannifolia* (Anacardiaceae) distributed in Yunnan and Guizhou provinces

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of China, and obtained fourteen microsatellite primers, nine out of fourteen loci developed for *P. weinmannifolia* amplified in *P. chinensis* (Chen et al., 2011).

Knowledge about presence or absence of null alleles is critical in order to assess a marker system's behavior in subsequent analyses. One main cause of microsatellite null alleles is poor primer annealing due to nucleotide sequence divergence (point mutations or indels) in one or both flanking primers. Microsatellite null alleles are not a natural characteristic of a specific gene, and are essentially different from isozyme null alleles. There have been few studies on germplasm resources at the molecular level, genetic diversity, and genetic structure. Therefore, we isolated and characterized 12 microsatellite markers for *P. chinensis* that were successfully applied to describe the genetic diversity and population structure of this species when subject to landscape fragmentation.

Materials and Methods

Genomic DNA was extracted from silica gel-dried *P. chinensis* leaves collected from Thousand Island Lake (TIL) (29°22'–29°50'N, 118°34'–119°15'E) (Zhejiang, China) using a Plant Genomic DNA Kit (Tiangen, Beijing, China). Samples were collected from different individuals that were randomly distributed to avoid sampling the same clone. A microsatellite-enriched genomic library based on magnetic beads was constructed following the protocol of Tong et al. (2012). Total genomic DNA (about 300 ng) was digested with the restriction enzyme *Mse*I (New England Biolabs, Beverly, Massachusetts, USA) for 1 h at 37°C, and the fragments were immediately linked with an *Mse*I-adapter pair (F:5'-TACTCAGGACTCAT-3', R:5'-GACGATGAGTCCTGAG-3'). After ligation, the products were diluted in a 1:10 ratio and amplified with an *Mse*I-N primer (5'- GATGAGTCCTGAGTAAN-3'). The PCR conditions were as follows: initial denaturation at 95°C for 3 min, followed by 20 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min, with a final extension cycle at 72°C for 8 min. Then a 5'-biotinylated probe, $(AG)_{15}$, and streptavidin-coated magnetic beads (Promega, Madison, Wisconsin, USA) were used to hybridize and capture the PCR product. Enriched products were amplified with the *Mse*I-N primers and the PCR products were purified using a TIANgel Midi purification Kit (Tiangen, Beijing, China). Purified DNA fragments were ligated into the pMD19-T vector (TaKaRa, Dalian, China) and then transformed into DH5α competent cells (TaKaRa).

A total of 210 clones were tested by PCR using the primer combination M13-47, M13-48, and $(AG)_{15}$. A total of 104 unique clones were identified and sequenced on a ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA) and 77 clones contained simple sequence repeats, of which 42 sequences were selected to design primers using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). The others were discarded because they were not suitable for designing primers. The optimum conditions of PCR and polymorphisms of these primers were tested with 12 individuals sampled from *P. chinensis* populations in TIL. The PCR reaction was performed under the following conditions: 95°C for 5 min; 30 cycles with each cycle lasting 30 s at 94°C, 30s at 51°C–59°C depending on the primer, and 30 s at 72°C, with a final extension of 72°C for 8 min. The 15 μL of PCR reagents included about 50 ng of genomic DNA, 0.25 mM of each dNTP, 0.1 μM of each primer, 1×PCR buffer (containing Mg^{2+} 1.5 mM), and 1 U of Taq DNA polymerase (Sangon, Shanghai, China). The products were resolved on 8 % (W/V) polyacrylamide denaturing gel. A total of 12 loci were screened out for either lack of products or monomorphism. We further tested the remaining 12 polymorphic loci, which were labeled with one of the following fluorescent dyes: HEX, ROX, TAMRA, or 6-FAM. There were 24 individuals from three *P. chinensis* populations. The PCR products were scanned on an ABI 3730 automated sequencer with an internal lane standard, and then analyzed using GeneMapper 4.0 (Applied Biosystems). Finally, we obtained 12 polymorphic loci for *P. chinensis* (Table 1). These microsatellite loci varied in allele size from 129 bp to 302 bp and all the sizes of the amplification products were matched based on sequences for all primers. The number of alleles per locus varied from three to sixteen with an average of 6.7 (Table 1).

To assess the efficiency of the selected loci, we used 24 individuals of *P. chinensis* from three different populations (eight individuals from each population) in TIL (Hangzhou, China) under the same PCR conditions mentioned above. The genetic diversity indexes, including the number of alleles, and the observed and expected heterozygosity, were analyzed using GENEPOP v4.0 (Rousset, 2008).The polymorphism information contents (PIC) were calculated using CERVUS v3.0.3 (Kalinowski et al., 2007). Deviations from the Hardy-Weinberg equilibrium (HWE) and from the linkage equilibrium were tested using GENEPOP v4.0 with sequential Bonferroni adjustment (Rice, 1989).

Results and discussion

The results showed that the mean number of alleles per locus was 4.0, 3.3, and 3.5 at the population level, respectively. The observed and expected heterozygosity per locus ranged from 0.1250 to 0.8750 and 0.2333 to 0.8917, respectively (Table 2). The average PICs were 0.5449 (range: 0.3589–0.7993), 0.4931 (range: 0.3047–0.7654) and 0.5442 (range: 0.1948–0.8157) within the three populations, respectively. No significant linkage disequilibrium ($P > 0.05$) was observed for each pair of loci. However, loci PC193 and PC205 in the *Mainland* population and loci PC193 in the *Medium Island* population were significantly deviated from HWE ($P<0.05$).

The 12 polymorphic microsatellite loci for *P. chinensis* were newly developed using a genomic enrichment library and characterized. These microsatellites are important tools for genetic studies on subjects such as genetic diversity, spatial genetic structure, mating system, and gene flow etc. in *P. chinensis*. Although microsatellite null alleles have the characteristics of universality, complexity and invisibility, using Hardy-Weinberg equilibrium tests, parent-offspring genotype analysis, and new primer design can test and estimate their frequency. Null

alleles have significant effects on the results of genetic analysis, potentially decreasing population genetic diversity and increasing genetic differentiation among populations.

Table 1
Characterization of 12 new polymorphic microsatellite loci in *Pistacia chinensis*.[a]

Locus	Primer sequences (5'-3')	Repeat motif	Size (bp)	A	T_a(℃)	Fluorescent dye [b]	GenBank accession no.
PC1	F:TATTGGAGAAGGAAGGGACG R:TCGTTTGAGATACCTGCTGC	$(GA)_7$	283–298	7	55℃	6-FAM	KT780354
PC19	F:AGGAAAACAGCAAACAAT R:GCTTTACTCACCTCCCAT	$(AG)_{20}$	149–177	4	51℃	6-FAM	KT780355
PC44	F:ATTGGAGAAGGAAGGGAC R:GAGAATGTCATCATCGTTTG	$(GA)_{11}$	161–187	4	56℃	HEX	KT780356
PC64	F:AAATAGGGAGAGGACTGGAG R:ACTCTCGTTACCTTGTGCTT	$(AG)_{16}$	255–282	10	54℃	HEX	KT780357
PC70	F:GCAACCAAAAATAAAAATCA R:CGAATCACTCCCACTCTAAG	$(TC)_{29}$	270–283	16	55℃	6-FAM	KT780358
PC96	F:TGAGAGTGAGAGAAGGTTGG R:AGTAAGGGAGATTTAGCGAA	$(CT)_{10}$	295–300	9	55℃	ROX	KT780359
PC106	F:ATGAAGAGGCTGTAGTTGAA R:ACCACAAGGACTAAAGAGAA	$(CT)_{13}$	178–181	3	56℃	TAMRA	KT780360
PC172	F: CTTCCCATTCACTTGTCC R:GGTTAGTTTGCTCGCTCT	$(GA)_{21}$	178–200	9	54℃	HEX	KT780361
PC189	F:TGAGTCCTGAGTAAGCAAATG R:TTAGAGGGCGTGAAGATGTA	$(TC)_8$	222–272	3	54℃	TAMRA	KT780362
PC193	F:TAAGGATGTTTGTGTGGAGG R:TAAGGTTTTACCAAATGCCA	$(GA)_{16}$	210–223	6	59℃	ROX	KT780363
PC196	F:CACGGTATCCATCTCCCTTT R:AGTAAGGTGGTTCGGATGCT	$(CT)_8$	129–159	5	59℃	TAMRA	KT780364
PC205	F:AAAGTATCTGTTATGTTGGG R:TGAGTCCTGAGTAAACACAC	$(AG)_{14}$	292–302	4	51℃	ROX	KT780365

Note: A = number of alleles observed; Ta = annealing temperature for each primer pair;

[a] All values are based on 24 samples representing two populations located in Thousand Island Lake in Zhejiang Province, China.

[b] Fluorescent dye: (i.e., 6-FAM, HEX, and TAMRA) used for fragment analysis.

Table 2
Genetic properties of the 12 newly developed microsatellites of *P.chinensis*.[a]

	Mainland population (n = 8)					*Medium* Island population (n = 8)					*Micro Island* population (n = 8)				
Locus	Na	Ho	He	P[b]	PIC	Na	Ho	He	P[b]	PIC	Na	Ho	He	P[b]	PIC
PC1	3	0.8750	0.6750	0.1616	0.5556	3	0.7500	0.6583	0.3862	0.5439	3	0.6250	0.6917	0.4378	0.5749
PC19	3	0.5000	0.5917	0.7325	0.4555	3	0.7500	0.5417	0.5062	0.4277	3	0.6250	0.6583	0.1049	0.5439
PC44	2	0.6250	0.5250	0.5637	0.3711	2	0.3750	0.5250	0.3865	0.3711	2	0.8750	0.5250	0.0866	0.3711
PC64	3	0.6250	0.6583	0.8866	0.5439	3	0.7500	0.6917	0.3398	0.5749	3	0.6250	0.7000	0.2998	0.5815
PC70	8	0.7500	0.8750	0.1558	0.7993	7	0.7500	0.8417	0.1178	0.7654	8	0.8750	0.8917	0.3026	0.8157
PC96	5	0.7500	0.8083	0.1447	0.7219	5	0.7500	0.8167	0.4309	0.7266	6	0.8750	0.8667	0.5509	0.7852
PC106	3	0.6250	0.5667	0.8013	0.4683	2	0.5000	0.4000	0.4250	0.3047	2	0.2500	0.2333	0.7815	0.1948
PC172	3	0.8750	0.5750	0.2526	0.4465	3	0.7500	0.5667	0.5062	0.4683	3	0.7500	0.6917	0.2773	0.5749
PC189	3	0.5000	0.6583	0.1049	0.5439	2	0.8750	0.5250	0.0866	0.3711	2	0.5000	0.4000	0.4250	0.3047
PC193	4	0.1250	0.6917	**0.0022***	0.5925	3	0.2500	0.4333	**0.0050***	0.3706	3	0.1250	0.5750	0.0755	0.4465
PC196	2	0.7500	0.5000	0.1266	0.3589	2	0.7500	0.5333	0.2193	0.3750	2	0.5000	0.5333	0.8501	0.3750
PC205	5	0.7500	0.7750	**0.0053***	0.6816	4	0.6250	0.7250	0.3970	0.6179	5	0.6250	0.8083	0.1084	0.7219

Note: Na = number of alleles; Ho = observed heterozygosity; He = expected heterozygosity; n = number of individuals in the population sampled; PIC = polymorphic information content.

[a] Locality and voucher information for the sampled populations: *Mainland* population = 29°42'39"N, 119°03'27"E; *Medium Island* population = 29°35'13"N, 118°55'20"E; *Micro Island* population = 29°34'59"N,118°55'07"E. All three populations are located in Thousand Island Lake (TIL) in Zhejiang Province, China.

[b] P-values for deviation from Hardy-Weinberg equilibrium. * Locus showed significant deviations from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.05$).

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