Conservation Genet Resour (2009) 1:81–83
DOI 10.1007/s12686-009-9019-6

TECHNICAL NOTE

Development of polymorphic microsatellite markers for *Ginkgo biloba* L. by database mining

Yuan-Yuan Li · Li-Ping Zang · Xiao-Yong Chen

Received: 20 April 2009 / Accepted: 6 May 2009 / Published online: 26 May 2009

Abstract *Ginkgo biloba* L. is a famous living fossil plant endemic to China. We report twelve polymorphic simple sequence repeat (SSRs) markers of *G. biloba* by mining expressed sequence tags (ESTs). One locus was significantly deviated from Hardy-Weinberg equilibrium. The number of alleles per locus ranged from 2 to 5. The PIC values were from 0.058 to 0.776. Observed and expected heterozygosity ranged from 0.050 to 0.776, and 0.055 to 0.799, respectively. These markers will be available for studies of population genetics, reproductive ecology and conservation genetics for *G. biloba*.

Keywords *Ginkgo biloba* · ESTs · Microsatellites · Genetic diversity

The genus *Ginkgo* (Ginkgoaceae) once contained at least 16 species widely distributing in Asia, North America and Europe, but now, only one species, *Ginkgo biloba*, survived after the Pleistocene glaciations (Willis and McElwain 2002). *G. biloba* is an endangered species and naturally distributes in China. It was widely cultivated for various aims of medicine use, food, timber, or ornament (Fan et al. 2004). Although genetic diversity had been analyzed by both nuclear and chloroplast DNA markers (Kuddus et al. 2002; Fan et al. 2004; Shen et al. 2005; Gong et al. 2008). No information based on microsatellite markers has been reported. To promote genetic analysis of diversity, origin, and evolution, a large number of microsatellite markers are desirable.

Simple sequence repeat (SSR) markers are powerful tools for reproducibility, co-dominant inheritance, relative abundance and highly polymorphism. A few of SSR markers have been developed in *G. biloba* for analyzing genetic diversity and mating patterns (Yan et al. 2006). Expressed sequence tag (EST) is sequenced portion of complementary DNA copies of message RNA, which represent part of the transcribed portion of the genome in given conditions. Large numbers of ESTs of *G. biloba* have been deposited in sequence database (Brenner et al. 2005), which provide abundant resource for marker development. EST-derived SSRs would take less expensive, effort and time comparing with traditional methods of SSR markers development, as well as they are theoretically transferable between taxa due to highly conserved exon sequences (Pérez et al. 2005). Here we report on the development of EST-SSR markers in the *G. biloba* by data mining for using in genetic enhancement projects.

The first 10,000 ESTs from Genbank data bases were scanned for microsatellites of trinuclotide motifs with a minimum of five repeats and dinuclotide motifs with a minimum of ten repeats using SSRHunter v.1.3 program (Li and Wan 2005). A set of 55 flanking SSR primer pairs from the non-redundant ESTs were designed using PRIMER3.0 software (Rozen and Skaletsky 2000). The major parameters for primer design were set as follows: primer length from 18 to 27 nucleotides, PCR product size from 100 to 300 bp, annealing temperature from 57 to 63°C and GC contents from 40 to 60%. Total DNA of *G. biloba* of 20 individuals was extracted from silica gel-dried leaves using a modified CTAB method (Fan et al. 2004).

Y.-Y. Li · L.-P. Zang · X.-Y. Chen (✉)
Department of Environmental Sciences, Shanghai Key Laboratory for Ecological Processes and Restoration, East China Normal University, 200062 Shanghai, China
e-mail: xychen@des.ecnu.edu.cn

Y.-Y. Li · X.-Y. Chen
Tiantong State Observation Station of Forest Ecosystems, 200062 Shanghai, China

Table 1 Twelve EST-SSR loci for *Ginkgo biloba* and PCR primers employed to screen 20 individuals

Primer ID	GeneBank accession no.	Repeat motif	Primer sequence (5′–3′)	Tm (°C)	Size (bp)	*A*	H_O	H_E	F_{IS}	PIC
Gb19	EX943410	$(CGA)_6$	F: CGGAAATATGGGAACGAAGA	55.1	155	3	0.403	0.569	0.292	0.560
			R: GAGGGGCCTTCTCCTTTAGA							
Gb30	EX934412	$(TGA)_5$	F: TTGCGTTTTAGCCCAAATTC	55.1	188	3	0.372	0.523	0.289	0.508
			R: TTAGCCCACCTTGCAGAACT							
Gb31	EX934456	$(CCT)_5$	F: ATTGGTGAGACGGAGAATGG	63.4	191	2	0.063	0.062	−0.016	0.058
			R: TCAGCGGTGATCGATAGTTG							
Gb32	EX934660	$(AGA)_5$	F: TCGGAGCTTTACGCTCAAAT	63	159	3	0.100	0.192	0.479	0.219
			R: CTGCACCAATTTCGTGGTCT							
Gb33	EX934694	$(TAT)_9$	F: GGCCACATTCTCTGCGTAGT	59.6	171	4	0.387	0.516	0.250	0.441
			R: ATTTCCGCAACTCACCATTC							
Gb37	EX934934	$(AT)_{18}$	F: CCAAACATCACTGCTAGACACA	63	162	4	0.050	0.238	0.790*	0.252
			R: TGTTGCATGGCTTTTCAATC							
Gb42	EX936042	$(CTT)_6$	F: TAAGCAGGCAAGGAAGAAGG	64	180	2	0.783	0.481	−0.628	0.219
			R: TGGATTTCTGCCATCGTACA							
Gb45	EX936879	$(CGA)_5$	F: ATGGTGGGGATGAGTACGAC	64	224	2	0.056	0.055	−0.018	0.058
			R: CCAAGCTAAAGGAAGAGAGGAA							
Gb46	EX937166	$(TCC)_5$	F: TCCCCCTGGAAACCTTTATC	63.4	187	5	0.306	0.740	0.586	0.732
			R: CTCAGGTCGATGGAAGGAAG							
Gb50	EX937397	$(CT)_{27}$	F: TGCAAAGTGCCTTTGTGTTC	63.4	233	5	0.733	0.799	0.083	0.776
			R: GGCACCCTTGTTTCTCCATA							
Gb62	EX933770	$(CAG)_8$	F: ATTCTCGACACACACCACCA	63.4	160	2	0.450	0.350	−0.286	0.349
			R: GCTGCTGCTCGTGAGGTATT							
Gb63	EX933770	$(AGC)_6$	F: TGATCCAGATGGTCAGTGCT	63.4	217	2	0.213	0.195	−0.092	0.241
			R: AAACGTTCAAGCAAGCGTCT							

* Significant departure from Hardy–Weinberg equilibrium ($P < 0.01$)

Tm (°C) annealing temperature; *A* number of observed alleles; H_O observed heterozygosity; H_E expected heterozygosity; F_{IS} inbreeding coefficient; *PIC* polymorphic information content

PCR amplification was carried out in 20 μl reactions of approximately 50 ng of genomic DNA, 0.1 μM of each primer, 1 × PCR buffer, 2.5 μM Mg^{2+}, 0.2 μM each dNTPs, 0.4 U of DNA *Taq* polymerase (Sangon). Cycling conditions were: 2 min denaturation at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55.1–64.0°C (Table 1), 30 s at 72°C; and a final extension of 72°C for 2 min. PCR products were resolved on 8% polyacrylamide denaturing gel, stained with silver and photographed by Bio-Rad DOC2000.

Fifty-five sequences are suitable for primer design, among them, 7 failed to have clear PCR products. After screening in the 20 individuals, 36 of 48 primer pairs were monomorphic. The 12 polymorphic loci yielded the predicted size ranges from 155–233 bp (Table 1). Linkage disequilibrium (LD) was tested using FSTAT v2.9.3.2 (Goudet 2001). Observed (H_O) and expected heterozygosity (H_E) were performed using the program TFPGA v1.3 (Miller 1997). Inbreeding coefficient (F_{IS}) was calculated by the equation of $(H_E - H_O)/H_E$, and bias from 0 was tested by χ^2 (Workman and Niswander 1970). The polymorphic information content (PIC) value was estimated according to the following formula: $PIC = 1 - \sum_{i=1}^{n} f_i^2$, where f_i is the frequency of the *i*th allele and n is the allele number. All of 12 loci produced a total of 37 alleles, and the number of alleles and genotypes varied from 2 to 5 and 2 to 9 per locus, respectively (Table 1). The PIC values are from 0.058 to 0.776 (Table 1). The observed and expected heterozygosity ranged from 0.050 to 0.783 and 0.055 to 0.799, respectively (Table 1). One locus, *Gb*37, was significantly deviated from Hardy-Weinberg equilibrium after sequential Bonferroni correction (Rice 1989). One of 66 pairwise exact tests rejected genotypic equilibrium between loci (*Gb*19 and *Gb*33) after sequential Bonferroni correction. These 12 polymorphic EST-derived microsatellite loci developed here provide a powerful tool for further studies in population genetics, reproductive ecology and conservation genetics of *Ginkgo biloba*.

Acknowledgments We thank Mrs Shu-Zhen Yang for helps in sample collection. This work was partly supported by Program for New Century Excellent Talents in University (NCET-05-0431) to XY Chen.

References

Brenner ED, Katari MS, Stevenson DW, Rudd SA, Douglas AW, Moss WN, Twigg RW, Runko SJ, Stellari GM, McCombie WR, Coruzzi GM (2005) EST analysis in *Ginkgo biloba*: an assessment of conserved developmental regulators and gymnosperm specific genes. BMC Gemomics 6:143–152

Fan XX, Shen L, Zhang X, Chen XY, Fu CX (2004) Assessing genetic diversity of *Ginkgo biloba* L. (Ginkgoaceae) populations from China by RAPD markers. Biochem Genet 42:269–278

Gong W, Zeng Z, Chen YY, Chen C, Qiu YX, Fu CX (2008) Glacial refugia of *Ginkgo biloba* and human impact on its genetic diversity: evidence from chloroplast DNA. J Integr Plant Biol 50:368–374

Goudet J (2001) FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3). Available at http://www2.unil.ch/popgen/softwares/fstat.htm

Kuddus RH, Kuddus NN, Dvorchik I (2002) DNA polymorphism in the living fossil *Ginkgo biloba* from the Eastern United States. Genome 45:8–12

Li Q, Wan JM (2005) SSRHunter: development of a local searching software for SSR sites. Hereditas (Beijing) 27:808–810

Miller MP (1997) Tools for population genetic analysis (TFPGA), 1.3: a windows program for the analysis of allozyme and molecular population genetic data. Distributed by the author

Pérez F, Ortiz J, Zhinaula M, Gonzabay C, Calderón J, Volckaert FAMJ (2005) Development of EST-SSR markers by data mining in three species of shrimp: *Litopenaeus vannamei*, *Litopenaeus stylirostris*, and *Trachypenaeus birdy*. Mar Biotech 7:554–569

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43: 223–225

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, Totowa, pp 365–386

Shen L, Chen XY, Zhang X, Fu CX, Qiu YX (2005) Genetic structure of *Ginkgo biloba* L. (Ginkgoaceae) based on cpDNA PCR-RFLPs: inference of glacial refugia. Heredity 94:396–401

Willis KJ, McElwain JC (2002) The evolution of plants. Oxford University Press, New York

Workman PL, Niswander JD (1970) Population studies on southwestern Indian tribes. II. Local genetic differentiation in the Papago. Am J Human Genet 22:24–49

Yan XF, Lian CL, Hogetsu T (2006) Development of microsatellite markers in ginkgo (*Ginkgo biloba* L.). Mol Ecol Notes 6:301–302