Applications in Plant Sciences

PRIMER NOTE

Development of microsatellite markers for a monotypic and globally endangered species, *Glyptostrobus pensilis* (Cupressaceae)

Gang-Tao Wang[1,2,3], Zheng-Feng Wang[1,2,4], Rui-Jiang Wang[1,2,4], Dan Liang[1,2], and Guo-Bin Jiang[1,2,3]

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[1] Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, People's Republic of China

[2] Guangdong Provincial Key Laboratory of Applied Botany, South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, People's Republic of China

[3] University of the Chinese Academy of Sciences, Beijing 100049, People's Republic of China

[4] Authors for correspondence: wangrj@scbg.ac.cn, wzf@scbg.ac.cn

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PREMISE OF THE STUDY: Microsatellite markers were developed to facilitate studies of genetic diversity and structure in *Glyptostrobus pensilis*, a critically endangered and monotypic conifer species.

METHODS AND RESULTS: Using restriction site–associated DNA sequencing (RAD-Seq), we developed 10 polymorphic and 27 monomorphic microsatellite markers. Polymorphism was characterized using 333 individuals from nine populations. The number of alleles per locus ranged from one to 14 at the population level. The levels of observed and unbiased expected heterozygosities varied from 0.058 to 0.844 and 0.219 to 0.583, respectively. Nine of these 10 polymorphic markers were successfully cross-amplified in *Taxodium distichum*, the species most closely related to *G. pensilis*.

CONCLUSIONS: These microsatellite markers can be used to reveal the genetic diversity in existing populations of *G. pensilis*, enabling its conservation and restoration.

KEY WORDS Cupressaceae; endangered species; genetic diversity; genetic markers; *Glyptostrobus pensilis*; RAD-Seq.

Glyptostrobus pensilis (Staunton ex D. Don) K. Koch (Cupressaceae) is known as "shui song" in Chinese and "water pine" or "Chinese swamp cypress" in English (Averyanov et al., 2009). As its names imply, *G. pensilis* is adapted to swamp habitats with an anoxic environment. The species is a relic conifer and has been recognized as monotypic based on its morphology.

In terms of biogeographic history, *G. pensilis* was widely distributed throughout the Northern Hemisphere from the Early Cretaceous until the early Pleistocene (LePage, 2007). However, it is currently restricted to southern China, southern Vietnam, and eastern Laos as a result of early Quaternary glaciations and subsequent desertification (Li and Xia, 2004). Recently, habitat destruction such as deforestation and urbanization has resulted in declines in both the number of individuals and the number of populations of this species. *Glyptostrobus pensilis* is now considered Critically Endangered according to the IUCN Red List (IUCN Red List Committee, 2011), and most of its wild populations contain only one or a few individuals. To conserve this rare and endangered species integratively, the population genetic diversity of *G. pensilis* should be carefully evaluated using as many populations as possible. A population genetic diversity analysis conducted by Li and Xia (2004) employed only a small fraction of the populations of this species in China and used dominant inter-simple sequence repeat (ISSR) markers. This method was later applied to compare genetic variation among four natural and artificial populations (Wu et al., 2011). Nguyen et al. (2013) also detected the genetic variation of *G. pensilis* using chloroplast microsatellites but only in the Vietnam populations. In this study, almost all the global water pine populations except those in Laos are sampled (Appendix 1) and used to characterize genetic variation in the newly developed microsatellite markers. These markers are also cross-amplified in *Taxodium distichum* (L.) Rich. (Appendix 1), the phylogenetically most closely related species in Cupressaceae (Hao et al., 2016).

METHODS AND RESULTS

We sampled a total of 333 individuals from China and Vietnam. In the field, most of the natural populations are small, containing only one or a few scattered individuals. For genetic diversity measurements, we grouped the populations and divided them into nine large populations based on their locations in the nation or province. All field-collected leaf materials were dried immediately in silica gel. In the lab, DNA was extracted from these materials using

TABLE 1. Characteristics of 37 microsatellite markers developed in *Glyptostrobus pensilis*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	GenBank accession no.
GP_19	F: GCCAGCAGATTATCACCCAG R: GGGCCACCAGAAGACATGC	$(GT)_9$	314–338	MH236836
GP_43	F: AGGTGCCTTGTCAACTAAATCC R: GGTCAACTTTGAATAAGGCCAAAC	$(AC)_9$	153–161	MH236849
GP_46	F: AAGGGTGGCTCATTTCCAG R: TCTAGCATTGAAACATAGTGGC	$(GAA)_7$	152–156	MH236852
GP_57	F: TTATATTAGTCATTTGTGGGCTCC R: TGGCGAGGTATAATTTGGGC	$(GT)_{11}$	207–212	MH236855
GP_58	F: AGAGGTAACTCCATCCATGTC R: GTCACATCCTATCTCAAGAATGAGC	$(TC)_{21}$	288–374	MH236856
GP_71	F: ACCTAGAAGGCAATAGGCCG R: AGGAGAAAGCATTCACTACAAGG	$(AC)_8$	199–201	MH236858
GP_75	F: TGGTTAGACTATGCTGGCAATC R: TCAGCCTTACTTCACAATGCTC	$(GA)_7$	149–153	MH236862
GP_80	F: TGGTTAGACCCATCCAAGCC R: AGAAGCACAGGTCATAGCC	$(CA)_{44}$	145–147	MH236864
GP_89	F: ACACTCACATCCTAGTCCGTC R: ATCGACCTTTATCATGCCATTC	$(GT)_8$	332–338	MH236868
GP_94	F: AGCATTTGGAACCTAAACAAGTCC R: ATGTCCTCAACATTCGCCC	$(AG)_{15}$	130–172	MH236871
GP_7	F: TGGGTCTGGATAATTGTGGC R: TCTCTGCAATAGGTCTGGTAAG	$(GT)_3AT(GT)_4TT(GT)_{39}$	332	MH236832
GP_8	F: ATCCTCCCTATCGTGACCC R: AGTGGGTGTTACATGCATCC	$(CTT)_7$	224	MH236833
GP_9	F: CGACTGATCGGTTCTTCGC R: CATCTCCAGTGGCATATCTCG	$(AT)_3AG(AT)_{12}AGATCT(AT)_8$	343	MH236834
GP_17	F: AATGGAGACAAGGACCATAGG R: GCCTTACAGCCATTTAAGTACC	$(GA)_8$	190	MH236835
GP_22	F: AAGAGGCGTTGCAGTGTTC R: GCCCTGCCGTATAGACTACC	$(GGA)_7$	232	MH236837
GP_26	F: ACATGTTTACCAAATTCAATGCCTC R: GAGGGAATTGGTGCCCTTC	$(CT)_7$	156	MH236839
GP_28	F: ACAACTCATTGGGTAAGTGGTC R: GGGATGGAAATCTAAGCAATGTC	$(AT)_8$	179	MH236840
GP_29	F: GGATGATGCAAAGGGACCG R: TCTTCCAAGCAAAGACTTCAGAC	$(AC)_8GTTATTTATAT(AC)_7$	370	MH236841
GP_31	F: CGGTTACCCTCCCATCTGC R: ACCAGCTACAAATTTATTCGCC	$(AC)_8$	394	MH236842
GP_32	F: AGGTACATAGGGTTGAGGGC R: GGTGAGAGGTGACAACCTAGAC	$(CT)_9$	192	MH236843
GP_35	F: GGACTTTGAGTTTGAAGGAGCC R: GCCATGAAAGAAGAAATTATAAGCC	$(GAA)_8$	251	MH236844
GP_36	F: TGGGTTATCTTCTAGTGCAACTC R: CCCAATATGGATACGGCTGG	$(AT)_9$	207	MH236845
GP_37	F: TCTTCTCCTTCACGAAATGAGC R: TGAACTAAACTGTGGTGCCTTAC	$(CT)_8$	194	MH236846
GP_39	F: TGAGAGAAGATTTCTATGGTATTGTCC R: TATTGAAGTGTTTGTGCCTTACAG	$(GT)_9$	153	MH236847
GP_41	F: ACTCTTGGAAAGGGATAAGTGG R: ATCCATCTTGTACTTGCATCAC	$(GT)_{13}$	175	MH236848
GP_44	F: TCAGGACCCAGCTCAAACC R: TCAGATCCTTATCTTCTTGAGGC	$(GT)_{12}$	185	MH236850
GP_47	F: ACATTGTGTTCCTTCTCTTAACCC R: ATGTTGGAAGATTGAACCCAGC	$(AC)_{15}$	176	MH236853
GP_56	F: TGGAATCTTTAGGGCTTTACTGC R: GCTTGTGACATCAGGGTTGG	$(CT)_8$	213	MH236854
GP_64	F: TTGCTTCACCTAGTGGGAC R: TGTTGGAGAGTTTGTACCTATTGAG	$(AC)_{10}$	184	MH236857
GP_72	F: CGGTTTGTGGATCTTAACTAGTGC R: AAGTGTTTGTGCCTCGCAG	$(GT)_8$	167	MH236859

(Continues)

TABLE 1 (Continued)

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	GenBank accession no.
GP_73	F: ACCATTGCATCTACAGCACG R: CCACACATCTAATGGTTTATTGAAG	$(GT)_9$	227	MH236860
GP_74	F: TATCGACCTGCTCCTAGCC R: ACTACTGATTTCATCCGGTCG	$(GT)_{13}$	203	MH236861
GP_78	F: CCTTTGCCTCAAATTAATCGCAC R: AGAATCACTTTAACTAGGGTGCTC	$(AC)_8$	160	MH236863
GP_83	F: TGGTCATGCTAGTTGTATCCC R: GCACTTTGATTCTTTACCAATTGTC	$(GT)_8$	177	MH236865
GP_84	F: CGTGCATCGAGATACTGAAGG R: TGATCGTATTGCACGCAACC	$(AT)_9$	152	MH236866
GP_88	F: ACTACTTTGTCGCTTGCATAC R: AGATCTGTGAAGTTTGACTTGG	$(AC)_9$	198	MH236867
GP_96	F: TGTCTTCACTTTAGGCTTTGGG R: TGGAAGTAGAAACCCTAGTATCCTC	$(TTC)_6TTTC$	173	MH236872

[a]For all loci, the annealing temperature was 53°C and the forward sequence was fluorescently labeled with FAM.

a modified cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991).

Restriction site–associated DNA sequencing (RAD-Seq; Baird et al., 2008) was used to obtain partial genomic DNA sequences of *G. pensilis*. The microsatellites were then selected and developed based on these sequences. Two samples, one from the South China Botanical Garden and the other from Conghua District, Guangzhou Province, China, were used to construct the RAD-Seq libraries with the restriction enzyme *Eco*RI (Promega Corporation, Madison, Wisconsin, USA), followed by 150-bp paired-end sequencing using a HiSeq X Ten genetic analyzer (Illumina, San Diego, California, USA). From the two samples, 35,615,442 and 35,297,882 raw sequences were obtained, respectively. The raw sequence data are available in the National Center for Biotechnology Information (NCBI) Sequence Read Archive database (accession no. SRR7133729 and SRR7133728). After filtering PCR duplicates and low-quality reads for each of these raw sequences, Rainbow 2.0.4 (Chong et al., 2012) was used to assemble the sequences separately. The two assembled sequences were subsequently combined and re-assembled by CAP3 (Huang and Madan, 1999), resulting in 3,285,999 contigs with a total length of 787,094,171 bp. The minimum and maximum lengths of the contigs were 80 bp and 2016 bp, respectively, with an average length of 173.69 bp and an N50 length of 325 bp. Microsatellites with dinucleotide and trinucleotide motifs with at least seven repeats were identified from these assembled sequences by MSATCOMMANDER 0.8.2 (Faircloth, 2008). Then, 100 microsatellites were chosen, and six individuals were initially used to characterize their polymorphisms.

We performed PCRs in a 20-μL volume with 0.2 mM dNTPs, 0.4 μM primers, 1× PCR buffer (2.5 mM Mg^{2+}), 50 ng of genomic DNA, and 1 unit of *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China). The conditions included an initial step of 95°C for 5 min; followed by 35 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final step of 72°C for 10 min. The PCR products were checked on a 2% agarose gel, and only the microsatellites with clear bands and correct sizes were retained. Subsequently, the allele size polymorphisms were analyzed by an ABI 3730 sequencer and determined by GeneMapper version 4.1 (Applied Biosystems, Carlsbad, California, USA). A total of 37 microsatellites showed clear allelic patterns, with 10 of them being polymorphic. Finally, we used an additional 327 individuals to test the full range of allelic variation in these 10 microsatellites.

All genetic diversity parameters, including the number of alleles per locus, observed heterozygosity, and unbiased expected heterozygosity were obtained with GenAlEx 6.5 (Peakall and Smouse, 2012). The fixation index was calculated using GENEPOP 4.3 (Rousset, 2008). The deviation from Hardy–Weinberg equilibrium (HWE) and genotypic linkage disequilibrium (LD) among all pairs of loci within populations were also estimated using GENEPOP 4.3 using the default parameters. Sequential Bonferroni correction (Holm, 1979) was applied to adjust the level of significance for the HWE and LD analyses.

In *G. pensilis*, 37 microsatellites were amplified successfully, 10 of which were polymorphic and 27 of which were monomorphic (Table 1). The number of alleles for *G. pensilis* ranged from one to 14 (Table 2). For the polymorphic loci, levels of observed heterozygosity and unbiased expected heterozygosity ranged from 0.058 to 0.844 and 0.219 to 0.583, respectively (Table 2). All 10 polymorphic loci showed deviation from HWE within one or more populations, mostly due to heterozygosity deficit. This is most likely the result of the artificial population groupings that were used (due to the very small population sizes and scattered distribution characters in *G. pensilis*), which might not follow their natural distributions. This may have resulted in a mixture of individuals with different genetic backgrounds, causing deviation from HWE by the Wahlund effect. We found no consistent deviation from LD for any loci within the populations. Nine of the 10 polymorphic markers successfully cross-amplified in six *T. distichum* individuals (Table 3).

CONCLUSIONS

In this study, 10 polymorphic and 27 monomorphic microsatellite markers were developed for *G. pensilis*. The cross-amplification test indicated that nine of the 10 polymorphic markers can be successfully amplified in the phylogenetically closely related *T. distichum*. These markers will offer valuable tools for future investigations of genetic diversity and structure, level of gene flow, and conservation genetic studies in these two species.

TABLE 2. Genetic diversities of 10 polymorphic microsatellites developed for *Glyptostrobus pensilis* in eight Chinese populations and one Vietnamese population.[a]

	JX ($n = 59$)			HN ($n = 6$)			Đắk Lắk ($n = 59$)			HK ($n = 6$)			FJ ($n = 81$)			GD ($n = 74$)			GX ($n = 31$)			ZJ ($n = 6$)			HB ($n = 11$)		
Locus	*A*	H_o	uH_e	*A*	H_o	uH_e	*A*	H_o	uH_e	*A*	H_o	uH_e	*A*	H_o	uH_e	*A*	H_o	uH_e	*A*	H_o	uH_e	*A*	H_o	uH_e	*A*	H_o	uH_e
GP_19	2	0.000	0.066*	2	0.000	0.303	1	0.000	0.000	2	0.333	0.303	2	0.000	0.048*	3	0.192	0.425*	4	0.000	0.649*	1	0.000	0.000	2	0.000	0.173
GP_43	2	0.983	0.504*	2	0.200	0.200	2	1.000	0.504*	1	0.000	0.000	2	0.938	0.501*	3	0.137	0.199	3	0.167	0.159	1	0.000	0.000	1	0.000	0.000
GP_46	1	0.000	0.000	2	0.167	0.409	3	0.237	0.217	2	0.333	0.485	3	0.086	0.106	3	0.297	0.467*	3	0.192	0.520*	3	0.167	0.318	3	0.273	0.394
GP_57	2	0.017	0.017	2	0.000	0.303	2	0.069	0.067	2	0.667	0.485	2	0.025	0.025	2	0.264	0.503*	4	0.379	0.475*	2	0.500	0.571	1	0.000	0.000
GP_58	3	0.068	0.187*	2	0.000	0.356	9	0.130	0.758*	8	0.667	0.894	2	0.049	0.072	14	0.479	t0.882*	6	0.464	0.779*	4	0.333	0.697	4	0.364	0.619
GP_71	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.167	0.530	2	0.000	0.472*	2	0.219	0.503*	2	0.067	0.282*	2	0.333	0.545	2	0.500	0.521
GP_75	2	1.000	0.504*	2	1.000	0.545	2	1.000	0.504*	2	1.000	0.545	2	0.827	0.488*	2	0.903	0.499*	2	0.931	0.506*	2	0.833	0.530	2	0.100	0.100
GP_80	1	0.000	0.000	2	0.833	0.530	2	1.000	0.504*	2	0.333	0.303	1	0.000	0.000	2	0.425	0.352	2	0.567	0.481	2	1.000	0.545	2	0.091	0.091
GP_89	2	0.017	0.017	3	0.167	0.439	2	0.017	0.017	2	0.500	0.409	3	0.025	0.108*	4	0.403	0.513*	3	0.400	0.674*	3	0.667	0.682	3	0.455	0.567
GP_94	2	0.017	0.017	2	0.200	0.200	1	0.000	0.000	3	0.333	0.530	4	0.188	0.260	7	0.250	0.545*	3	0.100	0.267*	1	0.000	0.000	3	0.000	0.329*
Overall	–	0.210	0.131	–	0.257	0.329*	–	0.345	0.257*	–	0.433	0.448*	–	0.214	0.208*	–	0.357	0.489*	–	0.327	0.479*	–	0.383	0.389*	–	0.178	0.279*

Note: A = number of alleles; F = fixation index; H_o = observed heterozygosity; n = sample size; uH_e = unbiased expected heterozygosity.
[a]See Appendix 1 for locality and voucher information.
*Significant deviation from Hardy–Weinberg equilibrium after Holm's sequential Bonferroni correction ($P < 0.05$).

TABLE 3. Cross-amplification of 10 polymorphic microsatellite loci developed for *Glyptostrobus pensilis* in *Taxodium distichum*.[a]

Locus	*A*	H_o	uH_e	*F*	Adjusted *P* value
GP_19	4	0.833	0.773	−0.087	0.526
GP_43	1	0.000	0.000	—	—
GP_46	2	0.833	0.530	−0.667	0.242
GP_57	3	0.750	0.679	−0.125	0.571
GP_58	6	0.800	0.844	−0.059	0.863
GP_71	—	—	—	—	—
GP_75	2	1.000	0.545	−1.000	0.069
GP_80	2	1.000	0.545	−1.000	0.069
GP_89	1	0.000	0.000	—	—
GP_94	2	1.000	0.545	−1.000	0.069
Overall	—	0.691	0.496	−0.488*	0.000

Note: A = number of alleles; F = fixation index; H_o = observed heterozygosity; uH_e = unbiased expected heterozygosity.
[a]See Appendix 1 for locality and voucher information.
*Indicates a significant deviation from Hardy–Weinberg equilibrium after Holm's sequential Bonferroni correction ($P < 0.05$).

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AUTHOR CONTRIBUTIONS

R.J.W. conceived and designed the project. R.J.W., G.T.W., and D.L. carried out the field collection. G.T.W., Z.F.W., and G.B.J. carried out the laboratory procedures. G.T.W. and Z.F.W. analyzed the data. All authors read and approved the final version of the manuscript.

DATA ACCESSIBILITY

The microsatellites and raw sequences developed in this article have been deposited in the National Center for Biotechnology Information (NCBI). The GenBank accession numbers for the microsatellites are provided in Table 1, and the accession numbers for the raw sequences in the NCBI Sequence Read Archive are SRR7133729 and SRR7133728.

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APPENDIX 1. Locality information for the *Glyptostrobus pensilis* and *Taxodium distichum* samples used in this study.[a]

Species	Population code	*N*	Collection locality	Voucher no.
Glyptostrobus pensilis (Staunton ex D. Don) K. Koch	JX	59	Shangrao, Jiangxi Province, China	IBSC799028
			Yingtan, Jiangxi Province, China	IBSC799072
	HN	6	Zixing, Hunan Province, China	IBSC799035, 799034, 799082
	HK	6	The Chinese University of Hong Kong, China	IBSC799085
	FJ	81	Ningde, Fujian Province, China	IBSC799064
			Sanming, Fujian Province, China	IBSC799019
			Quanzhou, Fujian Province, China	IBSC799016, 799075
			Fuzhou, Fujian Province, China	IBSC799068
	GD	74	Guangzhou, Guangdong Province, China	IBSC799061, 799020, 799014, 799078, 799079, 799041, 799042, 799054, 799083, 799084
			Zhuhai, Guangdong Province, China	IBSC799080, 799022
			Huaiji, Guangdong Province, China	IBSC799056
			Meizhou, Guangdong Province, China	IBSC799021, 799018, 799032
			Huizhou, Guangdong Province, China	IBSC799066, 799057, 799031, 799030
	GX	31	Tiandeng, Guangxi Province, China	IBSC799047
			Qinzhou, Guangxi Province, China	IBSC799048
			Guilin, Guangxi Province, China	IBSC799049
			Cangwu, Guangxi Province, China	IBSC799051
			Luchuan, Guangxi Province, China	IBSC799044
			Funing, Yunnan Province, China	IBSC799046
	ZJ	6	Hangzhou, Zhejiang Province, China	IBSC799050
			Shanghai, China	IBSC799069
	HB	11	Wuhan, Hubei Province, China	IBSC799053
			Xinyang, Henan Province, China	IBSC799055
	Đắk Lắk	59	Ea H'leo, Đắk Lắk Province, Vietnam	HN11357, 7111, 11946, 11950
Taxodium distichum (L.) Rich.	*T. distichum*	6	South China Botanical Garden, Guangzhou, Guangdong Province, China (23°10′51″N, 113°21′08″E)	IBSC799015

Note: N = number of individuals sampled.

[a]All voucher specimens were deposited in the South China Botanical Garden Herbarium (IBSC), Guangzhou, China, or the Vietnam Academy of Science and Technology Herbarium (HN), Hanoi, Vietnam.