Applications in Plant Sciences

PRIMER NOTE

Development of chloroplast microsatellite markers for *Glyptostrobus pensilis* (Cupressaceae)

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PREMISE: *Glyptostrobus pensilis* (Cupressaceae) is a critically endangered conifer native to China, Laos, and Vietnam, with only a few populations remaining in the wild.

METHODS AND RESULTS: Using a complete chloroplast genome sequence, we designed 70 cpSSR loci and tested them for amplification success and polymorphism in 16 samples. Ten loci were found to be polymorphic and their genetic diversity was characterized using a total of 83 individuals from three populations in China. A total of 43 haplotypes were present, the effective number of haplotypes varied from 4.55 to 13.36, and the haplotypic richness ranged from 8.04 to 16.00. Gene diversity ranged from 0.81 to 0.97 (average 0.89). The number of alleles per locus and population ranged from one to eight, and the effective number of alleles ranged from 1.00 to 3.90. All polymorphic loci were successfully amplified in the related species *Cryptomeria japonica* var. *sinensis*, *Taxodium distichum*, *T. ascendens*, and *Cunninghamia lanceolata*.

CONCLUSIONS: These newly developed chloroplast microsatellites will be useful for population genetic and phylogeographic analyses of *G. pensilis* and related species.

KEY WORDS chloroplast microsatellite (cpSSR); Cupressaceae; *Glyptostrobus pensilis*; haplotypes.

Glyptostrobus pensilis (Staunton ex D. Don) K. Koch, the only extant species in the genus *Glyptostrobus* Endl., is a relict conifer in the family Cupressaceae (Hao et al., 2016). In China, it is mainly distributed in the Pearl River delta region of Guangdong Province, the central region of Fujian Province, the lower reaches of the Minjiang River, and northeastern Jiangxi Province (Li and Xia, 2004). A few wild populations have recently been found in Laos and Vietnam, extending its latitudinal distribution from 28°N to 13°N (Averyanov et al., 2009; Thomas and LePage, 2011). The species preferred habitat of riverbanks and flood plains have been severely degraded by human activities (e.g., agriculture and rice cultivation) in many locations, which has led to a rapid decline of most *G. pensilis* populations (Li and Xia, 2004, 2005; Nguyen et al., 2013). Currently, the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species has evaluated *G. pensilis* as Critically Endangered (CR) (Thomas et al., 2011).

Chloroplast microsatellites have been widely used to investigate the population genetic structure and phylogeographic history of a range of tree species (Ruhsam et al., 2016; Gryta et al., 2017). Previous molecular studies of *G. pensilis* have only used nuclear markers such as inter-simple sequence repeats (Li and Xia, 2005; Wu, 2011), and recently Wang et al. (2019) developed 10 polymorphic nuclear microsatellite markers for this species. Compared with nuclear simple sequence repeats (SSRs), chloroplast SSRs (cpSSRs) are more likely to detect historical bottlenecks or genetic drift due to their uniparental inheritance, slower mutation rate, and lack of recombination (Ennos et al., 1999; Pleines et al., 2009; Li and Liu, 2012). Nguyen et al. (2013) analyzed *G. pensilis* populations from Vietnam using six cpSSRs; however, these loci were developed from *Pinus thunbergii* Parl. and designed for use in Pinaceae species (Vendramin et al., 1996). In this study, we developed new species-specific chloroplast microsatellite loci using the complete chloroplast genome of *G. pensilis* (Hao et al., 2016). Additionally, we tested the transferability of these loci in four related species: *Cryptomeria japonica* (Thunb. ex L. f.) D. Don var. *sinensis* Miq., *Taxodium distichum* (L.) Rich., *T. ascendens* Brongn., and *Cunninghamia lanceolata* (Lamb.) Hook.

METHODS AND RESULTS

We searched the complete chloroplast genome of *G. pensilis* (Hao et al., 2016; GenBank accession number KU_302768) for

TABLE 1. Characterization of 10 polymorphic chloroplast microsatellite loci developed in *Glyptostrobus pensilis*.[a]

Locus	Primer sequences (5′–3′)	Location	Repeat motif	Allele size range (bp)	GenBank accession no.
Gp_cp_1	F: (ROX) TGACACACGGGTCTGTATCA R: GCCTTTGGTGGGCTTGTTTT	ycf4 to psaI	$(AT)_{10}$	261–271	MK386658
Gp_cp_6	F: (FAM) GCTGTTCCCCTGTGCATCAT R: GATCAATTTGTGTCTGCTTCTGT	trnL to trnF	$(AT)_{11}$	195–203	MK386659
Gp_cp_7	F: (HEX) ACCTGTCTCAAATCGACTTCCC R: CTCCTCTTTCCAGACGAGACA	ycf3 to psaA	$(T)_{11}$	149–167	MK386660
Gp_cp_8	F: (HEX) TGAACCGATGACTTACGCCT R: AAATCGAATCCCCGTTGCCT	psb to trnE	$(TA)_{9}$	267–279	MK386661
Gp_cp_11	F: (TAMRA) AATCCTGAAAGTCGACTAGAATTAAGT R: GCTAAGAGCATCTTCGAATAAAAATAG	chlB to rps16	$(AT)_{23}$	363–414	MK386662
Gp_cp_12	F: (TAMRA) TTAAGTCGAGTGAGTCAGATGG R: TGCCCATAGGATGCCAAGTG	accD to clpP	$(T)_{11}$	379–437	MK386663
Gp_cp_13	F: (TAMRA) TGGGGGATCAAAATAACACAGA R: GTTTTCCAATGTGAATTTGAAAATCGA	rbcL to accD	$(AT)_{16}$	208–334	MK386664
Gp_cp_14	F: (FAM) TCCCCGCAGAACTATCGTTT R: AGGAAAGAATTTGGTAATCTTGGCT	ccsA to petA	$(T)_{12}$	208–214	MK386665
Gp_cp_17	F: (FAM) ACCTACCCAGAATTAGCAAGCC R: AGAATTGGCGGTTGCTTCCT	trnD to psbM	$(T)_{12}$	116–119	MK386666
Gp_cp_35	F: (HEX) TTTTCCTCTACCGCGAACCC R: ACTTCACCCCTCCTTGAATTCT	psaJ to rpl33	$(A)_{10}$	115–117	MK386667

[a]Optimal annealing temperature was 55°C for all loci.

microsatellite loci exhibiting a minimum of eight repeats as these loci are likely to exhibit a higher level of polymorphism (Ueno et al., 2012). For loci with a minimum of eight repeats, primers were designed using the online software Primer3Plus (Untergasser et al., 2007) using default parameters. In total, 70 cpSSR loci were selected and evaluated for their amplification efficiency and level of polymorphism using 16 *G. pensilis* DNA samples from different populations (Appendix 1). DNA was extracted from *G. pensilis* leaves using a modification of the cetyltrimethylammonium bromide (CTAB) method (Tsumura et al., 1995).

PCR amplification was carried out in volumes of 15 µL using the following protocol: 7.5 µL of 2× Taq PCR Master Mix (Tiangen, Beijing, China), 0.75 µL of forward primer (10 µM), 0.75 µL of reverse primer (10 µM), 3 µL of 20–50 ng/µL DNA template, and 3 µL of ddH_2O. The mixture was then cycled using the following profile: 94°C for 4 min; 34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; with a final extension at 72°C for 30 min. PCR products were visualized on a 1.6% agarose gel. All loci that could be amplified successfully were tested individually using 16 *G. pensilis* samples to establish their polymorphism. These amplifications were carried out using fluorescently labeled primers (FAM, HEX, TAMRA, and ROX; Applied Biosystems, Foster City, California, USA) and the same PCR protocol as detailed above. PCR products were run on an ABI 3730xL DNA Analyzer adding a GeneScan 500 LIZ internal size standard (Applied Biosystems) to size fragments. The software GeneMarker version 1.9 was used to score the electropherograms of all samples (Hulce et al., 2011). Sixty-five of the 70 cpSSR loci amplified successfully across the 16 test individuals, but 55 loci were monomorphic, and only 10 loci were polymorphic (Table 1, Appendix 2). These polymorphic loci were used to investigate the genetic diversity of 83 individuals across three Chinese *G. pensilis* populations (Appendix 1).

The software Haplotype Analysis version 1.05 (Eliades and Eliades, 2009) was used to calculate the following statistics: number of haplotypes (A), number of private haplotypes (P), effective number of haplotypes (N_e), haplotypic richness (R_h), and gene diversity (H_e). The software GenAlEx6.5 (Peakall and Smouse, 2012) was used to calculate the following parameters: number of alleles (N_a), effective number of alleles (N_e), Shannon's information index (I), and diversity (H).

TABLE 2. Haplotype diversity in three Chinese *Glyptostrobus pensilis* populations based on 10 polymorphic chloroplast microsatellite markers.[a]

Population	A	P	N_e	R_h	H_e	D^2_{sh}
DM (N = 33)	18	16	7.026	11.857	0.884	76.6
GZHN (N = 21)	17	15	13.364	16.000	0.971	470.5
PNSL (N = 29)	11	9	4.546	8.035	0.808	1.4
Mean	15.333	13.333	8.312	11.964	0.888	182.8

Note: A = number of haplotypes; P = number of private haplotypes; N_e = effective number of haplotypes; R_h = haplotypic richness; H_e = genetic diversity; D^2_{sh} = mean genetic distance between individuals; N = number of individuals sampled.
[a]Locality and voucher information are provided in Appendix 1.

A total of 43 haplotypes were detected in the three assayed populations. The number of haplotypes per population ranged from 11 to 18, the number of private haplotypes ranged from nine to 16, the effective number of haplotypes ranged from 4.55 to 13.36, the haplotypic richness ranged from 8.04 to 16.00, and the gene diversity ranged from 0.81 to 0.97 (Table 2). The number of alleles per locus ranged from one to eight per population, the effective number of alleles ranged from 1.00 to 3.90, Shannon's information index ranged from 0.00 to 1.52, and the diversity ranged from 0.00 to 0.74 (Table 3). The 10 polymorphic loci could also be successfully amplified in five individuals in each of the following four related species: *Cryptomeria japonica* var. *sinensis*, *Taxodium distichum*, *T. ascendens*, and *Cunninghamia lanceolata* (Table 4, Appendix 1).

CONCLUSIONS

In this study, we developed 10 polymorphic cpSSRs (as well as 55 pairs of monomorphic primers) that can be used to assess the

TABLE 3. Characteristics of 10 polymorphic chloroplast microsatellite markers in 83 individuals of three Chinese *Glyptostrobus pensilis* populations.[a]

	DM (*N* = 33)				GZHN (*N* = 21)				PNSL (*N* = 29)			
Locus	N_a	N_e	*I*	*H*	N_a	N_e	*I*	*H*	N_a	N_e	*I*	*H*
Gp_cp_1	3	1.824	0.765	0.452	2	1.960	0.683	0.490	2	1.890	0.664	0.471
Gp_cp_6	2	1.198	0.305	0.165	3	2.194	0.852	0.544	3	1.324	0.479	0.245
Gp_cp_7	3	2.139	0.883	0.533	3	2.845	1.071	0.649	3	1.979	0.779	0.495
Gp_cp_8	3	1.280	0.437	0.219	3	2.110	0.832	0.526	1	1.000	0.000	0.000
Gp_cp_11	5	1.806	0.917	0.446	5	3.084	1.301	0.676	1	1.000	0.000	0.000
Gp_cp_12	3	1.280	0.437	0.219	2	1.893	0.665	0.472	1	1.000	0.000	0.000
Gp_cp_13	8	2.515	1.371	0.602	6	3.903	1.524	0.744	3	1.235	0.398	0.190
Gp_cp_14	3	1.203	0.363	0.169	3	2.110	0.832	0.526	1	1.000	0.000	0.000
Gp_cp_17	2	1.424	0.474	0.298	3	2.384	0.940	0.580	2	1.071	0.150	0.067
Gp_cp_35	2	1.271	0.369	0.213	2	1.995	0.692	0.499	1	1.000	0.000	0.000
Mean	3.400	1.594	0.632	0.331	3.200	2.448	0.939	0.571	1.800	1.250	0.247	0.147

Note: *N* = number of individuals sampled; N_a = number of alleles; N_e = effective number of alleles; *I* = Shannon's information index; *H* = diversity.
[a]Locality and voucher information are provided in Appendix 1.

TABLE 4. Results of cross-amplification of 10 polymorphic chloroplast microsatellite markers developed for *Glyptostrobus pensilis* in four closely related species.[a,b]

Locus	*Taxodium distichum* (*N* = 5)	*Taxodium ascendens* (*N* = 5)	*Cryptomeria japonica* var. *sinensis* (*N* = 5)	*Cunninghamia lanceolata* (*N* = 5)
Gp_cp_1	254–256	250–254	258–262	254–256
Gp_cp_6	179	179	197	171
Gp_cp_7	149	149	133	177
Gp_cp_8	276	276	284	286
Gp_cp_11	379–383	381–383	375	371
Gp_cp_12	378	378	384	432
Gp_cp_13	303–305	303	299	299
Gp_cp_14	205	205	201–213	213
Gp_cp_17	117	115–117	117	117
Gp_cp_35	116–117	114–117	115–116	116–117

Note: *N* = number of individuals sampled.
[a]Locality and voucher information are provided in Appendix 1.
[b]Numbers shown represent the size in base pairs of the amplified fragments.

population genetic and phylogeographic structure of *G. pensilis* populations. The high number of private haplotypes in the three assayed populations suggests geographically isolated populations. Additionally, the 10 loci can be successfully amplified in four related species of *G. pensilis*.

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DATA ACCESSIBILITY

All polymorphic primer sequences were uploaded to the National Center for Biotechology Information (accession number: MK386658–MK386667; Table 1).

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APPENDIX 1. Sampling information for species in this study. All voucher specimens are deposited at the herbarium of Central South University of Forestry and Technology, Changsha, Hunan, China.

Species	Population code	Voucher no.	Collection locality	Geographic coordinates	Elevation (m)	*N*
Glyptostrobus pensilis (Staunton ex D. Don) K. Koch	PNSL	Lin170804	Pingnan, Fujian, China	27°0′27.87″N, 118°51′59.75″E	1260	29
	GZHN	Lin170411	Guangzhou, Guangdong, China	23°11′24.6″N, 113°21′38.13″E	40	21
	DM	Lin170729	Doumen, Guangdong, China	22°23′42.5″N, 113°15′14.65″E	20	33
Taxodium distichum (L.) Rich.		Li180522	Changsha, Hunan, China	28°8′16.48″N, 112°59′28.36″E	90	5
Taxodium ascendens Brongn.		Li180522	Changsha, Hunan, China	28°8′16.48″N, 112°59′28.36″E	90	5
Cryptomeria japonica (Thunb. ex L. f.) D. Don var. *sinensis* Miq.		Wang180720	Jiujiang, Jiangxi, China	29°32′59.77″N, 115°58′03.32″E	911	5
Cunninghamia lanceolata (Lamb.) Hook.		Li180522	Changsha, Hunan, China	28°8′16.48″N, 112°59′28.36″E	90	5

Note: N = number of individuals sampled.

APPENDIX 2. Characteristics of 55 monomorphic chloroplast microsatellite primers developed in *Glyptostrobus pensilis*.

Locus	Primer sequences (5′–3′)	Repeat motif	Product size (bp)
Gp_cp_2	F: ACATTGATTTCTAAAAGAGAGGAGTCA R: TCAGTGTCAGAAATTTGGCTGA	$(A)_{11}$	211
Gp_cp_3	F: TGATGAGCTACTCTACGTGCT R: ATCTGCCATTGTACCCGCAA	$(T)_{13}$	369
Gp_cp_4	F: ATAGATTCCGAGCGGCTGTG R: ACCGCTGAGTTATATCCCTTTCC	$(T)_{18}$	292
Gp_cp_5	F: GCGATCGTACCTTCATCGGA R: TCCTTTTTCAATATCGTTCCCTGG	$(T)_{20}$	230
Gp_cp_9	F: ATTTCTCGCCAAGCTGTCCA R: CGAGCAATGCCATCTCCTACT	$(AT)_{10}$	332
Gp_cp_10	F: CGAACCCGCATCGTTAGCTT R: GGTTGTTCACCTGAAATTAAGAGGA	$(A)_{15}$	280
Gp_cp_15	F: TCAAGCAAAGGTAGATGGTGAG R: TCTCAACCTTCATGTGGGAG	$(A)_{12}$	257
Gp_cp_16	F: ATGCTCTTTCGCAACGTTCG R: TGAACACAAAGAAAGGTAAGGTCT	$(A)_{12}$	201
Gp_cp_18	F: TCCGCTCAATTCCGTTACTC R: TCCATGATTGATTTTCCCTTCGT	$(T)_{12}$	145
Gp_cp_19	F: TCTTGCAAAATCCGGACCG R: TGAACCAAGTCAGTTCGCTTG	$(A)_{11}$	201
Gp_cp_20	F: CGAAAACCGTCGGGAAACAT R: GCTTCTTCCTTCCCGCCAT	$(A)_{11}$	260
Gp_cp_21	F: GGCTCGCGGGTATGTTAACT R: TCGGGCAATTTTGTCATGTACC	$(A)_{11}$	190

(Continues)

APPENDIX 2. (Continued)

Locus	Primer sequences (5′–3′)	Repeat motif	Product size (bp)
Gp_cp_22	F: AGGGGCAGAATCTAGGGTT R: CCGCTATTTTCCACGTTGAGC	$(A)_{11}$	194
Gp_cp_23	F: ATCCGCCTTGATTCCCGTTT R: ACAGGCGCTGTGGAAAGAT	$(A)_{11}$	263
Gp_cp_24	F: TCTCTTTTGCGTCCTTCCCC R: AAGAATTAGTTCGCCATGGGT	$(T)_{11}$	231
Gp_cp_25	F: TCCTTCGGGATTAATTCTTCATTCT R: AATCCTGAGCAGCCAAACC	$(T)_{11}$	264
Gp_cp_26	F: TTGTAGCTCTACGTGGCAC R: AGGCATAAACAAAAACAGGGCT	$(AT)_{11}$	263
Gp_cp_27	F: CGGGGGAATGATACCTGTCG R: ACGGAGACTTGATATTGATGCTC	$(T)_{10}$	138
Gp_cp_28	F: TCGTGAATTCGTTGGACAG R: TCCATCTGACTCACTCGACT	$(TA)_{10}$	213
Gp_cp_29	F: GAGCTTACTTGGGTACTGAGC R: CATCCGGCTCGAGCAATAGT	$(A)_{10}$	126
Gp_cp_30	F: TGAGTATCCGTTTCCTTTCTTTTGC R: TAAGTTTTCCCTTACTATAGTGTGTGT	$(A)_{10}$	201
Gp_cp_31	F: CGGGAAGAGTAGTATGAAGCTC R: GCATATGTGCGATGAATAGACTCC	$(A)_{10}$	233
Gp_cp_32	F: CCGAGAACGAACCGAATGGA R: GGGATTGACTGTTGGATTGGC	$(A)_{10}$	157
Gp_cp_33	F: ATTAGCGGGGAGTTCCATCC R: CGGACTTGTGATTCGTTTGATCT	$(A)_{10}$	226
Gp_cp_34	F: ACGCGGCGATCAATTGGATA R: CCTACAGAGCGTGATCCTGC	$(A)_{10}$	143
Gp_cp_36	F: TCATTTTTACCCAGGAATAGAAACAT R: GATGGCTTCATTTTATTCATAGTTTGT	$(A)_{10}$	156
Gp_cp_37	F: ACCCAAAAAGAGGAGACAAGC R: GAATGACTTCGGGGTGGGAG	$(A)_{10}$	165
Gp_cp_38	F: ACTTGGACGAACTCCCTATTGA R: CAGCCGGGATAGCTCAGTTG	$(AT)_{9}$	221
Gp_cp_39	F: TCTTATGTTCTTAGTAACACGCCT R: TGGAGTAGGAGGAAAATCCGT	$(A)_{9}$	125
Gp_cp_40	F: ATGTCTCGTTATCGCGGACC R: TGACCTGTTGATCCCTTGGC	$(A)_{9}$	248
Gp_cp_41	F: CTGCACATCTGTCCCTCTGT R: TGCTTTCATCCTCCCGCAAT	$(T)_{9}$	162
Gp_cp_42	F: TGCGATCGTAAGGAAATCCA R: TTCTCCCCTGAAGCCATTGG	$(A)_{9}$	211
Gp_cp_43	F: GGTTGATGGCTCTGGTCTTGA R: TGAATCCTTGTTGCTCGGCT	$(A)_{9}$	186
Gp_cp_44	F: CCATTCGATCCCTATCCGGTC R: CATCAACCACTCGGCCATCT	$(T)_{9}$	224
Gp_cp_45	F: AGTGAGGTAGATTACGCCTAATCT R: AGCCCAGTGTTCATTTTGAATATT	$(A)_{9}$	222
Gp_cp_46	F: GCGAGTCAAGCCGAAGTACA R: AATTTTTCGTTTCCTTCGTACTACT	$(A)_{9}$	132
Gp_cp_47	F: GAAGCAACCGCCAATTCTTCA R: TGTTCGGGTGAGAAAGGTGT	$(T)_{9}$	190
Gp_cp_48	F: TCTCTTACATATCTCTGGAAAAAGGA R: TGCTGCTCTGTCCCAACTAT	$(T)_{9}$	228
Gp_cp_49	F: AGCGAAGAATCCCTTGTCCTG R: ATCTGGGCCCTCCGTCTAAT	$(A)_{9}$	171
Gp_cp_50	F: CAGATACTGGCCGGGCTAGA R: CGCTCAGCCATCTCTCCTAG	$(A)_{9}$	140
Gp_cp_51	F: CGCCATCTTGGATGGAATGG R: TGTGGCGGGTATAGTTTAGTGG	$(T)_{9}$	233
Gp_cp_52	F: CGGCTTTTAAGTGCGACTATGG R: TGACTTAATCACCCGCACTC	$(A)_{9}$	298
Gp_cp_53	F: GGCACGAGAACTTGAAGATCG R: ATTGATTCATCGACCCGCGG	$(A)_{9}$	164

(Continues)

APPENDIX 2. (Continued)

Locus	Primer sequences (5′–3′)	Repeat motif	Product size (bp)
Gp_cp_54	F: TGCATAAGAATGAGCCAACTTGA R: TCATACGGCTTAAACAAGAACAC	$(T)_9$	187
Gp_cp_55	F: CAGGCATTTACTTTTTGTTTTGGAGT R: TTTGGGTGGAATGGGGATTG	$(T)_9$	134
Gp_cp_56	F: ATATTCCGCAAGAATTTTGGGTT R: TGCATTTGTCAACTTGTTTATCGAGA	$(T)_9$	202
Gp_cp_57	F: CGCACGGCTCCTAAGTGAT R: ACCCTAAGATGAGCATCGC	$(T)_9$	257
Gp_cp_58	F: TGTGTATTTGGCTTTGAAACGA R: TGTCTTTGTTTGCTCAATTTTGC	$(T)_9$	176
Gp_cp_59	F: TATTGGACCAGCGGTAGTGG R: ATAAGCAGTCCAAGGGGAGC	$(T)_9$	144
Gp_cp_60	F: ACGATTATTCAGATTGAGCTCCGA R: CCCCATTTACCTGTATGCTATACT	$(T)_9$	201
Gp_cp_61	F: GTTCAGCCAATAGGGGAGGG R: TAAGTCCCAGGTCCCGCAT	$(T)_9$	159
Gp_cp_62	F: TGTCTACGTGCATAAACTCTTTTC R: ACCACGCTCATCTCATGTAC	$(T)_9$	209
Gp_cp_63	F: CCACCTATGCCCATACGGTC R: TCGATTGACCTGAGGACCTT	$(T)_9$	127
Gp_cp_64	F: GGGTACCGGGTTCTATTGAAT R: TCGATCTATGCCGCCTTACT	$(A)_8$	149
Gp_cp_70	F: TCGAGCCGTATGAAGATAAACCT R: GCTCTTCCTTCGCTTCGAG	$(G)_{11}$	137

[a]Optimal annealing temperature was 55°C for all loci.