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ORIGINAL RESEARCH

Isolation and Characterization of 13 Microsatellite Loci from Korean *Quercus acuta* (Fagaceae)

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Abstract *Quercus acuta* is an evergreen broadleaf tree that grows in the warm-temperate regions of Korea and Japan. Its habitats and populations are being destroyed, and a new northernmost limit of distribution has now been reported. To further our scientific understanding of its conservation and phylogeography, we isolated and characterized 13 microsatellite loci. An analysis of diversity was conducted among 35 individuals on Hong-do Island of Jeollanam-do, South Korea. Variability of the markers was also tested for 11 individuals from Jeju-do. At the population level, alleles numbered 2 to 12 and the observed and expected heterozygosities ranged from 0.0909 to 0.9143 and from 0.0909 to 0.9364, respectively. Those 13 loci were also tested for cross-species amplification in three other evergreen *Quercus* species within the same subgenus *Cyclobalanopsis*. In all, 6 of 13 loci could be amplified for all three species. The microsatellite markers described here provide a powerful genetics tool for population, conservation, systematics, and phylogeographic studies, not only for *Q. acuta* but also for other evergreen *Quercus* species.

Keywords Evergreen broadleaf tree · Marker · Microsatellite · Phylogeography · Subgenus *Cyclobalanopsis* · *Quercus acuta*

Microsatellites are regions of DNA that contain a tandem repeat of two to six nucleotides. Because microsatellite loci are hypervariable and reproducible markers, they have been widely used for population, systematics, and conservation studies (Pardo et al. 2008; Hiraoka and Tomaru 2009; Honjo et al. 2009). Such loci are actively being developed for plant species (King and Roalson 2009; Nakagawa and Ito 2009; Tsuneki et al. 2009).

Quercus acuta is distributed only in Korea and Japan (Ohashi et al. 2006a) and grows in warm-temperate evergreen broadleaf forests. However, due to deforestation of those sites through artificial disturbances (Yeo and Lee 2004), habitats and populations of this species have been destroyed and trees are now restricted to conservation areas in Korea (Choi 2001). A shift in the northernmost limit of distribution for evergreen broadleaf species is thought to reflect recent climate change in temperate regions. A population located at Hampyeong-gun (N 35°03') in southwestern Korea was designated in 1962 as a natural monument that marked its northern boundary of occurrence. Since then, however, a new northernmost limit has been reported for this species at Nab-do Island (N 37°04') of Incheon, in the middle of the Korean Peninsula (Lee and Choi 2008). The limit in Japan was the Miyagi Prefecture (N 38°52') (Ohashi et al. 2006b). Based on these traits, this species is a suitable model that can provide information for conservation efforts while enhancing our scientific understanding of historical climate change. In this study, we isolated and characterized 13 microsatellite loci for *Q. acuta*.

Materials and Methods

Genomic DNA was extracted from fresh leaves of *Q. acuta* that were collected from Hong-do Island of Jeollanam-do, South Korea, using a G-spinTM 2p Kit for plant (Intron). Microsatellites were then developed according to the enrichment protocol described by Hammond et al. (1998) with minor modifications. Briefly, genomic DNA was digested with *Mbo*I (Promega). Subsequently, the resulting

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fragments were ligated to SAUL linkers (Hammond et al. 1998) by using the T4 DNA ligase (Promega). These ligated fragments were enriched for microsatellites with a cocktail comprising six biotinylated probes [$(AG)_{15}$, $(AT)_{15}$, $(AC)_{15}$, $(ACG)_{10}$, $(CAA)_{10}$, and $(ACAT)_7$] that were bound to streptavidin magnetic beads (Streptavidin MagneSphere® Paramagnetic Particles, Promega). This enrichment process was performed twice. The enriched fragments were then amplified and cloned using the pGEM®-T Easy Vector System (Promega). The size of the insert in 288 clones was evaluated by PCR using the linker primer, and clones showing insert sizes ranging from 400 to 800 bp were selected for sequencing. In all, 265 clones were subjected to double-stranded DNA sequencing that utilized BigDye Terminator version 3.1 and an ABI 3730xl sequencer (Applied Biosystems). Fourty-three PCR primer pairs were designed using the FastPCR version 5.4.51 software (Kalendar 2009). The M13 (-21) (5'-TGTAAAAC GACGGCCAGT-3') sequence tag method was used to label the 43 primers (Schuelke 2000). Loci characterizations and GenBank accession numbers for our sequences are listed in Table 1.

Of the developed primer pairs, 13 microsatellites were characterized with samples of 46 individuals collected from Hong-do Island of Jeollanam-do (n=35) and from Jeju-do (n=11), South Korea. Cross-species amplification was tested in other evergreen *Quercus* species from Jeju-do, *Quercus glauca*, *Quercus myrsinifolia*, and *Quercus salicina*, which belong to the same subgenus *Cyclobalanopsis*. DNA was extracted according to the method described above, and PCR was conducted with a GeneAmp® PCR System 2700 Thermal Cycler (Applied Biosystems). Each reaction mixture contained 200 μM dNTPs (GeneCraft), 1× PCR buffer with 1.5 mM MgCl2, 1 U of *Taq* DNA polymerase (TaKaRa), 10 ng of DNA, and an appropriate concentration of primers in a total volume of 30 μL. The mixture contained 0.08 μM forward M13 (-21) tagged primer, 0.3 μM reverse primer, and 0.3 μM M13 (-21)

Table 1 Characteristics of 13 polymorphic microsatellite loci developed from *Q. acuta*

Locus	GenBank accession no.	Repeat motif	Primer sequences (5'–3')	T_a (°C)	Allele size range	*A*
MSQa1	GU576568	$(TTG)_9$	F:TACCTGAAGTTTGCTACAG R:TCACGGATATTGTACATGGGTC	56	273–288	4
MSQa2	GU576570	$(TC)_9$ $(AC)_{11}$	F:AACTAAGGAGACTATCTTTCC R:TTGAGTCCAATGCAATACC	60	329–339	4
MSQa3	GU576571	$(AG)_{27}$	F:TGACCTATAAGCTTCATGTACG R:TCAACCTAGCAAGATTCCTC	60	327–359	14
MSQa4	GU576569	$(TC)_{13}$	F:ATGTTAAATGTTCATGCAC R:AATGCTACGGGCCTAGAAG	56	295–315	7
MSQa6	GU576572	$(GT)_{31}$	F:TAGGTTTCTCCATTGGCTAC R:ATACTTGACAACTACTCAG	56	312–362	14
MSQa8	GU576566	$(AG)_{25}$	F:ATACGTCCTGTTGAGATGA R:TGCCACTCCCTACCGTCAG	56	191–221	10
MSQa9	GU576563	$(TTG)_7$	F:AAAGAAGTTTCTGGCATTGG R:TGAATTCTGGTACCCAAGTC	52	118–130	4
MSQa10	GU576560	$(GT)_{11}$	F:ATGGCTGTATGAATAGTACTC R:TGACTAGATGACTGCAATGAG	52	331–343	4
MSQa11	GU576561	$(GT)_{11}$	F:ATGTGACCTTGAACACGGG R:TTAAAGGCCTAGAGGCTAG	56	253–271	7
MSQa12	GU576562	$(GA)_{14}$	F:AATTGTGCTTGTGAAATTG R:TTTGGTTCAAGCTCAACCC	52	204–312	9
MSQa13	GU576564	$(AG)_{14}$	F:AACCGAGCCCATGTTCATG R:ATTCATTCATACTTTTATGG	52	294–328	14
MSQa14	GU576567	$(AG)_{17}$	F:TTTAGTAATTGGCGTTAAAG R:ACTGGCAATTATCTAAAATC	52	260–278	8
MSQa15	GU576565	$(CAA)_{10}A_{17}$	F:TTTAGTTACAGTACATAGC R:AAAGACGCTGACCTTTTTAGG	56	288–323	12

All forward primers were M13 (5'-TGTAAAACGACGGCCAGT-3')–tailed at the 5' end

T_a PCR annealing temperature; *A* number of alleles

Table 2 Parameters of genetic diversity for two populations of *Q. acuta*

	Hong-do Island (n=35)				Jeju-do (n = 11)			
	A	*Ho*	*He*	*P*	*A*	*Ho*	*He*	*P*
MSQa1	4	0.1142	0.1113	1.0000	3	0.2727	0.4455	0.1119
MSQa2	4	0.5143	0.4475	1.0000	3	0.3637	0.3273	1.0000
MSQa3	11	0.9143	0.8824	0.1997	12	0.9092	0.9364	0.7513
MSQa4	7	0.7428	0.6899	0.8793	5	0.4545	0.8409	0.0071
MSQa6	11	0.8286	0.8298	0.5096	10	0.8182	0.8864	0.8276
MSQa8	10	0.8000	0.7441	0.3119	6	0.8183	0.6864	0.2690
MSQa9	4	0.4000	0.3416	0.7943	2	0.0909	0.0909	–
MSQa10	4	0.4000	0.3416	0.7932	3	0.3637	0.3864	0.2034
MSQa11	5	0.6286	0.6748	0.5783	6	0.8182	0.7591	0.9713
MSQa12	8	0.5429	0.6538	0.2061	5	0.7273	0.6182	1.0000
MSQa13	12	0.8857	0.8504	0.8652	11	0.9091	0.9000	0.2403
MSQa14	4	0.5143	0.6046	0.4174	7	0.6364	0.6500	0.7077
MSQa15	11	0.4572	0.8824	0.0000[a]	8	0.5454	0.8727	0.0145
Average	7.3	0.5956	0.6196		6.23	0.5874	0.6458	

n number of individual genotype, *A* number of alleles, *Ho* observed heterozygosity, *He* expected heterozygosity

[a] Significant deviation from the Hardy–Weinberg equilibrium after Bonferroni correction (P<0.0038)

labeled 6-FAM fluorescent dyes. The PCR conditions included an initial denaturation at 94°C for 2 minutes, followed by 38 cycles at 95°C for 30 seconds, 52 to 60°C for 45 seconds (annealing temperature depending upon locus, see Table 1), and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Fluorescently labeled PCR products were electrophoresed concurrently with the GeneScan™- 500LIZ™ Size Standard on an ABI 3730xl sequencer, and sizes were determined with GENEMAPPER version 3.7 (Applied Biosystems). Diversity statistics, deviations of Hardy–Weinberg equilibrium, and linkage disequilibrium were estimated by GENEPOP version 4.0 software (Rousset, 2008).

Results and Discussion

With our 43 primer pairs, we produced 13 polymorphic microsatellite loci that revealed clear and strong bands for each allele across two populations. Genotypic data were obtained for 46 individuals sampled from Hong-do Island and Jeju-do. Overall, the alleles numbered 2 to 12 (average of 6.77); their observed and expected heterozygosities (*Ho* and *He*) ranged from 0.0909 to 0.9143 and from 0.0909 to 0.9364, respectively (Table 2). After a Bonferroni correction, only 1 of those 13 loci (MSQa15) showed any significant deviation from the correction Hardy–Weinberg equilibrium in the Hong-do Island population (P<0.0038; Table 2). In contrast, a pair of loci (MSQa4 and MSQa9) had a significant linkage disequilibrium (P<0.0015; data not shown). Parameters for genetic diversity in each population are presented in Table 2.

We also tested these 13 loci for cross-species amplification with 3 other evergreen *Quercus* species and were able to amplify 6 loci for all 3 (Table 3). Among the 13, 8 were successfully amplified for *Q. glauca,* 7 for *Q. myrsinifolia*, and 11 for *Q. salicina* (Table 3). Therefore, the microsatellite markers described here are powerful genetics tool for population, conservation, systematics, and phylogeographic studies, not only for *Q. acuta* but also for other evergreen *Quercus* species.

Table 3 Cross-species amplification of 13 microsatellite loci developed for *Q. acuta*

Locus	Size of microsatellite alleles (bp)		
	Q. glauca	*Q. myrsinifolia*	*Q. salicina*
MSQa1	279/285	274	273/285
MSQa2	337	329/333	297/339
MSQa3	[a]	[a]	[a]
MSQa4	295/307	331/347	307/311
MSQa6	297/347	304	310
MSQa8	[a]	[a]	[a]
MSQa9	[a]	126/133	130/133
MSQa10	335/341	–	335
MSQa11	269/271	261	263/271
MSQa12	[a]	[a]	300/302
MSQa13	290	300/308	288/290
MSQa14	272/282	[a]	262/270
MSQa15	[a]	[a]	285

One individual from each taxon was tested

– PCR products were not available

[a] More than one locus was amplified

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