Development of Nuclear Microsatellites for the Arcto-Tertiary Tree Zelkova carpinifolia (Ulmaceae) Using 454 Pyrosequencing

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PRIMER NOTE

DEVELOPMENT OF NUCLEAR MICROSATELLITES FOR THE ARCTO-TERTIARY TREE *ZELKOVA CARPINIFOLIA* (ULMACEAE) USING 454 PYROSEQUENCING[1]

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- *Premise of the study:* The current study aimed at developing nuclear microsatellite markers for the relict tree species *Zelkova carpinifolia*, which is threatened in its natural range in the South Caucasus.
- *Methods and Results:* Pyrosequencing of an enriched microsatellite library on the Roche FLX platform using the 454 Titanium kit produced 86,058 sequence reads, most of which contained short tandem repeats. Eighty microsatellite loci identified using the software package QDD version 1 were selected and tested for proper PCR amplification. Of these, 13 allowed proper amplification and were shown to be polymorphic among a sample of 25 *Z. carpinifolia* specimens from various geographic origins.
- *Conclusions:* The set of microsatellite markers will be suitable for the assessment of genetic diversity in *Z. carpinifolia*. They will allow for an examination of phylogeographic patterns as well as of population structure and gene flow within this species.

Key words: conservation genetics; microsatellites; phylogeography; pyrosequencing; Ulmaceae; *Zelkova carpinifolia*.

Zelkova carpinifolia (Pall.) K. Koch. (Ulmaceae) is a representative species of the Arcto-Tertiary relict flora (Wang et al., 2001; Milne and Abbot, 2002; Kozlowski et al., 2012). It occurs naturally in Hyrcan and Colchic forests in Iran, Azerbaijan, Georgia, and Turkey (Browicz and Zielinski, 1982; Denk and Grimm, 2005; Kvavadze and Connor, 2005; Akhani et al., 2010). Its valuable timber has led to uncontrolled logging, which has resulted in a general population decline. Therefore, *Z. carpinifolia* was listed under the International Union for Conservation of Nature (IUCN) category "Near Threatened" (Güner and Zielinski, 1998). To plan conservation strategies for *Z. carpinifolia*, investigation of its genetic diversity is of great importance (Kozlowski et al., 2012). The phylogeography of the species needs to be understood as there may be specific refugia within the range of the species that harbor relatively high genetic diversities and regionally adapted genotypes may occur.

The first studies of genetic diversity in *Zelkova* Spach employed inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers (Fineschi et al., 2004). Primers targeting microsatellite loci in *Z. serrata* (Thunb.) Makino (Fukatsu et al., 2005) did not amplify well in *Z. carpinifolia* and revealed only limited polymorphism (unpublished data). The current study therefore aimed to develop polymorphic microsatellite markers for *Z. carpinifolia* using 454 pyrosequencing.

METHODS AND RESULTS

Plant material and DNA extraction*—Zelkova carpinifolia* samples were collected from six populations (10–20 individuals per population) in Azerbaijan and Iran (Appendix 1). Genomic DNA was extracted from silica gel–dried leaves using either the NucleoSpin Plant II kit (Macherey Nagel, Düren, Germany) following the manufacturer's protocol or a three-fraction cetyltrimethylammonium bromide (CTAB) method (Borsch et al., 2003).

***Microsatellite marker development*—**Construction of the enriched microsatellite libraries was carried out according to the protocol of Glenn and Schable (2005) with modifications. Genomic DNA was digested in 25-µL reactions containing 1× NEBuffer 3.1, 0.1 mg/mL bovine serum albumin (BSA), 0.05 M NaCl, 20 U *Eco*RI, 10 U *Mse*I restriction enzymes (New England Biolabs, Frankfurt, Germany), and 2.4 µg genomic DNA during a 2-h incubation at 37°C. Digested DNA was run on a 2% agarose gel, and 200–400-bp fragments were extracted using the Gel/PCR DNA Fragment Extraction Kit (Avegene, Zollikofen, Switzerland) following the manufacturer's protocol. To prepare double-stranded adapters, forward (*Eco*RI: 5′-CTCGTAGACTGCGTACC, *Mse*I: 5′-GACGATGAGTCCTGAG) and reverse (*Eco*RI: 5′-AATTGGTACGC-AGTCTACGAG, *Mse*I: 5′-TACTCAGGACTCATCGTC) adapters (Metabion, Martinsried, Germany) were mixed in equimolar amounts (10 µM), incubated at 95°C for 5 min, and allowed to slowly cool down to room temperature. Adapters were ligated to the restriction fragments in 40-µL reactions containing 0.8 µM of each of the double-stranded *Eco*RI and *Mse*I adapters, 1× T4 DNA

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TABLE 1. Characteristics of 13 nuclear microsatellite loci for *Zelkova carpinifolia.*

Locus	Primer sequences (5′–3′)	Fluorescent label	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank/ENA accession no.
ZMS_1	F: AAGGTTCATCCCATGAGCAC*	6-FAM	$(CTT)_7$	60	238–259	KF408341
	R: GTCCAGTCCGGCATCTTTT					
ZMS_2	F: CAAGCTGCCAATACTCCACA*	VIC	$(AGA)_7$	60	181–213	KF408342
	R: GGTTTGGTTTCTGCTTGGAA					
ZMS_3	F: CCCAACAGCACTTGAAGCTA*	6-FAM	$(CA)_8$	60	136–138	KF408343
	R: TTTGTGTAGTCTTTGGCCCC					
ZMS_4	F: ACCGTTGGAAACAAAACTCG*	NED	$(AG)_9$	52	105–131	KF408344
	R: AGGGCAGTAGAGCTTGATCG					
ZMS_5	F: TTTTGTTCGTTGTTGCATGTC*	PET	$(GAA)_6$	60	99–114	KF408345
	R: AGGCTTAGGTTGGGTGGTTT					
ZMS_6	F: TTTGGTGCTTAGCATGTGGA*	6-FAM	$(CT)_8$	58	101–120	KF408346
	R: TCTTTTGTTTGCGATAAGTCG					
ZMS_7	F: TCTCTGTTATGATCACGTCTCCA	NED	$(AC)_9$	60	120–128	KF408347
	R: GAGCTAAACCCCATTTCAAATATAA*					
ZMS_8	F: ACGAACAAGATCGACAGAGG*	PET	$(TC)_{12}$	60	188–204	KF408348
	R: ATTGGATGGCACACGTAATTC					
ZMS_9	F: CAGGTGATTGGAATTGGAAAA*	6-FAM	$(AC)_7$	62	171–196	KF408350
	R: CCTCACAGAGTACGAGGGATG					
ZMS_10	F: CTGCGAGGGCAGTAGGTAAG*	VIC	$(GA)_8$	62	175–189	KF408351
	R: TCCTAGATCCTTTGATATCTTTCTCA					
ZMS_11	F: AGTAGGCTTGGAAGCAAATGA	VIC	$(GT)_9$	58	96–115	KF408352
	R: TTGGAACTGCAATCGCTTT*					
ZMS_12	F: CTTCCAATCATTCCGAGAGC*	PET	$(CA)_7$	58	100–104	KF408353
	R: TGACATCTCAAAGGTTGCCA					
ZMS_13	F: AACAAAGAATCTTCATCCCC*	NED	$(TG)_9$	58	110–116	KF408354
	R: GCATTTGGTTTTACCTAAAGTTACAG					
ZMS_14	F: GCGGTTGAGAGGAGAAAGAA	6-FAM	$(AGG)_{10}$	57	105†	HG737351
	R: ACCAAACCCATCAACCATTG*					
ZMS_15	F: GCTCCTCTTCAGCCAAACAC*	6-FAM	$(AGG)_{12}$	60	128†	HG737352
	R: CTTCATCCTCGTTTGCATCA					
ZMS_16	F: GCCGGTACTGATTCCTTCAA*	6-FAM	$(AAG)_5$	60	233†	HG737353
	R: GGACCATCTCACCAACATCC					
ZMS_17	F: AGCCCGTGCTTTTGAATATG	6-FAM	$(GA)_8$	55	167†	HG737354
	R: GGATAAGGCTATTTTGGCCC*					
ZMS_18	F: GCCACGTAAACAGAGTAAGGG*	6-FAM	$(TG)_7$	58	103†	HG737355
	R: GCATGTTGGTGCTTTGAGAA					

Note: ENA = European Nucleotide Archive; T_a = optimal annealing temperature.

*Labeled primer.

†Expected size.

ligase buffer, and 600 U T4 DNA ligase (New England Biolabs). Reactions were performed at 16°C for 12 h and successful ligation was tested by PCR amplification in 50-µL reactions containing 4 µL of the adapter-ligated DNA, 1× *Taq* DNA polymerase buffer, 1× PCR enhancer solution, 150 µM of each dNTP, 2 U *Taq* DNA polymerase (PeqLab, Erlangen, Germany), 35 ng/µL BSA, 0.5 µM *Eco*RI primer (5′-CTCGTAGACTGCGTACCAATTC), and 0.5 µM *Mse*I primer (5′-GACGATGAGTCCTGAGTAA) (Metabion) using the following temperature profile: 95°C for 2 min; 20 cycles of 95°C for 20 s, 60°C for 20 s, 72°C for 1.5 min; and 72°C for 10 min. Two mixtures of 3′-biotinylated oligonucleotides [1: $(AG)_{12}$, $(TG)_{12}$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$ and 2: $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$] were used to generate two separate libraries of adapter-ligated genomic DNA enriched for repetitive motifs. Hybridization of biotinylated oligonucleotides and adapter-ligated genomic DNA, followed by the capturing of hybridized DNA on Dynabeads (Life Technologies, Darmstadt, Germany) and subsequent washing and resuspension, followed the protocol of Glenn and Schable (2005). Recovery PCR was performed with 2 µL of enriched DNA in the same reaction mixture and the temperature profile as described above, but with 25 cycles. The enrichment procedure was repeated and the second recovery PCR was carried out with an *Eco*RI oligonucleotide primer extended with a Roche 454 pyrosequencing adapter (5′-GCCTCCCTCGCGCCATCAG-3′) and a specimen-specific barcode sequence and an *Mse*I oligonucleotide primer extended with a Roche 454 B sequencing adapter (5′-GCCTTGCCAGCCCGCTCAG-3′; Appendix 2). Two enrichment libraries were prepared for each of six *Z. carpinifolia* specimens (population ZE053_IR, Appendix 1). Enrichment libraries were purified using the Amplicon Library Preparation Protocol (Roche, Branford, Connecticut, USA) according to the manufacturer's instructions. Lib-L Kits and the GS FLX Titanium Sequencing Kit XLR70 (Roche) were used for the library sequencing on the Roche GS FLX System. The resulting DNA sequences were analyzed using the R package SeqinR (Charif and Lobry, 2007). The 454 pyrosequencing yielded 86,058 total reads ranging from 40 to 577 bp with an average length of 138 bp.

***Microsatellite screening*—**Screening for microsatellite loci and primer design was performed using QDD software version 1 (Meglécz et al., 2010), which identified 136 microsatellite loci containing di-, tri-, and tetranucleotide repeat motifs. A total of 80 microsatellite loci with 5–45 repeats, including three loci with tetranucleotide repeats, 17 loci with trinucleotide repeats, and 60 loci with dinucleotide repeats, were selected randomly, and oligonucleotide primer combinations with a GC content of 35–60% and melting temperature (T_m) ranging between 57°C and 60°C were tested for proper PCR amplification on genomic DNA extracted from three specimens (Table 1). A gradient PCR was carried out for each primer pair in a 25-µL reaction volume containing 20–40 ng DNA, 0.4 µM each of forward and reverse primer (Eurofins MWG Operon, Ebersberg, Germany), 1× TaqBuffer S, 1.5 mM $MgCl_2$, 250 µM of each dNTP, and 0.75 U Hot Taq polymerase (PeqLab). The temperature profile was as follows: 96°C for 2 min, 54 ± 8°C for 30 s, 72°C for 30 s; followed by 34 cycles of 95°C for 30 s, 54 ± 8°C for 30 s, 72° for 30 s; and 72°C for 15 min. PCR performance was assessed by gel electrophoresis. Of the initial 80 loci tested, 40 primer pairs failed to amplify a product. The reactions with the other 40 primer pairs were repeated in 50-µL reaction volumes. PCR products were run on 1.5% agarose gel and extracted using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel) following the manufacturer's protocol. PCR products were sequenced at Macrogen (Seoul, Korea) using standard Sanger sequencing to confirm locus-specificity of the oligonucleotide primers. Only the 20 primer pairs that specifically amplified the targeted microsatellite loci were retained for further analyses. Polymorphism

TABLE 2. Results of initial primer screening of specimens from five populations of *Zelkova carpinifolia*.

Locus	ZE001_AZ (N = 5)			ZE007_AZ (N = 5)			ZE013_AZ (N = 5)			ZE017_AZ (N = 5)			ZE021_AZ (N = 5)			Total (N = 25)		
	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	PIC
ZMS_1	2	0.60	0.4667	4	1.00	0.7778	2	0.80	0.5333	4	1.00	0.7111	4	0.60	0.6444	5	0.80	0.6294	0.5536
ZMS_2	3	1.00	0.7111	4	1.00	0.7333	4	0.60	0.5333	4	0.80	0.7333	3	0.80	0.6444	8	0.84	0.7053	0.6439
ZMS_3	2	1.00	0.5556	2	0.80	0.5333	2	0.80	0.5333	2	0.20	0.5556	2	0.20	0.4667	2	0.60	0.4971	0.3685
ZMS_4	3	0.80	0.6000	4	0.80	0.8000	4	0.80	0.8000	5	1.00	0.8444	5	0.40	0.8000	8	0.76	0.8188	0.7811
ZMS_5	3	0.40	0.3778	2	0.40	0.3556	1	0	0	1	0	0	3	0.60	0.5111	5	0.28	0.2596	0.2455
ZMS_6	2	0	0.3556	2	0	0.3556	3	0	0.6222	5	0.40	0.8667	5	0.20	0.8222	8	0.12	0.8049	0.7599
ZMS_7	3	1.00	0.6444	4	1.00	0.7778	4	0.60	0.6444	2	0.80	0.5333	3	0.80	0.6000	4	0.84	0.7094	0.6413
ZMS_8	5	1.00	0.8222	4	1.00	0.7333	4	1.00	0.7333	3	0.80	0.6000	4	0.20	0.6444	7	0.80	0.7861	0.7351
ZMS_9	3	0.40	0.6222	3	0	0.7111	4	0.60	0.7333	3	0.60	0.5111	3	0.20	0.5111	5	0.36	0.7127	0.6506
ZMS_10	2	0.20	0.5556	4	0.60	0.7333	3	0.40	0.6000	2	0.20	0.4667	4	0.80	0.7778	5	0.44	0.6588	0.6065
ZMS_11	1	0	0	3	0.40	0.7111	2	0	0.3556	5	0.40	0.8222	2	0	0.3556	6	0.16	0.5037	0.4526
ZMS_12	2	0.60	0.4667	2	0.40	0.3556	2	0.20	0.2000	2	0.20	0.2000	1	0	0	3	0.28	0.2539	0.2316
ZMS_13	3	0.60	0.6444	3	1.00	0.6444	2	0.80	0.5333	2	0.20	0.2000	2	0.20	0.2000	4	0.56	0.5102	0.4431

Note: A = number of alleles; H_e = expected heterozygosity (Nei's unbiased gene diversity); H_o = observed heterozygosity; N = sample size; PIC = polymorphism information content.

assessment used genomic DNA extracted from 25 *Z. carpinifolia* specimens (five specimens per population; Appendix 1) and fluorescently labeled forward or reverse primers (Applied Biosystems, Warrington, United Kingdom), and the same reaction conditions as in the previous step. Two primer pairs did not amplify a product in all individuals and were excluded from further analysis. The rest of the PCR products were sent to Macrogen for fragment analysis.

***Microsatellite marker data analysis*—**Electropherograms from the fragment analysis were analyzed using GeneMarker software version 1.95 (SoftGenetics, State College, Pennsylvania, USA). Analyzed amplification products with unscorable peaks were not considered to be useful in the studies of variability due to the possible misinterpretation of data. Thirteen primer pairs produced polymorphic and heterozygous loci (accession numbers KF408341–KF408348, KF408350–KF408354). The Excel Microsatellite Toolkit (Park, 2001) was used for the calculation of the number of alleles, observed (H_o) and expected (H_e) heterozygosities, and polymorphism information content (PIC) values. Allele sizes ranged from two to eight alleles per locus across 25 individuals from five different populations, with H_o and H_e varying between 0.12 and 0.84, and 0.25 and 0.81, respectively (Table 2).

CONCLUSIONS

In the current study, we used 454 pyrosequencing to obtain sequence reads for microsatellite primer development in *Z. carpinifolia*. After thorough evaluation, we present 13 primer pairs for amplifying polymorphic microsatellite loci in *Z. carpinifolia*. These 13 loci are proposed to be used for the microsatellite screening of naturally distributed *Z. carpinifolia* populations, but they may also cross-amplify in closely related species. Analysis of the obtained allele frequencies and knowledge of the individual genotypes will help us to understand the population structure and relationships between and within different populations of *Zelkova* species.

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Appendix 1. Locations and voucher information for populations of *Zelkova carpinifolia* used in this study. Vouchers are deposited at the herbarium of the Botanischer Garten und Botanisches Museum Berlin-Dahlem (B), Berlin, Germany.

Population	Locality	Geographic coordinates	Collection no.
ZE001_AZ	Zuvand Mtn., Lerik, Azerbaijan	38°43′33″N, 48°25′53″E	EM01
ZE007_AZ	Azfilial, Lankaran, Azerbaijan	38°40.938′N, 48°47.222′E	EM13
ZE013_AZ	Tezekend, Lankaran, Azerbaijan	38°39.091′N, 48°48.284′E	EM20
ZE017_AZ	Zungulesh, Astara, Azerbaijan	38°27.175′N, 48°47.575′E	EM28
ZE021_AZ	Khanbulan, Lankaran, Azerbaijan	38°39.809′N, 48°46.649′E	EM33
ZE053_IR*	Gorgan, Golestan, Iran	36°43′35″N, 54°34′49″E	

*Used for construction of the genomic library.

Appendix 2. *Eco*RI-A and *Mse*I-B primers used for PCR recovery of the enriched DNA library.

Primer name	Primer sequence (5′–3′)[a]
EcoRI_A001	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ACG AGT GCG T**CT CGT AGA CTG CGT ACC AAT TC
EcoRI_A002	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ACG CTC GAC A**CT CGT AGA CTG CGT ACC AAT TC
EcoRI_A003	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **AGA CGC ACT C**CT CGT AGA CTG CGT ACC AAT TC
EcoRI_A004	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **AGC ACT GTA G**CT CGT AGA CTG CGT ACC AAT TC
EcoRI_A005	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ATC AGA CAC G**CT CGT AGA CTG CGT ACC AAT TC
EcoRI_A006	CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG **ATA TCG CGA G**CT CGT AGA CTG CGT ACC AAT TC
MseI_B	CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG GAC GAT GAG TCC TGA GTA A

[a]Specimen-specific barcodes (tags) are shown in bold.