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PRIMER NOTE

MICROSATELLITE MARKERS FOR RUSSIAN OLIVE (*ELAEAGNUS ANGUSTIFOLIA*; ELAEAGNACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for the plant species *Elaeagnus angustifolia* to assist in future investigations of genetic variability in its native and invasive ranges and the precise origins of the United States/Canada invasion.
- *Methods and Results:* Eleven polymorphic microsatellite markers were developed. The number of alleles observed for each locus ranged from three to 11.
- *Conclusions:* These microsatellites have sufficient potential variability to define population structure and origins of the Russian olive invasion.

Key words: Elaeagnaceae; *Elaeagnus angustifolia*; population genetics; simple sequence repeat (SSR) markers.

Elaeagnus angustifolia L. (Elaeagnaceae; common name: Russian olive) is a diploid ($2n = 12$) tree species native to southern Europe and Asia (Little, 1961) that has escaped cultivation in western North America and is considered noxious in Colorado, New Mexico, and Wyoming. Russian olive has increased its frequency in riparian forests as well as wet pastures, ditches, and overflow channels (Lesica and Miles, 1999) and is now the fourth most dominant riparian tree in the western United States (Friedman et al., 2005). Although this invasive tree enhances habitat for some wildlife species, the resulting loss of dominant native riparian woody plants such as *Populus* spp. and *Salix* spp. reduces the habitat of cavity-nesting and insectivorous birds (Olson and Knopf, 1986). The objective of developing microsatellite loci for *E. angustifolia* is to enhance the efficacy of classical biological control that is being developed for this invasive species. Population biology tools, often molecular-based, provide insight into variation within invasive species in their introduced range that may be driven by founding events, bottlenecks, or distinct origins of genotypes and lineages. Our microsatellites will eventually be used in a larger project to determine the precise origins of invasive *E. angustifolia*, which is where potentially effective, coevolved biological control agents may be found. Microsatellites will also be used to determine the geographic distribution of genetic diversity in the invasion, which can assist in determining whether different biological control agents are required in different regions of an invasion due to variation in host plant resistance.

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METHODS AND RESULTS

Total genomic DNA was extracted from silica-dried leaf tissue using a DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). The library enrichment process was performed as in Andres and Bogdanowicz (2011). In short, genomic DNA was digested with the restriction enzyme *Hinc*II (New England Biolabs, Beverly, Massachusetts, USA) at 37°C for 1 h and ligated to a double-stranded SNX linker (Integrated DNA Technologies, Coralville, Iowa, USA). Digested, ligated DNA fragments were enriched for microsatellites by hybridization to 3′-biotinylated oligonucleotide repeat probes $(GT)_8$ (i.e., GTGTGTGTGTGTGT), $(TC)_{9.5}$, $(GTT)_{6.33}$, $(TTC)_7$, $(GTA)_{8.33}$, $(GTG)_{4.67}$, $(TCC)_5$, $(TTTG)_{2.5}$, $(TTTC)_6$, $(TTAC)_{6.75}$, and $(GATG)_{4.25}$ (Integrated DNA Technologies). Enriched fragments were captured by streptavidin-coated magnetic beads (New England Biolabs), amplified by PCR (30 cycles of 94°C for 50 s, 55°C for 45 s, 72°C for 60 s), and ligated to a Roche/454 rapid library adapter (Roche, Branford, Connecticut, USA). The sample was submitted to the Sequencing and Genotyping Facility at the Cornell Life Sciences Core Laboratory Center (CLC) for Titanium 454 (Roche) sequencing.

The .sff file (raw data) was imported to SeqMan Pro (Lasergene version 8.1.1, DNASTAR, Madison, Wisconsin, USA) and assembled (average Q score = 16 for quality trimming, mer size = 120, minimum match percentage = 94, repeat handling = on). Post assembly, sequences smaller than 150 bp were discarded; both multiread and single-read contigs above this size were kept. We used the program MSATCOMMANDER version 1.0.3 (Faircloth, 2008) to scan the data for all di-, tri-, tetra-, penta-, and hexameric microsatellites and to design primers. Minimum perfect repeat lengths were six for dimers, and five for all other repeat types selected. Minimum, optimum, and maximum primer lengths were set to 22, 23, and 24 nucleotides, respectively. All other settings in MSATCOMMANDER were kept at defaults. Of the original 996 contigs, 253 contained di-, tri-, tetra-, penta-, or hexameric microsatellites. Of these, 102 were suitable for primer design, and 83 out of the 102 produced no primer warnings in MSATCOMMANDER (e.g., high self-complementarity). Seventeen out of these 83 that had successful primer design were duplicates of other contigs in this study, leaving us 66 primer pairs for exploration. Forty-seven of these primer pairs amplified PCR product, and 11 of these provided scorable and polymorphic microsatellite products. One primer of each pair was modified by MSATCOMMANDER by adding either a "CAG" (5′-CAGTCGGGCGTCATCA-3′) or "M13R" (5′-GGAAACAGCTATGACCAT-3′) tail to the 5′ end of either the forward or reverse primer (tails are boldfaced in Table 1).

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TABLE 1. Characteristics of 11 microsatellite loci of *Elaeagnus angustifolia.*

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
Ro20	F: TCCCATACCCAGCACAAGG	$(AAG)_6$	233–272	56	KC516766
	R: **CAGTCGGGCGTCATCA**GCCATGAGGAATTGAAGGGTG				
Ro97	F: **GGAAACAGCTATGACCAT**CGGACTGCTTTGAACCCTC	$(ACC)_6$	160–199	53	KC516767
	R: AAGCCACTCAGCCTATCTAC				
Ro218	F: **CAGTCGGGCGTCATCA**GTGGAAATGCGCTGGATCG	$(AAAG)_{12}$	358–390	56	KC516768
	R: AACCCAAGTCCAAATGCCC				
Ro236	F: GTGCCTTATTCCTCGCCAC	$(AGCCTC)_4$	269–299	56	KC516769
	R: **GGAAACAGCTATGACCAT**CCAAATGCAGGCCCAAAGG				
Ro325	F: **GGAAACAGCTATGACCAT**CCAAGAGCAGCGCATTC	$(ACC)_4$	198–210	54	KC516770
	R: ACTGGCCGGTGACAAGG				
Ro367	F: **CAGTCGGGCGTCATCA**GTAGGAAGTTGGAGCTCTGC	$(AG)_{13}$	330–360	55	KC516771
	R: GTTGGAATTGCCGGTTGGG				
Ro721	F: **CAGTCGGGCGTCATCA**GGGTGGTCCTCTGACATTTG	$(ACC)_7$	271–286	55	KC516772
	R: TGCGCTCTGTATGGAATGC				
Ro749	F: **CAGTCGGGCGTCATCA**GGTACCACGGTTCAGTGTATTTC	$(AAGAAT)_5$	195–225	55	KC516773
	R: CACCAAACGGGCATTCTCC				
Ro833	F: GGGAGCTTACCAACCTTGC	$(AAG)_7$	264–300	54	KC516774
	R: **GGAAACAGCTATGACCAT**GCAATCGAAGTCCGTGC				
Ro851	F: GGTCTAAGAGCTTCAAGTGGC	$(AAAAT)_4$	244–254	52	KC516775
	R: **CAGTCGGGCGTCATCA**GGAGATTCTGACCTGTGATAAG				
Ro887	F: CAATCGCGAGTAGGGAACC	$(AAG)_5$	355–373	53	KC516776
	R: **CAGTCGGGCGTCATCA**CCCATTGATTAACTGTACACCTTG				

Note: T_a = annealing temperature.
[a]The forward and reverse primer sequences are shown with added tails set in boldface.

Singleplex PCR reactions occurred in 10-μL volumes containing 2 μL of DNA template, 1× PCR buffer (Bioline, Taunton, Massachusetts, USA), 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.1 μM CAG- or M13R-tailed primer, 0.2 μM of 6-FAM–labeled CAG or M13R universal primer, 0.2 μM of each non-tailed primer, 0.25 U *Taq* polymerase (Bioline), and H_2O. Cycling was as follows: denaturing at 94°C for 120 s; 30 cycles of 94°C for 50 s, annealing temperature (Table 1) for 60 s, 72°C for 120 s; and 72°C for 300 s. The PCR product was diluted 1 : 50 with H_2O, and 0.25 μL of this dilution was added to 9.25 μL of Hi-Di formamide (Applied Biosystems, Foster City, California, USA) and 0.5 μL of GeneScan 600 LIZ Size Standard (Applied Biosystems). This mixture was loaded into an ABI 3130 Genetic Analyzer, and electropherograms were scored with GeneMapper version 4.0 (Applied Biosystems).

Results presented here represent five populations (n = 10 plants per population) of Russian olive; two from Kazakhstan (native range; herbarium voucher no. 10117 [44.30076°N, 76.73158°E], voucher no. 10136 [43.77344°N, 78.24388°E]) and three from the United States (invasive range; Arizona: voucher no. 10860 [35.67822°N, 109.67793°W], Montana: voucher no. 10055 [45.84672°N, 109.93708°W], Utah: voucher no. 12243 [38.77396°N, 112.03393°W]). Herbarium specimens are deposited under the voucher numbers listed above at the U.S. Department of Agriculture–Agricultural Research Service (USDA-ARS; Sidney, Montana), and plants were identified to species using descriptions in Haining and Gilbert (2007). Overall, the number of alleles per locus ranged from three to 11; observed heterozygosities were between zero and 0.800, and expected heterozygosities between zero and 0.799 (CERVUS version 3.0; Kalinowski et al., 2007) (Table 2). When considering all 50 genotyped plants, two loci (Ro367, Ro833) showed evidence of null alleles (MICRO-CHECKER version 2.2.3; van Oosterhout et al., 2004). Using CERVUS, we found that two loci (Ro749, Ro833) significantly deviated from Hardy–Weinberg equilibrium, and for one locus (Ro325), Hardy–Weinberg equilibrium could not be calculated because there were not enough samples with allelic variation. Testing for linkage disequilibrium showed that no significant linkage was found when Bonferroni correction was applied (GENEPOP version 3.4; Raymond and Rousset, 1995).

When considering variation by range, although sampling is still preliminary, it is interesting to note that average observed heterozygosities are similar between ranges (native = 0.455 across loci, introduced = 0.476 across loci), as are average numbers of alleles per locus (native = 4.545 alleles/locus, introduced = 4.455 alleles/locus). Looking at individual loci, some are more heterozygous in the native range, as might be expected given bottlenecks in population size that are often associated with introductions. Interestingly, others (e.g., Ro325, Ro749, and Ro833) are less heterozygous in the native range samples, indicating that other populations than the two native populations sampled here contributed to the invasion (Table 2).

TABLE 2. Results of initial primer screening for populations from the native and invasive ranges of *Elaeagnus angustifolia*.

	Native range				Invasive range				
Locus	n	A	H_o	H_e	n	A	H_o	H_e	Total A
Ro20	20	5	0.650	0.672	30	4	0.533	0.552	6
Ro97	20	6	0.700	0.691	30	4	0.267	0.395	7
Ro218	20	7	0.700	0.799	30	6	0.467	0.678	8
Ro236	20	5	0.500	0.527	30	4	0.500	0.620	6
Ro325	20	1	0.000	0.000	30	3	0.267	0.269	3
Ro367	20	7	0.500	0.503	30	6	0.567	0.704	11
Ro721	20	5	0.800	0.647	30	4	0.433	0.453	6
Ro749	20	2	0.100	0.097	30	5	0.767	0.795	6
Ro833	20	4	0.200	0.599	30	6	0.367	0.614	7
Ro851	20	3	0.600	0.497	30	2	0.300	0.463	3
Ro887	20	5	0.250	0.760	30	5	0.767	0.721	7
Average		4.545	0.455	0.527		4.455	0.476	0.569	6.364

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of samples genotyped.

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CONCLUSIONS

The 11 microsatellite loci described here are the first developed for this species, and were found to amplify reliably and have sufficient variation to eventually study the population structure and origins of the North American Russian olive invasion.

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