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TECHNICAL NOTE

Isolation and characterization of 22 nuclear and 5 chloroplast microsatellite loci in the threatened riparian plant *Myricaria germanica* (Tamaricaceae, Caryophyllales)

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Abstract The German tamarisk (*Myricaria germanica)* is a riparian shrub threatened in Europe, where populations are largely confined to the upstream areas of rivers. To study gene movement in this species within and among catchments, we isolated and characterized 22 nuclear and 5 chloroplast microsatellite loci and tested their polymorphism based on 40 individuals of *M. germanica* and four individuals of *Myricaria rosea*. For *M. rosea,* 15 markers amplified, and of these, eight were polymorphic. All markers were polymorphic for *M. germanica*. The polymorphic markers allow the characterization of gene flow patterns at various spatio-temporal scales.

Keywords Conservation · Riparian plants · German Tamarisk (*Myricaria germanica)* · Microsatellites

Myricaria germanica Desv. is a shrub growing on gravel banks of braided rivers (Bill et al. 1997). This charismatic riparian species has suffered a severe decline due to channelization of rivers and habitat degradation and is threatened in Europe. The plant used to occur widespread along the upper and middle reaches of European rivers, but is now largely confined to the headwaters of rivers which have not been regulated at all, or remain near natural (Endress 1975). To assess whether populations of *M. germanica* are functionally connected through contemporary gene flow between main channels and tributaries within catchments, to assess gene flow across catchments, and to infer biogeographic patterns, we developed microsatellite markers for this species for which none were available previously.

An enriched library was developed by ecogenics GmbH (Zurich, Switzerland) from size-selected genomic DNA ligated into SAULA/SAULB-linker (Armour et al. 1994) and enrichment was performed by magnetic bead selection with biotin-labelled (GT)13, (CT)13, (AAC)10 and (ATC)10 oligonucleotide repeats (Gautschi et al. 2000a; Gautschi et al. 2000b). Of the 370 recombinant colonies screened, 87 positive clones were sequenced and primers were designed for 47 microsatellite inserts (GenBank accessions HQ680637-HQ680688; HQ687489-HQ687492). After PCR optimization for a melting temperature of 55°C for all primers, 20 microsatellite inserts could be amplified reliably, and their polymorphism was determined (Table 1).

To obtain chloroplast markers, DNA sequences were generated with universal chloroplast primers for psbC-trnS; trnC-trnD; trnH-trnK (Demesure et al. 1995), trnL (Taberlet et al. 1991), and psbA-trnH (Sang et al. 1997). These loci contained microsatellite motives with 1-bp repeat units that exhibited length polymorphisms, as well as insertion/deletion polymorphisms. Primers were designed with annealing temperatures of $T_M = 52°C$ for all loci, optimizing fragment lengths for multiplex PCR (Table 1). The locus trnC-trnD contained a 12bp-insertion/deletion polymorphism, for which a separate set of primers was designed (trnC.2-F: 5′-GCC CCC TAT GAC AGA AATA-3′; trnC.2-R: 5′-6FAM-AAT TGA AAG AAA CCA TCC AA-3′, $T_M = 52°C$, end concentration 200nM), which can be included in multiplex PCR 3. We also tested three universal chloroplast microsatellite markers developed for plants (ccmp2, ccmp3, ccmp6, Weising and Gardner 1999), but found no polymorphism in *M. germanica*.

To determine the variability of the microsatellite loci, 40 individuals were analyzed which had been sampled along

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Table 1 Primer sequences and characterisation of 20 nuclear and five chloroplast microsatellites in *Myricaria germanica*

Locus	Organelle	Primer sequence (5′–3′)	Repeat motif	Dye	Mpl	Size range	N_A	Ho	He	*P*	GenBank accession	Conc (nM)	*M. rosea*
Mg442	nuDNA	F: TTTGCTTGCTTGTTATAGAGG	$(GT)_{14}$	NED	2	273–281	3	0.425	0.491	1.000	HQ680640	100	Yes[P]
		R: ACAACAACGACAGGACTACC										100	
Mg444	nuDNA	F: TTTTGAGCACACAAAGCTAA	$(GT)_{14}(GA)_{19}$	NED	2	393–398	3	0.150	0.186	1.000	HQ680641	300	Yes
		R: GCGAGCCTGATAAAGTTAAA										300	
Mg451	nuDNA	F: TCTACAAAACCCATCAATCC	$(TG)_{14}(AG)_{13}$	6FAM	1	452–469	6	0.150	0.190	1.000	HQ680644	250	Yes
		R: CATCGGTTCTCAAGTACTCC										250	
Mg452	nuDNA	F: GTCTGCACTCCTACGTCTTC	$(CA)_{17}$	VIC	1	425–441	4	0.550	0.600	1.000	HQ680645	200	Yes
		R: TAAGGAGAAGAAGGGAGGTT										250	
Mg455	nuDNA	F: ACAGAACGGTGTCGTTTAAT	$(GT)_{10}$	PET	2	175–181	4	0.625	0.656	1.000	HQ680646	150	Yes
		R: ATTTGATTGGGATACACGTC										150	
Mg457	nuDNA	F: CATCACCATCATTAGCATTG	$(AC)_{14}(AT)_3(ACAT)_2$	6FAM	1	223–225	2	0.125	0.162	1.000	HQ680649	200	
		R: GGGCAATGAAATATAGCAAG										200	
Mg459	nuDNA	F: ACGGTGATGTTGGTAAATGT	$(CA)_{14}(TA)_6$	6FAM	1	161–175	4	0.350	0.409	1.000	HQ680650	150	
		R: TGTGTAGCTTATGGTGGAAA										150	
Mg461	nuDNA	F: CTCCCCTTTCTTTCCCTAA	$(GT)_{11}$	VIC	2	174–176	2	0.275	0.309	1.000	HQ680652	60	
		R: GTTACGAGTGGTGGTGATTT										60	
Mg462	nuDNA	F: TCCCATAGTTTTATGATGTGG	$(GT)_{13}$	NED	2	137–145	4	0.325	0.378	0.972	HQ680653	60	Yes
		R: AATGTTATGTGAAGATGCCC										60	
Mg480	nuDNA	F: AAGGTCCAAGGCTGTCC	$(GT)_{13}(GA)_4(GT)_1$	PET	1	290–300	2	0.025	0.025	1.000	HQ680654	250	Yes
		R: CTAAGTTCACGGGAGTTTGA	$(GA)_8(GT)_8(GA)_{18}$									250	
Mg482	nuDNA	F: ATCTATGTCAATCGGACGC	$(AC)_{14}$	VIC	1	144–154	3	0.250	0.343	0.000	HQ680656	100	
		R: TAAACCCCAGACACTCAAAC										100	
Mg488	nuDNA	F: AATTAGGAAGCGAGGGGGA	$(GA)_{22}$	NED	1	112–138	4	0.450	0.528	1.000	HQ680661	100	Yes[P]
		R: CTCTGCCTATATAATGAGGAGC										100	
Mg489	nuDNA	F: GTTGAGGTCACACAATGACA	$(AC)_{15}$	NED	1	202–210	2	0.100	0.096	1.000	HQ680662	200	
		R: CATTCCTATGCTAGTCCCAC										250	
Mg493	nuDNA	F: CACCATAGTCTAGTAGGAGATGC	$(CA)_{13}(A)_{12}$	PET	1	194–230	6	0.450	0.493	1.000	HQ680665	120	Yes[P]
		R: ATGAGTCTGAAGAGAGTGGG										120	
Mg495	nuDNA	F: GATAGATGATGTTCGGCGT	$(GT)_{18}(GA)_{12}$	NED	1	403–405	2	0.225	0.240	1.000	HQ680667	150	
		R: GGGTTAGTGTCTGGCTAGTG										150	
Mg498	nuDNA	F: AAGTAATGATATGGCTCGCA	$(GT)_{23}(AT)_5$	PET	1	161–163	2	0.075	0.202	0.055	HQ680669	150	Yes[P]
		R: TGATGTGCTTATACACCCAA										150	
Mg499	nuDNA	F: CATCCATCCTCACTGTTTCT	$(CT)_{10}(CA)_{10}$	VIC	1	346–354	3	0.400	0.509	0.750	HQ680670	300	Yes[P]
		R: ATGCTGACCCTCTTGTATGT										300	

Table 1 continued

Locus	Organelle	Primer sequence (5′–3′)	Repeat motif	Dye	Mpl	Size range	N_A	Ho	He	*P*	GenBank accession	Conc (nM)	*M. rosea*
Mg500	nuDNA	F: GGAAACATGCCTAAACAAAA	$(GT)_{20}$	NED	1	286–296	3	0.375	0.406	1.000	HQ687489	300	
		R: TGAACGATTGTATTATCCCG										300	
Mg502	nuDNA	F: TCCCTAGTCTCCCTTAGTCC	$(AC)_{11}$	6FAM	2	147–159	4	0.425	0.531	1.000	HQ680671	120	Yes[P]
		R: AGCTAGCTAACCAAAACCATT										120	
Mg504	nuDNA	F: CATCAAGCACAGACACTTGA	$(AC)_{12}$	6FAM	2	386–398	3	0.500	0.557	1.000	HQ680673	250	Yes[P]
		R: ATGACAGAACCTTATGTGCC										250	
Mg507	nuDNA	F: TTAAGAAAAGAAAAGCAGCC	$(GT)_{20}$	VIC	2	239–251	4	0.425	0.533	1.000	HQ680675	150	Yes[P]
		R: GGAGGAAGCAAGTGAGTTTA										150	
Mg549	nuDNA	F: TAGCTAGTTGCTTCATTCCC	$(ATAC)_{11}$	6FAM	1	280–312	4	0.575	0.566	1.000	HQ687490	150	Yes
		R: ACATAACATGAACAGGGAGG										150	
psbA	cpDNA	F: TTCCCTCTAGACCTAGCTGC	T_9	NED	4	375–382	3	–	0.537	–	HQ680684	100	Yes
		R: CGCGCATGGTGGATTCACAAATC[a]										100	
psbC	cpDNA	F: AGACAAGAGATCCAATGCTT	T_{13}	6FAM	3	257–261	3	–	0.645	–	HQ680685	160	Yes
		R: GGTTCGAATCCCTCTCTCTC[b]										160	
trnC	cpDNA	F: AGGGTTTACTGAATGGACCT	T_7	6FAM	3	207–208	2	–	0.328	–	HQ680686	200	Yes
		R: AAGTTATTTTGCCTTTGCAC										200	
trnH	cpDNA	F: GATCACTTGGCTACATCCGC	$(*)(TA)_5(x)A_6$	6FAM	3	325–334	3	–	0.537	–	HQ680687	240	Yes
		R: CTGCTATTGAAGCTCCATCT										240	
trnL	cpDNA	F: GAGACTCAACGGAAGCTGT	T_{13}	6FAM	3	386–387	2	–	0.328	–	HQ680688	200	Yes
		R: GGGGATAGAGGGACTTGAAC[c]										200	

Primer sequence (*F* forward primer, *R* reverse primer), repeat motif, fluorescent dye used in PCR, multiplex number (Mpl), size range [bp], N_A observed number of alleles, H_o observed heterozygosity, H_e expected heterozygosity (for the haploid cpSSR loci, H_e can be interpreted as gene diversity), *P*, *P* value of the test for Hardy–Weinberg equilibrium adjusted with Holm's correction; GenBank accession; primer end concentration in PCR reaction; *M. rosea*, indication if the marker amplifies with *M. rosea*, [P] polymorphic among four samples of *M. rosea*. (*), Indel of 8 bp; (x) = TTATTAGTAAAGTTAAAAAACGT

[a] From Sang et al. (1997)

[b] From Demesure et al. (1995)

[c] From Taberlet et al. (1991)

the Lech river in Austria. Total genomic DNA was extracted from leaf tissue, using the DNeasy 96 plant kit (Qiagen) according to the manufacturer's protocol. PCR was carried out in 5 μL reaction volumes containing 1× Multiplex PCR mix (Qiagen), primers in varying concentrations (see Table 1), 1–10 ng of diluted genomic DNA, and ddH_2O. For the fourth PCR reaction, we used the JumpStartREDTaq Ready Mix (Sigma–Aldrich). Amplifications were performed on a Veriti thermal cycler (Applied Biosystems), and the cycling conditions were 95°C for 15 min to activate the hot start DNA polymerase, followed by 35 cycles of denaturation for 5 s at 95°C, primer annealing at the locus-specific annealing temperature for 10 s, elongation at 72°C for 45 s, and a final extension at 60°C for 30 min (multiplex 1 and 2). For multiplex 3, we used the following cycling protocol: activation of the polymerase at 95°C for 15 min, 34 cycles of 95°C for 10 s, 52°C for 1 min, and 72°C for 1 min, and a final extension of 30 min at 72°C. The fourth PCR reaction containing the cpDNA locus psbA differed by a shorter initial activation of the polymerase: 2 min at 95°C. Fragments lengths were determined by capillary electrophoresis on an ABI 3130 sequencer (Applied Biosystems), and the samples were genotyped using GENEMAPPER version 3.7 (Applied Biosystems). We also tested amplification of the markers with four samples of *Myricaria rosea,* collected in a single population.

We used Arlequin version 3.11 (Excoffier et al. 2005) to test if the nuSSR loci were unlinked and in Hardy–Weinberg equilibrium, and to characterize the variability of both nuSSR and cpSSR. All nuclear markers were polymorphic, with two to six alleles (mean 3.4). The observed heterozygosity H_o ranged from 0.025 to 0.625, the expected heterozygosity H_e from 0.025 to 0.656. Three of the nuclear loci were not in Hardy–Weinberg equilibrium (Mg482, Mg498, and Mg499). With the exception of Mg482, none of the nuclear loci showed a significant deviation from Hardy–Weinberg equilibrium when we applied Holm's correction of *P*-values for multiple tests (Table 1). Fifteen of 231 pairwise combinations of loci were linked, but none of the significances remained when *P*-values were adjusted with Holm's correction.

The present markers show a potential to be transferable to other species of the genus *Myricaria*: 15 nuSSR markers amplified with *M. rosea*, and 8 of these were polymorphic. All cpSSR markers amplified with *M. rosea*, but they did not show polymorphism, which is not surprising given that we analyzed few samples that were collected from the same population.

In the microsatellite loci located on the chloroplast DNA, we found between two and three alleles across the 40 samples from Lech in Tirol. Gene diversity ranged from 0.328–0.654. We did not test linkage disequilibrium for the physically linked chloroplast loci.

The microsatellites reported here will be utilized to study contemporary and historical gene movement of *M. germanica* along major rivers in Switzerland, Austria (Lech, Drau) and Italy (Tagliamento).

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