Development, Characterization, and Cross-Amplification of 16 Microsatellite Primers for Atriplex tatarica (Amaranthaceae)

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PRIMER NOTE

DEVELOPMENT, CHARACTERIZATION, AND CROSS-AMPLIFICATION OF 16 MICROSATELLITE PRIMERS FOR *ATRIPLEX TATARICA* (AMARANTHACEAE)[1]

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- *Premise of the study:* Microsatellite primers were developed to characterize the genetic diversity and structure of the annual herb *Atriplex tatarica* (Amaranthaceae) and to facilitate ecological and evolutionary studies of *A. tatarica* and its relatives.
- *Methods and Results:* Sixteen novel microsatellite primers were developed for *A. tatarica* based on high-throughput sequencing of enriched libraries. All markers were polymorphic, with the number of alleles per locus ranging from three to 25 and observed and expected heterozygosity ranging from 0.08 to 0.74 and 0.10 to 0.87, respectively. In addition, some of these loci were successfully amplified and showed polymorphisms in four *Atriplex* and seven *Chenopodium* species.
- *Conclusions:* The microsatellite markers published here will be useful in assessing genetic diversity, structure, and gene flow within and across populations of *A. tatarica*, as well as in other species of *Atriplex* and the related genus *Chenopodium.*

Key words: Amaranthaceae; *Atriplex*; *Chenopodium*; cross-amplification; microsatellites.

The genus *Atriplex* L. (Amaranthaceae) numbers about 270 species (McArthur and Sanderson, 1984) distributed mainly in the deserts and semideserts in southwestern North America, in southern Australia, in southern Central Asia, in southwestern South America (Osmond et al., 1980; McArthur and Sanderson, 1984), or in coastal and solonchak regions of the Northern Hemisphere (Osmond et al., 1980). Most previous population studies in *Atriplex* used allozymes (Mandák et al., 2005, 2006a, 2006b), and highly variable microsatellites have been employed only in the study of the Australian species *A. nummularia* Lindl. (Byrne et al., 2008). To date, no nuclear simple sequence repeat markers (SSRs) have been developed specifically for *A. tatarica* L. and successfully cross-amplified to closely related *Atriplex* and *Chenopodium* L. species to enable population-level assessment of various representatives of the genus. Given the number of species in both the genus and the whole family, we expect that these markers will have broad applicability for conservation and population-level analyses.

Atriplex tatarica is an annual diploid ($2n = 2x = 18$) with a mixed mating system (Mandák et al., 2005) and is native to a wide area of Eurasia (Kochánková and Mandák, 2008). Along with 13 other species, it belongs to the section *Sclerocalymma* (Asch.) Asch. & Graebn. The distribution center of this section is located in southern Central Asia (Kochánková and Mandák, 2008). In Europe the species has a continental distribution. The northwestern border of its current continuous European range runs through the Czech Republic (southern Moravia), southern Slovakia, eastern Poland, and central Belarus, and its expansion in these countries has recently been reported (Kochánková and Mandák, 2008). The species possesses remarkable heterocarpy, which is morphologically manifested in the shape and size of bracteoles and in the size and color of fruits. Heterocarpy enables colonizing species such as *A. tatarica* to survive both major disturbances and unfavorable conditions (by ensuring that at least some seeds persist) and to expand during periods of favorable conditions (by ensuring that some seeds effectively spread and germinate) (Doudová et al., 2017). In this paper, we report the development and characterization of 16 novel microsatellite loci for *A. tatarica*. Additionally, we cross-amplified these loci in four and seven species of the genera *Atriplex* and *Chenopodium*, respectively.

METHODS AND RESULTS

***Microsatellite development*—**Total genomic DNA of *A. tatarica* was extracted from 20–25 mg of silica gel–dried leaf tissue from seven samples of different population origin (Appendix 1) using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). These samples were used by GenoScreen (Lille, France) to develop microsatellite loci following the protocol of Malausa et al. (2011) based on GS FLX Titanium pyrosequencing (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) of microsatellite-enriched DNA libraries. Microsatellite enrichment was carried out using eight microsatellite probes [$(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(AGG)_8$, $(ACG)_8$, $(AAG)_8$, $(ACAT)_6$, $(ATCT)_6$]. The sequencing yielded 32,229 reads, and 1956 of these contained microsatellite

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TABLE 1. Characteristics of 16 polymorphic microsatellite loci of *Atriplex tatarica*.

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent label	Concentration in multiplex reaction (μM)	GenBank accession no.
Multiplex 1						
AT22	F: GTGCAACGAAGATGGATTTG	$(TGT)_7$	114–134	6-FAM	0.1	MF598744
	R: TGAGAAAGTGGAGCAAGAAGTG					
AT28	F: CTGGACAGTTGTGAAAGCTCA	$(AG)_6$	161–222	6-FAM	0.6	MF598747
	R: CGTACCTATTGAGGTTCTCTCCC					
AT15	F: GGTATTCGTCGATTTCCCCT	$(TGT)_8$	101–134	VIC	0.1	MF598741
	R: CGAGTTCGACTCGCCTAAGA					
AT20	F: GGTGGGTTTAATGGTGGAAA	$(AGA)_7$	139–150	NED	1.0	MF598743
	R: CCTGAGTCGCGTTTGTACCT					
AT17	F: GGTGTTACCTACGCTTTCATTC	$(TG)_8$	100–118	PET	0.2	MF598742
	R: ATTTGCAATTCCATGAGCCT					
AT07	F: TGGTCGTGGTAACCCTCTTC	$(TGT)_{11}$	153–188	PET	0.2	MF598740
	R: AGCTCAGCGCAATTCAGTTT					
AT38	F: ATGGGCCAACATATTTTCCA	$(AC)_6$	102–104	6-FAM	0.1	MF598750
	R: GCATGTTTAGTTGGTAGCAAGG					
AT27	F: GCAATCAGAGGCAGAACTCC	$(TGC)_6$	218–233	VIC	0.1	MF598746
	R: CCATGCTTGTTGAGTTTCTTCA					
AT44	F: TTCGATCGTTCTGTCGTAACC	$(TG)_5$	165–180	VIC	0.1	MF598753
	R: TCCTTTCTAGACCACCCCAA					
Multiplex 2						
AT04	F: TGTGAAAGTTGTCTTTGTGCAAC	$(TCT)_{14}$	94–200	6-FAM	0.4	MF598738
	R: CGAGTAAGACGATAGAGGAAGAGG					
AT05	F: TTCTTGAGGGCTTTCTTGGA	$(TGT)_{12}$	86–108	VIC	0.1	MF598739
	R: TGACCCTTTTGCTTTTAGGG					
AT25	F: TAACTGGTGTTGGTCGGTGA	$(AGG)_6$	266–283	NED	0.2	MF598745
	R: TAGGATCTTCTTCGGGTTCG					
AT30	F: GAGGCAGAGACTGAAGGTGG	$(GAA)_6$	136–150	PET	0.1	MF598748
	R: TTCTTCATTCCTCCGTTTCG					
AT31	F: GAAGCCATGAAGGAGGTGAG	$(GT)_6$	137–161	NED	0.2	MF598749
	R: CTGCCAGCAGCAATCAATTA					
AT41	F: TTGCACCAATGCAATCCTAA	$(TACA)_5$	190–230	PET	0.2	MF598751
	R: AATAAGGCCAAGAGATCCCG					
AT42	F: GAGGTCTGGATCTGAGACGG	$(CATA)_5$	172–188	VIC	0.1	MF598752
	R: CTTCCCCTTGCTTCCATAAA					

[a]Annealing temperature was 55°C for both multiplexes.

motifs. Primers were designed based on reads of the positive strands using QDD software (Meglécz et al., 2010).

***Biological validation*—**Forty-seven candidate loci possessing perfect repeat motifs and different expected amplicon lengths within the 100–400-bp interval were selected and tested for amplification from all seven individuals. The PCR reactions were performed in 5-μL reaction volumes containing 1 μL of genomic DNA, 0.1 μM of both primers, and 1× QIAGEN Multiplex PCR Master Mix (QIAGEN). Reactions were performed with the following conditions: an initial denaturation step at 95°C for 15 min; followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The PCR products were checked on 2% agarose gels. Of the markers that amplified successfully from all seven individuals, 24 were selected and used for initial polymorphism tests. In this step, PCRs were performed as described above, but only the forward primers were labeled by fluorescent dyes (6-FAM, VIC, PET, NED; Applied Biosystems, Foster City, California, USA). The PCR products were diluted 5×, and 1.0 μL of the dilution was added to a mix of 12.0 μL Hi-Di Formamide (Applied Biosystems) and 0.1 μL GeneScan 500 LIZ Size Standard (Applied Biosystems) for sequencing on an ABI PRISM 3130 Automated Capillary DNA Sequencer (Applied Biosystems). In the end, 16 polymorphic markers with well-scorable peaks were selected and combined into two multiplexes (Table 1). The sequences of the 454 reads containing these microsatellite loci have been deposited in the GenBank database of the National Center for Biotechnology Information (NCBI) (Table 1). These two multiplexes (Table 1) were tested for polymorphism in 120 individuals from six geographically well-separated populations collected across Europe (Appendix 1).

Using the same reaction conditions as specified above, the primers were tested on DNA extracted from *A. oblongifolia* Waldst. & Kit. (15 individuals tested), *A. patula* L. (15), *A. prostrata* DC. (3), *A. sagittata* Borkh. (20), *C. bonus-henricus* L. (7), *C. hybridum* L. (6), *C. polyspermum* L. (4), *C. pumilio* R. Br. (6), *C. rubrum* L. (4), *C. suecicum* Murr (6), and *C. urbicum* L. (4) (Appendix 1).

***Microsatellite data analysis*—**Allele size was determined using GeneMarker 2.6.4 (SoftGenetics, State College, Pennsylvania, USA). FSTAT 2.9.3 (Goudet, 1995) was used to calculate summary statistics for SSR loci such as the average number of alleles per locus and Weir and Cockerham's parameter f (F_{IS}; Weir and Cockerham, 1984) as a measure of departure from within-population random mating. Observed and expected heterozygosities were calculated using GENEPOP (Rousset, 2008), and the deviation from Hardy–Weinberg equilibrium was determined based on 10,000 permutations in FSTAT 2.9.3 (Goudet, 1995). MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004) was used to test for evidence of stuttering, allele dropout, and the presence of null alleles at each locus. The Brookfield 1 equation (Brookfield, 1996) was used to calculate null allele frequencies.

We identified 143 alleles at 16 microsatellite loci, with an average of 8.9 alleles per locus. The summary statistics for genetic variability across and within populations are presented in Table 2. The deficit of heterozygotes, computed over all populations and loci, was significant, as indicated by a relatively high inbreeding coefficient (f = 0.171). Eight out of 16 loci were not in Hardy–Weinberg equilibrium (Table 2), which might be due to high levels of self-pollination and the strong bottleneck effect of newly founded expanding populations. No signs of stuttering or large allele dropout were detected. The average null allele frequency for each locus calculated using the Brookfield method detected the presence of null alleles at five loci (Table 2).

Fifteen microsatellite loci were successfully cross-amplified from some of the species tested (Table 3). The cross-amplification was more successful with closely related species of the genus *Atriplex* than of *Chenopodium* (Table 3).

TABLE 2. Genetic characterization of 16 newly developed polymorphic microsatellite loci across six populations of *Atriplex tatarica*.[a]

		Bulgaria (11A, $n = 20$)				Poland (25A, $n = 20$)				Germany (30A, $n = 20$)				Romania (44A, $n = 20$)				Hungary (62A, $n = 20$)				Czech Republic (70A, $n = 20$)				Overall ($n = 120$)			
Locus	*B*	*A*	H_o	H_e	*f*	*A*	H_o	H_e	*f*	*A*	H_o	H_e	*f*	*A*	H_o	H_e	*f*	*A*	H_o	H_e	*f*	*A*	H_o	H_e	*f*	*A*	H_o	H_e	*f*
Multiplex 1																													
AT38	0.124	2	0.10	0.26	0.62*	1	ND	ND	ND	1	ND	ND	ND	2	0.25	0.22	−0.12	1	ND	ND	ND	2	0.10	0.10	−0.03	3	0.08	0.10	0.28*
AT22	0.124†	4	0.40	0.61	0.36*	5	0.75	0.70	−0.07	4	0.37	0.58	0.38*	6	0.55	0.61	0.10	2	0.25	0.30	0.16	4	0.70	0.64	−0.10	7	0.50	0.61	0.12*
AT28	0.223†	7	0.41	0.84	0.52*	6	0.65	0.77	0.16	5	0.68	0.75	0.10	5	0.50	0.60	0.17	4	0.25	0.59	0.59*	6	0.55	0.67	0.18	12	0.51	0.80	0.28*
AT15	−0.030	7	0.65	0.62	−0.05	8	0.75	0.78	0.04	8	0.63	0.77	0.18	9	0.85	0.82	−0.04	4	0.65	0.57	−0.14	3	0.40	0.47	0.14	11	0.66	0.79	0.02
AT44	−0.005	2	0.11	0.10	−0.03	3	0.15	0.14	−0.04	2	0.05	0.05	0.00	1	ND	ND	ND	2	0.00	0.12	1.00*	2	0.16	0.31	0.50	5	0.08	0.13	0.37*
AT27	−0.008	3	0.15	0.14	−0.04	4	0.55	0.47	−0.18	3	0.37	0.32	−0.17	4	0.35	0.38	0.07	5	ND	ND	ND	1	ND	ND	ND	6	0.24	0.24	−0.09
AT20	0.227†	5	0.35	0.77	0.55*	6	0.53	0.73	0.28*	7	0.53	0.85	0.39*	4	0.85	0.75	−0.14	1	0.30	0.62	0.52*	5	0.60	0.74	0.20	8	0.53	0.83	0.30*
AT17	0.000	4	0.90	0.71	−0.27	4	0.32	0.63	0.51*	5	0.63	0.63	0.01	4	0.20	0.27	0.27	5	0.60	0.57	−0.06	4	0.70	0.68	−0.03	5	0.56	0.67	0.04
AT07	0.197†	4	0.40	0.76	0.48*	5	0.06	0.69	0.92*	5	0.21	0.71	0.71*	6	0.50	0.74	0.33*	3	0.16	0.23	0.33	4	0.25	0.65	0.62*	12	0.26	0.71	0.58*
Multiplex 2																													
AT04	0.010	5	0.55	0.58	0.05	8	0.50	0.82	0.40*	12	0.65	0.90	0.29*	6	0.20	0.74	0.74*	2	0.35	0.46	0.24	13	0.50	0.88	0.44*	25	0.46	0.87	0.38*
AT05	−0.074	5	0.65	0.55	−0.19	5	0.70	0.60	−0.17	5	0.40	0.43	0.07	7	0.50	0.50	0.00	4	0.55	0.45	−0.23	6	0.60	0.66	0.09	8	0.57	0.68	−0.07
AT42	−0.096	3	0.60	0.47	−0.28	6	0.60	0.71	0.16	6	0.89	0.77	−0.16	6	0.85	0.73	−0.18	2	0.50	0.57	0.12	5	0.60	0.57	−0.06	8	0.67	0.71	−0.06
AT31	0.018	8	0.80	0.86	0.07	6	0.42	0.63	0.34	9	0.85	0.86	0.01	6	0.85	0.81	−0.05	3	0.70	0.62	−0.14	6	0.80	0.78	−0.03	9	0.74	0.84	0.03
AT25	−0.015	3	0.45	0.44	−0.02	5	0.78	0.68	−0.14	5	0.68	0.71	0.04	6	0.60	0.79	0.25*	3	0.05	0.05	0.00	5	0.70	0.62	−0.14	7	0.54	0.67	0.02
AT30	−0.014	4	0.50	0.49	−0.02	5	0.50	0.68	0.27	3	0.63	0.52	−0.22	3	0.30	0.27	−0.10	2	ND	ND	ND	5	0.40	0.49	0.19	6	0.39	0.46	0.05
AT41	0.246†	6	0.29	0.74	0.61*	4	0.40	0.60	0.34	2	0.11	0.11	−0.03	5	0.06	0.45	0.87*	2	0.39	0.47	0.19	5	0.35	0.66	0.47*	11	0.27	0.61	0.48*
Mean		4.50	0.46	0.56	0.19*	5.06	0.51	0.64	0.21*	5.13	0.51	0.60	0.15*	5.00	0.49	0.58	0.15*	2.81	0.37	0.43	0.16*	4.75	0.49	0.59	0.17*	8.94	0.44	0.61	0.17*
s.d.		1.79	0.23	0.23	0.25	1.73	0.21	0.16	0.20	2.85	0.25	0.27	0.16	2.85	0.27	0.21	0.17	1.28	0.23	0.19	0.34	2.67	0.21	0.19	0.18	5.03	0.20	0.25	0.21

Note: *A* = number of alleles; *B* = null allele frequency averaged over all populations using the Brookfield 1 equation (Brookfield, 1996); *f* = inbreeding coefficient; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals sampled; ND = not determined.

[a]Codes presented with populations correspond to voucher accession numbers. Voucher and locality information are provided in Appendix 1.

*Significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

†Loci with null alleles.

TABLE 3. Results of cross-amplification (allele size ranges) of 16 microsatellite loci developed for *Atriplex tatarica* tested in seven *Chenopodium* and four other *Atriplex* species.[a]

Locus	*A. oblongifolia* (*n* = 15)	*A. patula* (*n* = 15)	*A. prostrata* (*n* = 3)	*A. sagittata* (*n* = 20)	*C. bonus-henricus* (*n* = 7)	*C. hybridum* (*n* = 6)	*C. polyspermum* (*n* = 4)	*C. pumilio* (*n* = 6)	*C. rubrum* (*n* = 4)	*C. suecicum* (*n* = 6)	*C. urbicum* (*n* = 4)
Multiplex 1											
AT38	102–108	106	—	100–104	102–108	—	—	100–106	108	—	108
AT22	114–125	114–118	118	118	128–148	—	118–147	111–121	121	110–113	—
AT28	152–200	200–201	—	187–233	206	—	219	160–219	—	160–163	—
AT15	127	104	—	124	—	—	—	113–138	—	111	—
AT44	146–154	144–146	142	151	160–183	157	160–186	153	155	153	155
AT27	218–230	230	230	227–230	199–218	213	218	213–218	207	218	207
AT20	173	119–173	—	129	—	—	—	150	—	—	—
AT17	127	—	—	134	—	—	123	—	—	—	—
AT07	—	—	—	—	—	—	—	—	—	—	—
Multiplex 2											
AT04	90–204	91–126		91–204	232	254	131	—	—	—	—
AT05	92	—	86	92	104	—	104	—	—	—	—
AT42	180–187	180–184	—	186–184	—	—	—	—	—	—	196
AT31	141–143	143	141–191	143–179	148–150	159	—	—	—	145–161	—
AT25	—	—	227	—	215	271	288	—	—	301	—
AT30	134–145	134–148	139	134–145	—	—	—	99–117	—	—	—
AT41	190–198	194–198	194–210	190–198	187	—	—	—	—	—	—

Note: *n* = number of individuals used.
[a]Locality and population information are provided in Appendix 1.

CONCLUSIONS

Sixteen polymorphic microsatellite loci were developed for *A. tatarica*. These markers will be valuable for investigating the population genetic structure, mating system, and phylogeographic pattern of this species. The cross-species amplification of these markers indicates that they may be widely useful in related Amaranthaceae species. We conclude that the SSRs described here will facilitate ecological and evolutionary studies of *A. tatarica* and related species.

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Appendix 1. Geographic information on *Atriplex* and *Chenopodium* populations in this study.

Species	Voucher specimen accession no.[a]	*N*	Collection locality	Latitude (°N)	Longitude (°E)
Atriplex tatarica L.	*B. Mandak 1A*[b]	1	Győr, Hungary	47.70633	17.48802
	B. Mandak 3A[b]	1	Nagyhegyes, Hungary	47.51795	21.35765
	B. Mandak 5A[b]	1	Siculeni, Romania	46.44174	25.76171
	B. Mandak 7A[b]	1	Constanty, Romania	44.28178	28.55689
	B. Mandak 9A[b]	1	Dragodana, Romania	44.72501	25.34959
	B. Mandak 11A[b,c]	20	Sofia, Bulgaria	42.74245	23.25354
	B. Mandak 12A[b]	1	Popovitsa, Bulgaria	42.13607	25.05463
	B. Mandak 25A[c]	20	Wielgolas Brzeziński, Poland	52.20053	21.41598
	B. Mandak 30A[c]	20	Berlin, Germany	52.30090	13.19406
	B. Mandak 44A[c]	20	Tețcani, Romania	47.03009	26.86970
	B. Mandak 62A[c]	20	Hegyháthodász, Hungary	46.93466	16.66857
	B. Mandak 70A[c]	20	Velké Němčice, Czech Republic	49.01046	16.68755
Species used for cross-amplification					
A. oblongifolia Waldst. & Kit.	*B. Mandak 930*	5	Cluj-Napoca, Apahida, Romania	46.81814	23.74997
	B. Mandak 932	5	Vlădeni. Romania	47.41469	27.37454
	B. Mandak 936	5	Horoměřice, Czech Republic	50.13175	14.35309
A. patula L.	*B. Mandak 927*	5	Ladná, Czech Republic	48.80956	16.89302
	B. Mandak 933	5	Vlădeni, Romania	47.40971	27.37309
	B. Mandak 928	5	Cluj-Napoca, Barai, Romania	46.85796	23.90922
A. prostrata DC.	*B. Mandak 95*	3	Helnæs, Denmark	55.12278	10.03095
A. sagittata Borkh.	*B. Mandak 929*	5	Cluj-Napoca, Apahida, Romania	46.79999	23.75494
	B. Mandak 931	5	Vlădeni, Romania	47.41469	27.37454
	B. Mandak 934	5	Praha-Suchdol, Štěpnice, Czech Republic	50.12898	14.36737
	B. Mandak 935	5	Litohlavy, Czech Republic	49.77035	13.55190
Chenopodium bonus-henricus L.	*B. Mandak 379*	3	Kladrubce, Czech Republic	49.48618	13.68160
	B. Mandak 383	3	Plužná, Czech Republic	50.47151	14.79743
	B. Mandak 394	1	Praha, Czech Republic	50.11596	14.43306
C. hybridum L.	*B. Mandak 287*	3	Velké Němčice, Czech Republic	48.99786	16.67289
	B. Mandak 295	3	Kunszentmiklós, Hungary	46.98325	19.25014
C. polyspermum L.	*B. Mandak 311*	2	Virt, Slovakia	47.74325	18.32589
	B. Mandak 394	2	Cehnice, Czech Republic	49.22747	14.03011
C. pumilio R. Br.	*B. Mandak 327*	3	Praha, Czech Republic	50.08723	14.40689
	B. Mandak 362	3	Bachkovo, Bulgaria	41.94260	24.85230
C. rubrum L.	*B. Mandak 281*	2	Rejšice, Czech Republic	50.31997	14.97881
	B. Mandak 312	2	Virt, Slovakia	47.74325	18.32589
C. suecicum Murr	*B. Mandak 264*	3	Rumšiškės, Lithuania	54.87940	24.19795
	B. Mandak 328	3	Švermov, Czech Republic	50.17681	14.10547
C. urbicum L.	*B. Mandak 335*	4	Hortobágy, Hungary	47.58125	21.15842

Note: *N* = number of individuals used.

[a] One voucher was collected from each sampled population. Herbarium vouchers are deposited in the author's collection at the Czech University of Life Sciences in Prague. Collector name and unique population number in our database are indicated.

[b] Individual used to create a DNA genomic library.

[c] Populations used for evaluating the quality of developed microsatellite loci.