Applications in Plant Sciences

PRIMER NOTE

Development of 15 microsatellite markers in *Acer triflorum* (Aceraceae) and cross-amplification in congeneric species

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PREMISE OF THE STUDY: *Acer* (Aceraceae) is an important genus in forest ecosystems in the Northern Hemisphere. In China, 151 species have been reported, and approximately 61 species are endemic. Thus, China is considered to host the greatest diversity of *Acer*, but markers are needed to evaluate the genetic structure and genetic diversity of these populations of wild *Acer* species.

METHODS AND RESULTS: Using an enriched genomic library, we developed and characterized 15 microsatellite primers for *A. triflorum*, 10 of which were polymorphic. The number of alleles varied from one to nine. The levels of observed heterozygosity and expected heterozygosity per locus ranged from 0.000 to 1.000 and 0.000 to 0.826, respectively. Most primers also successfully amplified in *A. ginnala*, *A. griseum*, *A. mandshuricum*, *A. pseudosieboldianum*, *A. sinopurpurascens*, *A. tegmentosum*, and *A. ukurunduense*.

CONCLUSIONS: These markers from *A. triflorum* will provide an opportunity to study genetic diversity and genetic structure in the genus *Acer*.

KEY WORDS *Acer triflorum*; Aceraceae; genetic studies; northeastern China; simple sequence repeat (SSR) markers.

The genus *Acer* L. belongs to the family Aceraceae, which comprises deciduous or evergreen small trees or shrubs, with more than 200 species distributed in the forests of the Northern Hemisphere. Of these, 151 species are located in China (Fang, 1981). Maple trees usually have an upright branching structure, and they play an important role in landscaping around the world. Moreover, species of the genus *Acer* also have value as timber and for medicinal and edible use (Wei and Liang, 2005). Several studies have inferred the phylogenetic relationships of maple trees collected from northeastern China using inter-simple sequence repeat (ISSR) markers and DNA sequences (Liu et al., 2010). Compared to ISSRs, microsatellite or simple sequence repeat (SSR) markers are currently the most practical, informative, and widely used tools in population genetic studies (Chan et al., 2014; He et al., 2017). However, microsatellite markers for *A. triflorum* Kom. and closely related species are currently not available. Therefore, we isolated and identified genomic microsatellites from the species *A. triflorum* and tested their transferability in congeneric species. These markers will be useful for studying genetic diversity and population structure across the genus *Acer*. Genetic studies of these valuable species are an important and necessary step in their conservation and management (Gordon et al., 2012; Lopes et al., 2014).

METHODS AND RESULTS

Leaf material of *A. triflorum* was sampled from five locations of China, namely Fusong (FS), Dandong (DD), Dunhua (DH), Benxi (BX), and Tonghua (TH), for a total sample size of 88 individuals (Appendix 1). Genomic libraries enriched for microsatellite motifs were constructed as described in detail in Zane et al. (2002). Genomic DNA was extracted from dried leaves of three individuals of *A. triflorum* collected from the FS population using the Plant Genomic DNA kit (TianGen, Beijing, China) following the manufacturer's protocols. Approximately 300 ng of genomic DNA were digested separately with the restriction enzyme *Mse*I (New England Biolabs, Beverly, Massachusetts, USA), then ligated to the *Mse*I adapter pair (forward: 5′-TACCAGGACTCAT-3′; reverse: 5′-GACGATGAGTCCTGAG-3′) (Vos et al., 1995). The diluted digestion-ligation mixture (1:10) was then amplified with the *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′) by PCR (5 min at 95°C; followed by 20 cycles of 94°C for 30 s, 53°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 7 min).

For enrichment, PCR products were denatured and hybridized to a 5′-biotinylated $(AC)_{15}$ probe, and DNA fragments containing microsatellite motifs were captured by streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). DNA fragments with motifs were purified

using a Gel Extraction Kit (TaKaRa Biotechnology Co., Dalian, Liaoning, China), ligated into the pMD-18 vector (TaKaRa Biotechnology Co.), and transformed into *Escherichia coli* DH5α competent cells (TaKaRa Biotechnology Co.). The positive clones were identified and tested by PCR using $(AC)_{10}$ and $M13^{+}/M13^{-}$ (forward: 5′-GTAAAACGACGGCCAG-3′; reverse: 5′-CAGGAAACAGCTATGAC-3′) as primers, respectively. Overall, 131 positive clones with microsatellite motifs were sequenced, of which 67 clones were found to have sufficient flanking regions (at least 30 bp in length) to design primer pairs using Primer Premier software (PREMIER Biosoft International, Palo Alto, California, USA). The conditions for primer design were performed according to Li et al. (2011). In total, we identified 15 primer pairs that successfully amplified (Table 1).

These primers were assessed in 88 individuals from five populations of *A. triflorum* and from five individuals from one population of each congeneric species (*A. ginnala* Maxim., *A. griseum* (Franch.) Pax, *A. mandshuricum* Maxim., *A. pseudosieboldianum* (Pax) Kom., *A. sinopurpurascens* W. C. Cheng, *A. tegmentosum* Maxim., and *A. ukurunduense* Trautv. & C. A. Mey.) (Appendix 1). The PCR was set up in 20-μL volumes, each containing 20–50 ng of template DNA, 0.4 μM of each primer, 0.2 mM of each dNTP, 1× PCR buffer (2.5 mM Mg^{2+}), and 1 unit of *Taq* polymerase (TaKaRa Biotechnology Co.). The PCR cycling parameters were as described above, but with annealing temperatures as given in Table 1. The amplified products were separated on a 6% polyacrylamide gel and visualized using silver staining. Overall, five of these primers were found to be monomorphic in *A. triflorum* (Table 2). The primers that successfully amplified for the majority of samples across the populations were used to test genetic diversity of the other congeneric species (Table 3).

Population genetic diversity analyses for these microsatellite loci were performed using GENEPOP (Raymond and Rousset, 1995) with the default settings and assumptions to determine the number of alleles per locus (A), observed heterozygosity (H_o), and expected heterozygosity (H_e). Additionally, departures from Hardy–Weinberg equilibrium (HWE) were tested using GenAlEx 6.5 (Peakall and Smouse, 2012).

In total for *A. triflorum*, A ranged from one to nine, and H_o and H_e levels varied from 0.000 to 1.000 and 0.000 to 0.826, respectively (Table 2). A few loci were found to significantly deviate from HWE: two in the FS population, four in the DD and BX populations, five in the DH population, and one in the TH population ($P < 0.001$; Table 2). However, the average A and H_e were low in the *A. ukurunduense* population compared with the populations of the other seven species (Tables 2 and 3). Selfing and inbreeding are likely to be major reasons for the reduction of genetic diversity (Lesica et al., 1988; Cole and Biesboer, 1992; Culley and Wolfe, 2001).

TABLE 1. Characteristics of 15 microsatellite loci developed in *Acer triflorum*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
ET206	F: GGCAATATGAGTTCAATG R: GGAAATCGCTAAATGTCTACAAAG	$(GT)_5$	172–196	50	MG554391
EA205	F: ATAACAGTTCACAGCACA R: AATATCACCTCACGTCTT	$(GT)_9$	194–208	50	MG554396
EA261	F: AGTAAACCATGAAAGGACACACAAG R: GAAAGTCTCCACCACAAT	$(GT)_6$	169–171	48	MG554401
ET513	F: CCTACGCAATGTGCTCTA R: CACGCTTCTGTATTCTTT	$(GT)_9$	240–336	52	MG554397
EA517	F: CAAATACGAAAACATACG R: TAGGACCTCATACCTCTTAC	$(GT)_6$	217–245	50	MG554394
EA560	F: TAAGAGCAAGAGCGAAAG R: ATCCAGGAGAAGAATAGG	$(AC)_6$	292–318	50	MG554389
ET660	F: AACCGTTTCAAGTTCTAG R: CTCACCCTTCCATATTCT	$(GT)_{10}$	171–193	50	MG554392
EA557	F: GCTCCCTCTGGTTCCAAT R: CTTCCATCAAAATCCTAACACTGCA	$(AC)_{11}$	140–168	54	MG554388
EA523	F: CCATTCTCACCCTTCCAT R: ATCCGTCAACCGTATCAAGTTCTAG	$(AC)_7$	168–192	54	MG554387
EA206	F: AGGAAATAAGGAAGCAGT R: GAGTAAAATCAGTTGGTGTCA	$(AC)_9$	130–169	54	MG554390
EA520*	F: GAAGAAAGTCTCCACCAC R: TGAGTAAACCATGAAAGG	$(CA)_6$	172	50	MG554393
EA555*	F: GAGTAAGAAGACGAAGAA R: TGAACCATGAAAGGACAC	$(CA)_7$	180	54	MG554395
ET514*	F: GAAGAAAGTCTCCACCAC R: TGAGTAAACCATGAAAGG	$(CA)_7$	174	50	MG554398
EA244*	F: GAAGAAAGTCTCCACCAC R: GCTCAAGTCCAAAAACACAAATACG	$(CA)_7$	335	48	MG554399
ET608*	F: TCCCATAGTTGAAGGTCC R: GGTCTTGAACAAGCCAAACATTGTG	$(CA)_4$	274	52	MG554400

Note: T_a = annealing temperature.
*Monomorphic loci.

TABLE 2. Genetic diversity in five *Acer triflorum* populations based on the 10 developed polymorphic microsatellite markers.[a]

	Fusong (n = 12)			Dandong (n = 19)			Dunhua (n = 20)			Benxi (n = 19)			Tonghua (n = 18)		
Locus	***A***	H_o	H_e[b]	***A***	H_o	H_e[b]	***A***	H_o	H_e[b]	***A***	H_o	H_e[b]	***A***	H_o	H_e[b]
ET206	3	0.167	0.236**	4	0.526	0.495	6	0.809	0.642**	4	0.118	0.321***	4	0.500	0.467
EA205	3	0.080	0.040**	3	0.579	0.576	3	0.600	0.594	2	0.235	0.428	4	0.333	0.544
EA261	1	0.000	0.000	2	0.000	0.193***	2	0.047	0.285***	1	0.000	0.000	1	0.000	0.000
ET513	3	1.000	0.650**	3	1.000	0.565***	4	0.952	0.696*	4	1.000	0.633*	4	1.000	0.637**
EA517	2	0.000	0.290***	2	0.474	0.508	2	0.476	0.502	2	0.000	0.478***	2	0.111	0.489**
EA560	4	1.000	0.681	3	1.000	0.649**	4	0.905	0.686***	5	0.789	0.653	3	0.944	0.595**
ET660	4	1.000	0.667***	6	1.000	0.821**	5	1.000	0.727***	6	0.947	0.826***	6	1.000	0.738
EA557	2	0.333	0.290	3	0.632	0.553	3	0.333	0.296	2	0.222	0.203	3	0.167	0.160
EA523	4	1.000	0.600	6	0.833	0.702***	9	0.714	0.784***	4	0.842	0.680	4	1.000	0.681
EA206	4	0.667	0.717	6	0.105	0.580***	7	0.550	0.756***	5	0.056	0.468***	7	0.278	0.681***
EA520	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
EA555	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ET514	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
EA244	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000
ET608	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.
[a]Locality and voucher information are provided in Appendix 1.
[b]Asterisks indicate significant deviation from Hardy–Weinberg equilibrium: *P < 0.05, **P < 0.01, ***P < 0.001.

TABLE 3. Genetic diversity in seven congeneric species based on the 15 microsatellite markers developed for *Acer triflorum*.[a]

	A. ginnala (n = 5)			*A. griseum* (n = 5)			*A. mandshuricum* (n = 5)			*A. pseudosieboldianum* (n = 5)			*A. sinopurpurascens* (n = 5)			*A. tegmentosum* (n = 5)			*A. ukurunduense* (n = 5)		
Locus	***A***	H_o	H_e[b]	***A***	H_o	H_e[b]	***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[b]
ET206	3	0.500	0.714	2	0.750	0.536	1	0.000	0.000	3	0.333	0.600	2	0.000	0.429	3	0.750	0.750	2	0.000	0.429
EA205	5	0.250	0.893**	4	0.500	0.643	3	0.500	0.464	4	0.500	0.750	1	0.000	0.000	2	0.250	0.250	4	0.500	0.786
EA261	2	0.000	0.429	1	0.000	0.000	1	0.000	0.000	—	—	—	3	0.250	0.464	2	1.000	0.571	2	0.500	0.429
ET513	—	—	—	1	0.000	0.000	3	1.000	0.679	3	0.333	0.600	—	—	—	—	—	—	3	0.500	0.607
EA517	3	0.250	0.464	1	0.000	0.000	2	0.250	0.250	2	0.500	0.429	2	0.250	0.250	—	—	—	—	—	—
EA560	3	0.250	0.679	2	1.000	0.571	5	1.000	0.893	5	1.000	0.857	1	0.000	0.000	2	1.000	0.571	3	0.000	0.714*
ET660	3	1.000	0.679	2	0.750	0.536	2	1.000	0.571	4	0.500	0.821	2	1.000	0.571	1	0	0	4	1.000	0.786
EA557	3	0.500	0.679	6	0.500	0.929*	1	0.000	0.000	4	0.750	0.821	5	0.750	0.857	—	—	—	4	0.250	0.750*
EA523	4	0.500	0.750	2	0.750	0.750	2	0.750	0.536	4	1.000	0.750	2	1.000	0.571	1	0	0	3	0.750	0.750
EA206	4	0.500	0.750	5	1.000	0.857	3	1.000	0.714	4	0.750	0.750	2	0.500	0.571	2	0.250	0.250	5	0.750	0.893
EA520	—	—	—	1	0.000	0.000	1	0.000	0.000	—	—	—	—	—	—	—	—	—	—	—	—
EA555	—	—	—	1	0.000	0.000	1	0.000	0.000	2	0.250	0.250	1	0.000	0.000	1	0.000	0.000	—	—	—
ET514	—	—	—	1	0.000	0.000	1	0.000	0.000	—	—	—	—	—	—	—	—	—	—	—	—
EA244	—	—	—	1	0.000	0.000	3	1.000	0.679	—	—	—	3	1.000	0.679	—	—	—	—	—	—
ET608	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000

Note: — = no product was observed; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.
[a]Locality and voucher information are provided in Appendix 1.
[b]Asterisks indicate significant deviation from Hardy–Weinberg equilibrium: *P < 0.05, **P < 0.01, ***P < 0.001.

CONCLUSIONS

In this research, 15 microsatellite markers were developed, which may be useful in studies of genetic diversity and spatial population genetic structure of *A. triflorum*. Furthermore, the results of genetic diversity studies may be used to formulate conservation strategies to prevent commercial exploitation of other *Acer* species.

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APPENDIX 1. Voucher information for *Acer* species used in this study.

Species	Collection locality[a]	Geographic coordinates	*N*	Voucher specimen accession no.[b]
Acer triflorum Kom.	Fusong, Jilin Province	42°33′14.04″N, 128°00′32.77″E	12	NENU20170704001
A. triflorum	Dandong, Liaoning Province	40°25′44.64″N, 124°05′32.71″E	19	NENU20170510001
A. triflorum	Dunhua, Jilin Province	43°33′51.07″N, 127°49′47.16″E	20	NENU20170613001
A. triflorum	Benxi, Liaoning Province	41°19′42.25″N, 124°53′52.06″E	19	NENU20170509001
A. triflorum	Tonghua, Jilin Province	41°07′52.68″N, 126°11′58.48″E	18	NENU20170621001
A. ginnala Maxim.	Wuchang, Heilongjiang Province	44°56′17.19″N, 127°10′26.70″E	5	NENU20110619001
A. griseum (Franch.) Pax	Luoyang, Henan Province	33°42′15.36″N, 111°44′14.61″E	5	NENU20170615001
A. mandshuricum Maxim.	Dunhua, Jilin Province	43°33′51.07″N, 127°49′47.16″E	5	NENU20170613002
A. pseudosieboldianum (Pax) Kom.	Wuchang, Heilongjiang Province	44°56′17.19″N, 127°10′26.702″E	5	NENU20110802001
A. sinopurpurascens W. C. Cheng	Linan, Zhejiang Province	30°19′04.94″N, 119°27′17.13″E	5	NENU20170428001
A. tegmentosum Maxim.	Tonghua, Jilin Province	41°7′52.68″N, 126°11′58.47″E	5	NENU20110611001
A. ukurunduense Trautv. & C. A. Mey.	Hailin, Heilongjiang Province	44°31′03.1″N,128°51′38.6″E	5	NENU20110708001

Note: N = number of individuals sampled.
[a]Collection sites are located in China.
[b]Specimens are deposited at Northeast Normal University (NENU), Changchun, Jilin, China.