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PRIMER NOTE

DEVELOPMENT OF EST-SSR MARKERS FOR THE INVASIVE PLANT *TITHONIA DIVERSIFOLIA* (ASTERACEAE)[1]

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- *Premise of the study: Tithonia diversifolia* (Asteraceae) is an invasive plant species that can outcompete natives and thus poses a great threat to biodiversity in introduced areas. Here, expressed sequence tag–simple sequence repeat (EST-SSR) markers were developed and characterized.
- *Methods and Results:* Sixteen polymorphic microsatellite loci were isolated from *T. diversifolia* using transcriptome sequencing and bioinformatic screening. The number of alleles per locus varied from two to four alleles in 48 individuals from three populations. Most of these primers also amplified in *T. rotundifolia* and some even in *Parthenium hysterophorus*.
- *Conclusions:* These markers are useful for investigating the genetic structure and evolutionary process of *T. diversifolia*, which may provide important information for better management.

Key words: Asteraceae; EST-SSR; invasive species; *Tithonia diversifolia*; transcriptome sequencing.

Tithonia diversifolia (Hemsl.) A. Gray (Asteraceae), commonly known as Mexican sunflower, is a perennial herb or shrub. It is native to Mexico and Central America and has been introduced into many countries in Asia, Africa, and the Pacific islands (CABI, 2016). In China, *T. diversifolia* was first recorded in southern Yunnan Province in 1936; it is now found in 53 counties of Yunnan and is expanding rapidly in southern China (Wang et al., 2004). *Tithonia diversifolia* was originally cultivated as an ornamental plant or green manure in villages and farms, but subsequently escaped and invaded into diverse habitats. As a pioneering species, *T. diversifolia* can produce numerous seeds and form dense stands, which suppress the growth of native species significantly and pose a great threat to biodiversity (CABI, 2016). Intersimple sequence repeat (ISSR) markers have been developed to assess genetic diversity in *T. diversifolia* (Yang et al., 2012), but dominant markers like ISSRs are less powerful in genetic and evolutionary studies. Here, expressed sequence tag–simple sequence repeat (EST-SSR) markers were isolated and characterized. These markers will be useful for genetic and evolutionary studies, providing important information (e.g., mating system, invasion routes) for better management of *T. diversifolia*.

METHODS AND RESULTS

Total RNA was extracted from a seedling collected from Yuxi (24.5246°N, 102.1235°E; herbarium ID YNU-YX106 at Yunnan University) using the Agilent Plant RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, California, USA). Sequencing by synthesis of the normalized cDNA library was performed with HiSeq 2000 (Illumina, San Diego, California, USA; sequencing performed by BGI, Shenzhen, China), which produced 48,619,098 clean reads. CLC Genomics Workbench 7.5.1 (CLC bio, Aarhus, Denmark) was used to run de novo assembly, resulting in 113,774 unigenes with an N50 length of 1289 bp. SSR detection was performed with MISA (Thiel, 2003) using unigenes as reference. The following search criteria were implemented: ≥6 repeat units for dinucleotides and ≥5 repeat units for tri-, tetra-, penta-, and hexanucleotides. We used the QDD version 3.1 pipeline (Meglécz et al., 2014) to remove redundant sequences and design primers for 5907 sequences with PCR product length longer than 80 bp. The default parameters were used.

We first randomly selected 130 primer pairs amplifying SSRs containing dinucleotide or trinucleotide motifs. The primers were tested in eight individuals of *T. diversifolia* collected from Yuxi (herbarium ID YNU-YX106), Lincang (herbarium ID YNU-LC011), and Jingxi (herbarium ID YNU-JX004) as preparatory screening. Primers that produced reproducible and clearly defined bands were further tested for polymorphism in three populations (48 individuals in total) from southern China (Appendix 1). PCR was conducted with a final volume of 20 µL containing 1 µL of template DNA (0.15 µg/µL), 2 µL 10× PCR buffer (Mg^{2+} plus), 0.4 µL $MgCl_2$, 0.4 µL dNTPs (2.5 mM), 0.2 µL of each primer (50 µM), and 1 unit *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China). The PCR program for amplification of all loci consisted of an initial denaturation at 94°C for 4 min, followed by 27 cycles of denaturation at 94°C for 45 s, annealing at specific temperature for 45 s (Table 1), extension at 72°C for 45 s, and a final extension at 72°C for 10 min. Amplification products were checked on 6% denaturing polyacrylamide gels using a pUC19 marker as a reference and were visualized by silver staining.

In total, 16 primer pairs successfully amplified products with expected sizes and showed clearly defined polymorphic banding patterns. The primer sequences, repeat motifs, allele ranges, and annealing temperatures are shown in Table 1. The number of alleles per locus (*A*) and the observed and expected heterozygosities (H_o and H_e) were calculated across the three populations using GenAlEx 6.5 (Peakall and Smouse, 2012). The number of alleles per locus varied from two to four alleles. One locus (Cl42) was monomorphic in two populations, but polymorphic in another population. At the population level, H_o and H_e ranged from 0.000 to 0.824 and from 0.000 to 0.643, respectively (Table 2). For 12 of the 16 loci, significant departures from Hardy–Weinberg equilibrium were detected in population LC or YX. This may result from nonrandom mating in the expanding populations. However, this pattern was not found in population GX, which may be due to the relatively

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Table 1. Characteristics of 16 polymorphic microsatellite markers in *Tithonia diversifolia.*

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank accession no.
Cl3	F: TGATTCCCCATCATCGAATAATA	$(TAA)_6$	58	166–202	KT862493
	R: TCCTATCTTCTCTCCGTTTCCAT				
Cl12	F: AATCACTTCACCATGAGGATGAC	$(CCA)_6$	58	207–216	KT862494
	R: GACAGGAAGGGTTCAAAATCCTA				
Cl23	F: AATAGGCTTTTCACCTTTTCCTC	$(TAT)_7$	59	159–162	KT862495
	R: TTGATTGGTAGTTGAAAACTTGC				
Cl28	F: CACACACTATAACCACAAACTCGAT	$(AG)_{10}$	60	220–244	KT862496
	R: ACTCCACCACACCATAAGATGAA				
Cl42	F: TTCTTTCACAATCGTTCATTTCA	$(TTA)_6$	60	227–230	KT862497
	R: GATCACCTGCTAAAATCACGAAC				
Cl52	F: TGGTTCTAGTCTTAACACGTGGG	$(AAT)_6$	60	214–220	KT862498
	R: ACAACTCCCCTGTATCCAAAAAT				
Cl53	F: CAAATACCATCCATCATCTCCAT	$(TCA)_7$	60	218–233	KT862499
	R: ATGATAATGATGAGCGTGACGA				
Cl76	F: GCTCCAGTTTCACCTAGAAAGAA	$(GAT)_6$	60	212–245	KT862500
	R: TCACACAATATTTCTAAAACTACATCAA				
Cl84	F: AACCGTTGTTTGATTACACTCGT	$(GAT)_6$	60	140–155	KT862501
	R: AGAAGGTTTCTTGAACTTGGAGG				
Cl92	F: TGGATCACCGTTTTCTTCTTAAA	$(AGC)_6$	60	103–112	KT862502
	R: ACCACCTATTCCAACATCTTCCT				
Cl95	F: TCAAAGTACACATCACTACCCCA	$(AT)_{10}$	60	160–172	KT862503
	R: AATAAGAAGAAGAAATGGCGGG				
Un1	F: TTTATTGAACCTGGTCGTTGAAG	$(ATC)_6$	60	172–181	KT862504
	R: AATATGACTAGGGTTCCGCCATA				
Un5	F: AGATGGAACAACCGAGTGTATTG	$(GTT)_7$	60	161–170	KT862505
	R: CACCACTCACCACTTCATAAACC				
Un6	F: TAATGGGCTCAGTAACACCTCTG	$(AGA)_6$	60	116–122	KT862506
	R: ATCACGATCGCAAACAGAAAC				
Un21	F: ATTAAGCTAGTTGCCGGAAAAAC	$(TTA)_6$	59	194–200	KT862507
	R: AAAAGTCGAGATTAGATCCCTCAG				
Un23	F: TCTTGGAACATGGAGATTCAACT	$(TCA)_6$	58	130–139	KT862508
	R: GAAGAGTGCACGAGTTCAGTAGG				

Note: T_a = annealing temperature.

small sample size (n = 10) or other unknown reasons. To evaluate the potential utility of the newly developed markers in other phylogenetically related species, cross-amplification experiments were performed in six individuals each of *T. rotundifolia* (Mill.) S. F. Blake and *Parthenium hysterophorus* L. The results are summarized in Table 3. Among the 16 markers from *T. diversifolia*, 12 could be cross-amplified in the congeneric *T. rotundifolia* and five could be cross-amplified in the more distantly related *P. hysterophorus*, another invasive species in tribe Heliantheae Cass.

CONCLUSIONS

We characterized 16 polymorphic EST-SSR markers specifically for *T. diversifolia* and demonstrated their utility. These markers are useful for investigating the genetic population structure, mating system, and invasion routes in this highly invasive plant, which may contribute to better management.

Table 2. Results of initial polymorphic microsatellite marker screening in three populations of *Tithonia diversifolia*.

	LC (N = 21)				YX (N = 17)				GX (N = 10)			
Locus	A	H_o	H_e	HWE[a]	A	H_o	H_e	HWE[a]	A	H_o	H_e	HWE[a]
Cl3	3	0.286	0.643	***	4	0.529	0.555	***	2	0.600	0.480	ns
Cl12	2	0.350	0.489	ns	2	0.294	0.438	ns	2	0.300	0.255	ns
Cl23	2	0.286	0.444	ns	2	0.353	0.457	ns	2	0.500	0.375	ns
Cl28	2	0.333	0.459	ns	3	0.412	0.552	***	2	0.200	0.480	ns
Cl42	2	0.095	0.363	***	1	0.000	0.000	—	1	0.000	0.000	—
Cl52	2	0.143	0.459	**	2	0.294	0.500	ns	2	0.500	0.495	ns
Cl53	3	0.143	0.541	***	2	0.235	0.498	*	2	0.400	0.500	ns
Cl76	2	0.381	0.499	ns	3	0.235	0.443	***	2	0.400	0.480	ns
Cl84	2	0.286	0.444	ns	2	0.353	0.498	ns	2	0.500	0.455	ns
Cl92	3	0.190	0.635	***	2	0.059	0.251	**	2	0.400	0.500	ns
Cl95	2	0.143	0.459	**	2	0.059	0.493	***	2	0.300	0.495	ns
Un1	2	0.667	0.444	*	2	0.824	0.484	**	2	0.700	0.455	ns
Un5	2	0.190	0.499	**	2	0.235	0.415	ns	2	0.300	0.495	ns
Un6	2	0.048	0.500	***	2	0.438	0.482	ns	2	0.600	0.500	ns
Un21	2	0.286	0.472	ns	2	0.235	0.484	*	2	0.200	0.320	ns
Un23	2	0.476	0.472	ns	2	0.706	0.498	ns	2	0.700	0.495	ns

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = sample size.
[a]Deviations from HWE at *P < 0.05, **P < 0.01, ***P < 0.001; ns = not significant.

TABLE 3. Cross-amplification length (in base pairs) of 16 microsatellite loci from *Tithonia diversifolia* in two related species of tribe Heliantheae.

Locus	*Tithonia rotundifolia* ($n = 6$)	*Parthenium hysterophorus* ($n = 6$)
Cl3	—	—
Cl12	201–207	204
Cl23	—	—
Cl28	216	—
Cl42	218–230	—
Cl52	208–220	202
Cl53	205	—
Cl76	—	—
Cl84	140	170
Cl92	103	—
Cl95	174–190	—
Un1	169	—
Un5	173	167
Un6	137	—
Un21	197	—
Un23	—	172–175

Note: — = failed amplification; n = sample size.

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APPENDIX 1. Voucher and location information for *Tithonia diversifolia*, *T. rotundifolia*, and *Parthenium hysterophorus* individuals used in this study. One voucher was collected for each population and all vouchers were deposited in Yunnan University, Kunming, China.

Plant materials	Population	Collection date	Locality (China)	Geographic coordinates	Herbarium ID
Tithonia diversifolia	LC	18 December 2014	Lincang, Yunnan	23.6027°N, 99.3761°E	YNU-LC011
Tithonia diversifolia	YX	5 November 2014	Yuxi, Yunnan	24.5246°N, 102.1235°E	YNU-YX106
Tithonia diversifolia	GX	26 July 2015	Jingxi, Guangxi	23.1380°N, 106.4055°E	YNU-JX004
Parthenium hysterophorus	—	8 August 2015	Honghe, Yunnan	23.3021°N, 103.4102°E	YNU-HH089
Tithonia rotundifolia	—	10 December 2015	Kunming, Yunnan	Cultivated in garden	YNU-TR001