**Development of EST-SSR Markers for the Invasive Plant Tithonia diversifolia (Asteraceae)**

Authors: Landi Luo, Pin Zhang, Xiaokun Ou, and Yupeng Geng
Source: Applications in Plant Sciences, 4(7)
Published By: Botanical Society of America
URL: https://doi.org/10.3732/apps.1600011
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 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.

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: ≥ 2 repeat units for dinucleotides and ≥ 2 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 5097 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 (Mg2+ plus), 0.4 μL MgCl2, 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 temperatures 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 (Ho and He) 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 anther population. At the population level, Hs ranged from 0.000 to 0.824 and Ho ranged from 0.000 to 0.645, 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
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 \(P. \) 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 \(Cass.\)

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

| Locus | Primer sequences \((5'-3')\) | Repeat motif | \(T_a\) \(^\circ\)C | Allele size range (bp) | GenBank accession no. |
|-------|-----------------------------|--------------|----------------|-----------------------|----------------------|
| Cl3   | F: \(\text{TATTCCCATCATGAAAAATA} \) | \(\text{(TAA)}_6\) | 58 | 166–202 | KT862493 |
|       | R: \(\text{GAGTGGGCTCAGACACCTGTT} \) | \(\text{(CA)}_8\) | 58 | 207–216 | KT862494 |
| Cl23  | F: \(\text{CACACTTTCTCAGCTTTT} \) | \(\text{(CAT)}_7\) | 59 | 190–196 | KT862495 |
|       | R: \(\text{TTGATTTGTTGGAATAACCC} \) | \(\text{(TA)\_7}\) | 59 | 190–196 | KT862496 |
| Cl42  | F: \(\text{TTCTTCCATCATTTTACCT} \) | \(\text{(CA)}_8\) | 60 | 214–223 | KT862497 |
|       | R: \(\text{TTCAGTTGTTGGAATAACCC} \) | \(\text{(TA)\_7}\) | 60 | 214–223 | KT862498 |

**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.*

| 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   | 0.268 | 0.444 | ns    | 2   | 0.353 | 0.457 | ns    | 2   | 0.500 | 0.375 | ns |
| Cl28  | 2   | 0.333 | 0.549 | ns    | 3   | 0.412 | 0.552 | ns    | 2   | 0.200 | 0.460 | 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  | 2   | 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   | 0.268 | 0.444 | ns    | 2   | 0.353 | 0.498 | ns    | 2   | 0.500 | 0.455 | ns |
| Cl92  | 2   | 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   | 0.353 | 0.484 | *     | 2   | 0.200 | 0.320 | ns |
| Un23  | 2   | 0.476 | 0.472 | *     | 2   | 0.706 | 0.498 | ns    | 2   | 0.700 | 0.495 | ns |

\(^a\) Deviations from HWE at \(*P < 0.05, **P < 0.01, ***P < 0.001\); ns = not significant.

Note: \(A\) = number of alleles; \(H_o\) = expected heterozygosity; \(H_e\) = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; \(N\) = sample size.

http://www.bioone.org/loi/apps
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) |
|-------|---------------------------------|-----------------------------------|
| CI3   | —                               | —                                 |
| CI12  | 201–207                         | 204                               |
| CI23  | —                               | —                                 |
| CI28  | 216                             | —                                 |
| CI42  | 218–230                         | —                                 |
| CI52  | 208–220                         | 202                               |
| CI53  | 205                             | —                                 |
| CI76  | 140                             | 170                               |
| CI92  | 103                             | —                                 |
| CI95  | 174–190                         | —                                 |
| Un1   | 169                             | —                                 |
| Un5   | 173                             | 167                               |
| Un6   | 137                             | —                                 |
| Un21  | 197                             | —                                 |
| Un43  | —                               | 172–175                           |

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

**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, 106.4053°E  | YNU-JX004    |
| *Parthenium hysterophorus* | —          | 8 August 2015    | Honghe, Yunnan    | 25.3021°N, 103.4102°E  | YNU-HH089    |
| *Tithonia rotundifolia* | —          | 10 December 2015 | Kunming, Yunnan   | Cultivated in garden    | YNU-TR001    |

**LITERATURE CITED**

CABI. 2016. Invasive Species Compendium. CAB International, Wallingford, United Kingdom. Website http://www.cabi.org/isc/datasheet/54020 [accessed 17 June 2016].

Mieglatz, E., N. Pech, A. Gilles, V. Dubut, P. Hingamp, A. Trilles, R. Grenier, and J.-F. Martin. 2014. QDD version 3.1: A user-friendly computer program for microsatellite selection and primer design revisited: Experimental validation of variables determining genotyping success rate. *Molecular Ecology Resources* 14: 1302–1313.

Peakall, R., and P. E. Smouse. 2012. GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—an update. *Bioinformatics* (Oxford, England) 28: 2537–2539.

Thiel, T. 2003. MISA—Microsatellite identification tool. Website http://pggr.ipk-gatersleben.de/misa/ [accessed 17 June 2016].

Wang, S., W. Sun, and X. Cheng. 2004. Attributes of plant proliferation, geographic spread and the natural communities invaded by the naturalized alien plant species *Tithonia diversifolia* in Yunnan, China. *Acta Ecologica Sinica* 24: 444–449.

Yang, J., L. Tang, Y. L. Guan, and W. B. Sun. 2012. Genetic diversity of an alien invasive plant Mexican sunflower (*Tithonia diversifolia*) in China. *Weed Science* 60: 552–557.