Development of expressed sequence tag-simple sequence repeat markers for genetic characterization and population structure analysis of *Praxelis clematidea* (Asteraceae)

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ABSTRACT. Invasive plants tend to spread aggressively in new habitats and an understanding of their genetic diversity and population structure is useful for their management. In this study, expressed sequence tag-simple sequence repeat (EST-SSR) markers were developed for the invasive plant species *Praxelis clematidea* (Asteraceae) from 5548 *Stevia rebaudiana* (Asteraceae) expressed sequence tags (ESTs). A total of 133 microsatellite-containing ESTs (2.4%) were identified, of which 56 (42.1%) were hexanucleotide repeat motifs and 50 (37.6%) were trinucleotide repeat motifs. Of the 24 primer pairs designed from these 133 ESTs, 7 (29.2%) resulted in significant polymorphisms. The number of alleles per locus ranged from 5 to 9. The relatively high genetic diversity ($H=0.2667$, $I=0.4212$, and $P=100\%$) of *P. clematidea* was related to high gene flow ($N_m = 1.4996$) among populations. The coefficient of population differentiation ($G_{ST} = 0.2500$) indicated

that most genetic variation occurred within populations. A Mantel test suggested that there was significant correlation between genetic distance and geographical distribution ($r = 0.3192$, $P = 0.012$). These results further support the transferability of EST-SSR markers between closely related genera of the same family.

Key words: *Praxelis clematidea*; Invasive plants; EST-SSR; Transferability; Genetic variation

INTRODUCTION

Praxelis clematidea (Griseb.) R.M. King & H. Rob. is an annual to short-lived perennial herb of the family Asteraceae (tribe Eupatorieae). Its stem is pubescent and its leaves emit an odor similar to cat urine when crushed. Its fruit bears a bristly pappus that facilitates wind and animal dispersal. Native to South America (Northern Argentina, Southern Brazil, Bolivia, Paraguay, and Peru; United States Department of Agriculture, January 19, 2016), the plant is now a noxious weed that has become naturalized in Florida, Australia, and southern China (Waterhouse, 2003; ALA, 2014; Gardner and Williges, 2015). Reports indicate that the plants are not eaten by livestock and that they are allelopathic (Pollock et al., 2004; Wang et al., 2006a; Chen et al., 2011). The presence of this species in invaded regions is a threat to agriculture and to the local flora (Qiu et al., 2011; ALA, 2014; United States Department of Agriculture, 2016).

The genetic diversity of a species is reflected in its ability to adapt to new habitats, since the higher the genetic diversity of an alien weed, the stronger its ability to adapt to the invaded environment (Müller-Schärer and Fischer, 2001). Therefore, studies on the genetic diversity of invasive plants are useful for understanding their potential for establishment, geographic patterns of invasion, and evolutionary responses to novel environments, as well as for directing management practices (Sakai et al., 2001).

Several types of molecular markers have been used to analyze the genetic variation of invasive species, including inter-simple sequence repeats (ISSRs; Chapman et al., 2004; Li et al., 2014; Wang et al., 2007, 2015) and random amplified polymorphic DNA (RAPD; Li et al., 2006; Li et al., 2011). Expressed sequence tag-simple sequence repeats (EST-SSRs), which involve ESTs and SSR-enriched genomic libraries, have also become an important resource for population genetic studies (Ellis and Burke, 2007; Yan et al., 2011). EST-SSRs are relatively inexpensive to develop (Varshney et al., 2005a) and in general, EST-SSRs have significantly more transferability across related species than do traditional genomic SSR markers, especially in plants (Decroocq et al., 2003; Gasic et al., 2009). Such transferability has previously been demonstrated for economically important crops (Decroocq et al., 2003; Thiel et al., 2003; Varshney et al., 2005b). An understanding of EST-SSR transferability is especially important when studying species for which the availability of ESTs is limited (Ellis and Burke, 2007).

In the present study, we developed *P. clematidea* EST-SSR markers by first testing the transferability of EST-SSRs from the closely related *Stevia rebaudiana* (Bertoni) Bertoni (Asteraceae, tribe Eupatorieae; Brandle et al., 2002). We then used these EST-SSRs to obtain valuable information on the genetic diversity and population structure of the highly invasive species *P. clematidea*.

MATERIAL AND METHODS

Plant materials and DNA extraction

A total of 107 *P. clematidea* samples from 14 natural populations were collected from three provinces in southern China (Table 1). All samples were identified by Dr. Qi-Zhi Wang and vouchers were deposited in the Huaqiao University Herbarium. Total genomic DNA was extracted from fresh leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987).

Table 1. Location, elevation, and populations of *Praxelis clematidea* sampled.

Province	City	Code	No. of samples	North latitude (°)	East longitude (°)	Elevation (m)	Voucher No.
Fujian	Xiamen	FXM	11	24.6076	118.0828	85	00003689
	Quanzhou	FQZ	19	26.0378	119.1848	187	00003690
	Jinjiang	FJJ	5	24.8091	118.5534	40	00003691
	Fuding	FFD	5	27.2838	120.2178	8	00003692
	Fuzhou	FFZ	4	24.9226	118.6294	10	00003693
Guangdong	Zhuhai	GZH	8	22.3690	113.5401	12	00003694
	Heyuan	GHY	12	23.7570	114.6992	121	00003695
	Chaozhou	GCZ	4	23.3917	116.3923	25	00003701
	Zhanjiang	GZJ	9	21.0848	110.1756	13	00003702
Hainan	Wanning	HWN	5	18.8231	110.3974	10	00003696
	Wenchang	HWC	10	19.5908	110.7888	7	00003697
	Haikou	HHK	4	19.9846	110.3262	30	00003698
	Danzhou	HDZ	5	19.5062	109.4959	138	00003699
	Qiongzhou	HQZ	6	19.0281	109.8470	220	00003700

Development of EST-SSRs

A total of 5548 ESTs from *S. rebaudiana* (Brandle et al., 2002) were downloaded from the dbEST database (<http://www.ncbi.nlm.nih.gov/dbEST>). SSRs within these ESTs were identified using the Simple Sequence Repeat Identification Tool (SSRIT; <http://www.gramene.org/db/markers/ssrtool>). The criteria used to identify SSRs were as follows: perfect di-, tri-, tetra-, penta-, and hexanucleotide motifs, with a minimum of eight, six, five, four, and three repeats, respectively. Using Premier 5.0 software (Gadberry et al., 2005), primers were designed using the following standards: 1) melting temperature (T_m) between 50 and 60°C; 2) primer length of 18 to 24 bp, with 20 bp being the optimum; 3) product size of 100 to 500 bp; and 4) a GC content between 40 and 60%. The primers were synthesized by Sangon Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Polymerase chain reaction (PCR) amplification

Each PCR assay was carried out in a total volume of 20 μ L, consisting of 2 μ L 10X PCR buffer, 2.0 mM Mg^{2+} , 0.2 mM dNTPs, 0.2 μ M each primer, 1.5 U *Taq* DNA polymerase (Takara, Dalian, China), and 30 ng template DNA. PCR amplification was performed as follows: an initial cycle at 94°C for 5 min, followed by 40 cycles of 94°C for 50 s, 53°C for 40 s, and 72°C for 70 s, and a final extension at 72°C for 5 min. Amplification products were separated by electrophoresis on 8% polyacrylamide gels. DNA fragments were documented with a digital camera immediately after silver staining (Bassam et al., 1991).

Data analysis

DNASTAR (Burland, 2000) was used to identify coding regions within the ESTs. BLASTX searches against the non-redundant protein database (NCBI, Bethesda, MD, USA) were used to assess putative gene function. Bands were scored based on their reproducibility and consistency; smeared and weak bands were ignored. Each sample was genotyped based on the presence (1) or absence (0) of a band. Genetic statistics were obtained using POPGENE 1.32 software (Yeh et al., 1997), which included unbiased measures of genetic identity (Nei, 1978) and genetic distance, percentage of polymorphic bands (P), number of alleles per locus (N_A), effective number of alleles (N_E), Nei's gene diversity (H ; Nei, 1973), Shannon's information index (I), total gene diversity (H_t), gene diversity within populations (H_s), and the coefficient of population differentiation ($G_{ST} = 1 - H_s/H_t$; Slatkin and Barton, 1989). The genetic variation of *P. clematidea* was further investigated by analysis of molecular variance (AMOVA) using Arlequin 3.0 software (Excoffier et al., 2005).

A matrix of genetic distance was generated and was used to construct an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram using the SHAN-clustering and TREE building programs in NTSYS-pc 2.10 (Rohlf, 1992). Significant correlations between genetic distance and geographic distance (km) were determined using a Mantel test in TFPGA 1.3 (http://www.marksgeneticssoftware.net/_vti_bin/shhtml.exe/tfpga.htm). Polymorphism information content (PIC) was calculated using a standard, published formula (Botstein et al., 1980).

RESULTS

Frequency and location of EST-SSRs

In total, 133 microsatellite-containing sequences were detected among the 5548 ESTs examined (2.4%). Characterization of SSRs in these ESTs indicated that hexanucleotide (56) and trinucleotide (50) repeat motifs were the most common (at 42.1 and 37.6%, respectively). Tetranucleotide (1) and pentanucleotide (4) repeats were detected at much lower frequencies (at 0.8 and 3.0%, respectively). Maximum repetitions of di-, tri-, tetra-, penta- and hexanucleotide repeat motifs were 57 (AG), 10 (ACC), 5 (TGTT), 6 (TTTAA), and 5 (CAAACC), respectively. DNASTAR revealed that 62 SSRs (46.6%) were located in untranslated regions (UTRs) and 71 SSRs (53.4%) were located in coding regions.

EST-SSR marker polymorphisms

From the 133 ESTs containing SSRs, only 24 primer pairs could be designed based on the criteria outlined above. Of these, seven (29.2%) resulted in significant polymorphisms among the *P. clematidea* samples (Table 2). These seven polymorphic EST-SSRs detected 47 alleles in 107 samples (mean = 6.7 alleles; range = 5 [srSSR716] to 9 [srSSR708] alleles). PIC values ranged from 0.7014 (srSSR716) to 0.7950 (srSSR704), with a mean of 0.7616. The two most polymorphic markers were srSSR704 and srSSR711, with PIC values of 0.7950 and 0.7895, respectively. With the exception of srSSR704, all markers were hexanucleotide or trinucleotide repeats. BLASTX searches revealed that EST accessions BG524316.1, BG524316.1, and BG523972.1 were hypothetical proteins and accessions BG521417.1 and BG526825.1 were unknown genes.

Table 2. Characteristics of the seven EST-SSRs markers used in this study.

EST accession	Primer	Motif (No. of repeats)	Primer sequence (5'→3')	Expected size (bp)	No. of alleles	PIC*
BG521417.1	srSSR704	AT ₍₁₃₎	F: CGTGATCCAACAAGCACCCA R: TGCCGCCGAAACAAGAAC	162	6	0.7950
BG526825.1	srSSR708	CAT ₍₆₎	F: ATCACTTACTGCGGGTCTTG R: ACGCCTTCTTTCTTCATTT	146	9	0.7449
BG526825.1	srSSR709	CAT ₍₆₎	F: ATCACTTACTGCGGGTCTTG R: AAACGCCCTTCTTTCTTCAT	148	7	0.7636
BG523972.1	srSSR711	TAA ₍₆₎	F: GAACCCGACCCGAGTATA R: TGAGGATGAAGACGATGC	120	6	0.7895
BG524316.1	srSSR716	AGATAC ₍₃₎	F: GAAAGCACCTTCGGTCAGC R: GATTTCGTCTCCACAACCTCCTC	135	5	0.7014
BG524316.1	srSSR717	AGATAC ₍₃₎	F: AAAGCACCTTCGGTCAGC R: GATTTCGTCTCCACAACCTCCTC	134	7	0.7805
BG526023.1	srSSR718	CAGTAA ₍₃₎	F: CGATGCTGTGATTTTCT R: CTTCATTCTTGGCTCA	197	7	0.7560
Mean					6.7	0.7616

*PIC = polymorphism information content.

Genetic diversity of *P. clematidea*

Nei's gene diversity (H) ranged from 0.2127 (Fujian) to 0.2881 (Hainan), Shannon's information index (I) ranged from 0.3347 (Fujian) to 0.4410 (Hainan), and the percentage of polymorphic bands (P) within the different groups ranged from 82.98% (Guangdong) to 91.49% (Hainan; Table 3). The Hainan group had the highest genetic diversity ($H = 0.2881$, $I = 0.4410$, and $P = 91.49\%$). At the species level, the genetic diversity parameters H , I , and P were 0.2667, 0.4212, and 100%, respectively (Table 3).

Coefficients of population differentiation (G_{ST}) within the Hainan, Guangdong, and Fujian groups were 0.2776, 0.2126, and 0.2446, respectively (Table 3). At the species level, G_{ST} was 0.2500, suggesting that 75% of the variation was distributed within populations and 25% was partitioned among populations. Gene flow (N_m) values were all >1 (Table 3), indicating frequent gene exchange among populations, both within groups and at the species level. The AMOVA results ($F_{ST} = 0.3100$) indicated that 69% of the genetic variation was partitioned within populations and 31% of the variation was partitioned among populations (Table 4).

Table 3. Genetic information for *Praxelis clematidea*.

		N_A	N_E	H	I	P	N_m	G_{ST}	Ht	Hs
Within groups	Hainan	1.9149	1.4731	0.2881	0.4410	91.49%	1.3010	0.2776	0.2838	0.2050
	Guangdong	1.8298	1.3789	0.2292	0.3548	82.98%	1.8518	0.2126	0.2195	0.1728
	Fujian	1.8936	1.3471	0.2127	0.3347	89.36%	1.5443	0.2446	0.2284	0.1725
Species level		2.0000	1.4219	0.2667	0.4212	100.00%	1.4996	0.2500	0.2787	0.2457

Number of alleles per locus (N_A), effective number of alleles (N_E), Nei's gene diversity (H), Shannon's information index (I), percentage of polymorphic bands (P), gene flow values (N_m), the coefficient of population differentiation (G_{ST}), total gene diversity (Ht), gene diversity within populations (Hs).

Genetic distance and genetic identity among populations

The genetic distance among populations ranged from 0.0203 (between GZH and GCZ) to 0.3276 (between HDZ and FQZ), with an average of 0.1141 (Table 5). The genetic identity among populations ranged from 0.7207 (between HDZ and FQZ) to 0.9800 (between

GCZ and GZH), with an average of 0.8946 (Table 5). The Mantel test indicated that there was a significant correlation between genetic distance and geographic distance ($r = 0.3192$, $P = 0.012$).

Table 4. Results of analysis of molecular variance (AMOVA).

Source of variation	d.f.	Sum of squares (SSD)	Variance components	Percentage of variation (P)	P value ^a	F-statistic
Among groups	2	100.3920	0.8776	11.39	$P < 0.005$	$F_{CT} = 0.1139$
Among populations within groups	11	177.1460	1.5106	19.61	$P < 0.001$	$F_{SC} = 0.2213$
Within populations	93	494.2750	5.3148	69.00	$P < 0.001$	$F_{ST} = 0.3100$
Total	106	771.8130	7.7030			

^aSignificance tests with 5040 permutations were carried out. F_{CT} = genetic variation among groups; F_{SC} = genetic variation among populations within group; F_{ST} = genetic variation among populations.

DISCUSSION

Characterization and transferability of EST-SSR markers for *P. clematidea*

The number of SSRs is known to be dependent upon SSR search criteria, the database-mining tools used, the size of the dataset, and the study species (Varshney et al., 2005a). The results presented herein show that hexanucleotide repeats are the most frequent. This finding, however, is not consistent with previous reports, which have suggested that trinucleotide repeats are the most common motif occurring in plants (Varshney et al., 2005a; Eujayl et al., 2004). With the exception of srSSR704, the other six markers were designed against expressed genes with tri- and hexanucleotide repeats, which will not disrupt the triplet code of coding sequences. The amplification of seven *S. rebaudiana* EST-SSRs (29.2%) in *P. clematidea*, both members of Asteraceae tribe Eupatorieae (Noyes, 2007), with PIC values ranging from 0.7014 to 0.7950 and an average of 0.7616 (Table 2), revealed polymorphism and good transferability between genera.

Genetic variation analysis

Compared with other noxious invasive species of the Asteraceae, such as *Mikania micrantha* Kunth ($I = 0.2518$, $H = 0.2404$, and $P = 85.65\%$; Wang et al., 2007) and *Chromolaena odorata* (L.) R.M. King & H. Rob. ($H = 0.0406$, $I = 0.0623$, and $P = 12.31\%$; Ye et al., 2004), *P. clematidea* has relatively higher genetic diversity at the species level. This result is consistent with data obtained using ISSR markers ($H = 0.2791$, $I = 0.4226$, and $P = 100.00\%$; Wang et al., 2015) and reflects a greater potential to adapt to an invaded environment.

Most genetic variation in *P. clematidea* exists within populations, which is consistent with results of previous studies using nuclear ribosomal DNA internal transcribed spacer (nrDNA ITS) sequences and ISSR data (Wang et al., 2013, 2015). *Praxelis clematidea* can reproduce both sexually (producing multiple generations in one year) and asexually, whereby new roots and stems sprout from plants that have fallen over (Powell, 1985; Wang et al., 2006b; Li et al., 2011; United States Department of Agriculture, 2016). Its bristly achenes with pappus can disperse long distances by wind, as well as by attaching themselves to animals, feathers, clothing, and machinery; hydro seeding can also spread the plants widely (United States Department of Agriculture, 2016). As a consequence, gene exchange can occur over large distances, as reflected by high gene flow ($N_m = 1.4996$) among populations and relatively high genetic diversity (Li et al., 2014).

Table 5. Genetic distance and genetic identity between populations of *Praxelis clematidea*.

Population	HHK	HDZ	HWN	HWC	HQZ	GZH	GZJ	GHY	GCZ	FQZ	FJJ	FFZ	FFD	FXM
HHK		0.9097	0.9675	0.9100	0.8759	0.8721	0.8304	0.8374	0.8548	0.8119	0.7804	0.7925	0.8732	0.8410
HDZ	0.0946		0.9077	0.8420	0.8160	0.8162	0.7475	0.7280	0.7730	0.7207	0.7245	0.7800	0.7953	0.7844
HWN	0.0331	0.0969		0.9692	0.9132	0.9402	0.9065	0.8354	0.9069	0.8633	0.8547	0.8811	0.9262	0.9136
HWC	0.0943	0.1720	0.0313		0.8932	0.9508	0.9107	0.8847	0.9475	0.9131	0.8602	0.8931	0.9597	0.9382
HQZ	0.1325	0.2034	0.0908	0.1192		0.9532	0.8801	0.8299	0.9015	0.8731	0.8741	0.8782	0.8837	0.8859
GZH	0.1368	0.2031	0.0616	0.0504	0.0479		0.9635	0.9146	0.9800	0.9519	0.9380	0.9269	0.9581	0.9724
GZJ	0.1858	0.2911	0.0981	0.0935	0.1278	0.0371		0.8921	0.9616	0.9519	0.9601	0.9203	0.9381	0.9760
GHY	0.1774	0.3175	0.1799	0.1226	0.1865	0.0893	0.1142		0.9257	0.9093	0.8898	0.8833	0.9414	0.9553
GCZ	0.1569	0.2575	0.0977	0.0540	0.1038	0.0203	0.0391	0.0772		0.9539	0.9442	0.9171	0.9680	0.9728
FQZ	0.2084	0.3276	0.1470	0.0909	0.1357	0.0493	0.0492	0.0951	0.0472		0.9309	0.8721	0.9187	0.9593
FJJ	0.2480	0.3222	0.1570	0.1506	0.1346	0.0640	0.0407	0.1168	0.0574	0.0716		0.9293	0.9095	0.9587
FFZ	0.2326	0.2484	0.1266	0.1130	0.1298	0.0759	0.0831	0.1241	0.0865	0.1369	0.0734		0.9385	0.9595
FFD	0.1356	0.2291	0.0767	0.0412	0.1236	0.0428	0.0639	0.0604	0.0325	0.0848	0.0948	0.0635		0.9706
FXM	0.1731	0.2429	0.0904	0.0638	0.1211	0.0280	0.0243	0.0669	0.0276	0.0416	0.0422	0.0414	0.0299	

Net's index of genetic distance (below diagonal) and genetic identity (above diagonal). Population codes are given in Table 1.

The higher genetic diversity of *P. clematidea* compared with that reported for other noxious invasive plants (Wang et al., 2015), its multiple invasion origins (Wang et al., 2013; Li et al., 2014), and its wide ranging distribution in China (Qiu et al., 2011) suggest that this weed should be on the Alert List for Environmental Weeds in China. We believe the management of this weed should be regulated, with controls implemented for its eradication and prevention of its further introduction.

Conflicts of interest

The authors declare no conflict of interest.

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