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Development of SSR markers for genetic analysis of Silverleaf Nightshade (*Solanum elaeagnifolium*) and related species

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Abstract Silverleaf nightshade (2n=2x=24) is a serious weed of the family Solanaceae, for which no specific markers are available to date. In order to investigate the extent and distribution of genetic diversity among accessions of silverleaf nightshade, we developed twenty-three simple sequence repeats (SSR) markers from publicly available nucleotide and EST databases for silverleaf nightshade. Eleven of them were single-locus polymorphic markers. The number of alleles among these loci ranged from 2 to 4. The observed and expected heterozygosity ranged from 0 to 0.97 and 0.07 to 0.64, respectively. Fourteen SSR markers enabled to amplify alleles in morphologically similar species quena. These results proved that the SSR markers that we developed could be useful for (i) determining genetic diversity and structure among natural populations of silverleaf nightshade and (ii) identification of silverleaf nightshade and quena ecotypes. This is the first set of species-specific SSR markers identified in silverleaf nightshade, which could contribute to the better understanding of genetic diversity of silverleaf nightshade and related species.

Key words *EST-SSR, genomic-SSR, weed, silverleaf nightshade, quena*

Introduction

Silverleaf nightshade (*Solanum elaeagnifolium* Cav.) is a summer growing perennial weed native to the American continent. It occurs in many countries including the United States of America, Australia, Argentina, Brazil, Chile, India, Israel, Greece, Morocco, South Africa and Spain (Stanton et al. 2009). This invasive weed reproduces both sexually (outcrossing) and vegetatively. In Australia, silverleaf nightshade causes up to 77% yield lost in cereal crops (Stanton et al. 2009). However little is known about its pollination biology and genetic diversity. Improved management of this weed would require a better understanding of genetic diversity in silverleaf nightshade, since genetically diverse weed species will affect the choice of appropriate control strategies, such as the selection of biocontrol agents (Dekker 1997).

Silverleaf nightshade is often morphologically confused with an Australian native species quena (*Solanum esuriale* Lindl.) and microscopic examination is required to distinguish these two species (Bean 2004; Zhu et al. 2011). Quena is non-invasive and easier to control than silverleaf nightshade, thus correct identification is critical for silverleaf nightshade management (Johnson et al. 2006). Currently, there is limited genomic resource available for silverleaf nightshade. Several marker systems such as restriction fragment length polymorphism, amplified fragment length polymorphism, random amplified polymorphism DNA, diversity array technology, simple sequence repeat (SSR) and single-nucleotide polymorphism have been utilised for genetic analysis of various plant species (Zhou 2005). SSRs have been the marker of choice due to their abundance, high rate of polymorphism and reproducibility, high-transferability across species, codominance, genetic stability and suitability for higher throughput analysis using highly parallel automated systems (Ellis and Burke 2007; O'Hanlon et al. 2000; Swapna et al. 2011).

Many SSR markers have been developed and widely used in *Solanum* species for genetic diversity studies (Kwon et al. 2009) and species and variety identification, especially in the three main *Solanum* crops: eggplant, potato and tomato (Ghislain et al. 2009). Some of these SSRs were transferred to other Solanaceae species such as naranjilla (*S. quitoense* Lam.) and bush tomato (*S. central* J. M. Black) for genetic diversity investigation (Torres et al. 2008; Waycott et al. 2011). However, only a few cross-species SSR markers were available for the molecular characterization of silverleaf nightshade ecotypes (Zhu et al. 2012). In the public databases, 181 nucleotide and expressed sequence tag (EST) sequences of silverleaf nightshade are available from NCBI Genbank (www.ncbi.nlm.nih.gov), which can be exploited to develop molecular markers for genetic analysis.

In the present study, we developed a suite of SSR markers from publicly available EST and nucleotide

sequences in order to determine the extent of natural population diversity in a subset of silverleaf nightshade accessions. These markers will provide a valuable tool to understand the genetic diversity and structure of silverleaf nightshade and to assist in the identification of silverleaf nightshade and quena ecotypes.

Materials and Methods

Plant Material and DNA Extraction

Thirty-nine samples of silverleaf nightshade were collected from nine locations across south-eastern Australia (Table 1). In addition, two samples of quena were also collected to investigate the transferability of primer-pairs between these two morphologically similar *Solanum* species. Genomic DNA from each sample was extracted from the frozen leaf material using the standard phenol/ chloroform method (Sambrook et al. 1989).

Identification of Microsatellites

A total of 169 ESTs and 12 nucleotide sequences derived from *S. elaeagnifolium* were sourced from the NCBI database (www.ncbi.nlm.nih.gov). Vector sequences (if any) were trimmed using VecScreen (www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html). ESTs were then assembled to eliminate redundancy using CD-HIT Suite with a 90% sequence similarity threshold (Huang et al. 2010), while the nucleotide sequences were compared and integrated using BlastN (blast.ncbi.nlm.nih.gov). The generated non-redundant sequences were further used to detect the SSR motifs using SSRIT software (Temnykh et al. 2001) with the criteria of at least 4 and 3 repeat units for di-, tri- and higher order nucleotides, respectively.

Primer Design

Primer pairs were designed based on the flanking sequences of the detected SSR motifs using Primer Premier 5.0 (www.premierbiosoft.com) with a length of 18-30 bp, amplification product size of 100-350 bp and melting temperature ranged from 55 °C to 60 °C (Table 2). The 5' end of the forward primer of each SSR primer-pair was tailed with M13 sequence (Raman et al. 2005), which allows an inexpensive way to perform high throughput fragments analysis (Rampling et al. 2001; Schuelke 2000).

PCR Amplification and SSR Analysis

The PCR protocol was modified from Raman et al. (2005). Amplification was carried out in 12 µL of reaction mixture consisting of 50-100 ng of template DNA, 1.2 µL of 10× buffer (contains Tris·Cl, KCl, $(NH_4)_2SO_4$ and 15 mM $MgCl_2$), 6 mM $MgCl_2$, 240 mM of each dNTP's, 0.4 unit of HotStar Taq (Qiagen, Australia), 0.15 µM of forward primer (Sigma Aldrich, Australia), 0.3 µM of reverse primer and 0.3 µM of M13 primer (D4, D3 or D2,

Beckman Coulter, USA). After an initial denaturation of 4 min at 94 ºC, 30 cycles for 30 s at 94 ºC, 30 s at 55-60 ºC (depending on the primers, Table 2), and 30 s at 72 ºC were performed, followed by a final extension of 10 min at 72 ºC. Then 0.6, 0.7 and 0.8 µL of PCR products generated by D4, D3 and D2 (Beckman Coulter, USA), respectively, were mixed with 0.4 µL of DNA size standard kit 400 (Beckman Coulter, USA) and 28.6 µL of loading solution (Beckman Coulter, USA), and separated on a CEQ 8000 genetic analysis system (Beckman Coulter Inc) as described previously (Raman et al. 2005).

Data Analysis

Only single locus markers were further used for calculating observed and expected heterozygosity and genetic similarity. The observed and expected heterozygosity were calculated on the polymorphic primers using POPGENE version 1.32 software (Yeh and Boyle 1997). A similarity matrix was calculated based on Jaccard's coefficient using Similarity for Qualitative data (SIMQUAL) in NTSYS-pc 2.1 (Rohlf 2000). An Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrogram was calculated using the same software to illustrate genetic relationships between the samples.

Results and Discussion

SSR Motif Frequency and Distribution

A total of 63 non-redundant sequences were identified through CD-HIT and BlastN analysis, including 56 ESTs and seven nucleotide sequences. Seventeen of the 56 EST sequences contained 25 SSR motifs, with about 30% of the non-redundant ESTs containing at least one SSR motif. One in 3.3 non-redundant ESTs contained at least one SSR. The number of di- and tri- EST-SSR motif varied from 12 and 11, respectively. The remaining two motifs consisted of one penta- and one hexa-nucleotide motifs. In addition, four of the seven nucleotide sequences contained six SSR motifs (data not shown).

The number of dinucleotide (48%) and trinucleotide (44%) repeats in ESTs were similar in this study and there were no obviously abundant motifs found in di- and tri- repeats. However, compared to other EST-SSR studies in other Solanaceae species, trinucleotides were the most frequent motifs, with AT and GA, and AAG and AAT were the most common di- and trinucleotide motifs (Stagel et al. 2008; Nunome et al. 2009; Feingold et al. 2005). The density and frequency of SSRs estimated in this study might be affected by the limited ESTs available in silverleaf nightshade expressed genome.

SSR Amplification and Polymorphism

Out of the 25 EST-SSR motifs and six nucleotide SSR motifs, a total of 26 primer pairs were designed that

included 20 EST-SSR and six genomic-SSR primer pairs (Table 2). The remaining five SSR motifs (data not shown) were inappropriate for primer design because of insufficient flanking sequence of the SSR loci. All 26 primer pairs were further used to amplify genomic DNA of 39 silverleaf nightshade samples. Of these primer pairs examined, three (SLNZ 3, SLNZ 13 and SLNZ 16) did not produce any amplification products, while the other 23 primer pairs produced repeatable and reliable alleles (Table 3). Twenty one of them produced 1 or 2 discrete fragments in each individual and have been considered as single locus markers. Among these 21 primer pairs, ten were monomorphic, while the others 11 SSRs (comprising ten EST-SSRs and one genomic-SSR) were polymorphic. By contrast, two primer pairs SLNZ 7 and SLNZ 22 amplified multiple bands (three or more). Amplification of multiple alleles might be caused by the duplication of genomic regions (Senthilvel et al. 2008), which is common in plants (Yu et al. 2004; Sharma et al. 2009).

The EST- and genomic-SSRs reported here is the first set of specific SSR markers for silverleaf nightshade. The number of alleles detected by single locus markers ranged from 2 to 4 with an average of 2.6 per locus. The observed heterozygosity ranged from 0 to 0.97 and the expected heterozygosity ranged from 0.07 to 0.64, with average of 0.15 and 0.26, respectively (Table 3). The level of polymorphism detected in this study is much lower than our previous cross-species SSR study (average expected heterozygosity at 0.53) in silverleaf nightshade (Zhu et al. 2012). This is probably due to the conserved nature of EST-SSR markers. Most of the SSRs developed here are EST-SSR which is usually less polymorphic than genomic-SSRs (Cho et al. 2000). However, EST-SSR markers derived from cDNA provide a valuable resource for identification and developing gene-associated SSR markers.

Cross Species Transferability of SSRs

Those 23 markers produced bands were further tested for cross transferability in quena. Fourteen of them (61%) were successfully transferred including eight EST-SSRs and six genomic-SSRs (Table 3) with four of them (SLNZ 8, SLNZ 10, SLNZ 15 and SLNZ 22) polymorphic. This within subgenus SSR transferable ratio is lower than cross-species SSR investigation in other species. Rossetto (2001) reviewed that SSR primer-pairs showed an average 89.8% success rate when applied within subgenera (such as *Magnolia* and *Vitis*). However, the transferability of SSR primer pairs within Solanaceae species is usually much lower than others. For instance, Torres et al. (2008) achieved 27% transferable rate when transferred SSR markers from potato (*S. tuberosum* L.) to naranjilla. When SSR markers from tomato and eggplant (*S. melongena* L.) have been transferred to bush tomato, a transferable rate of 60% was detected (Waycott et al. 2011). In addition, we have observed an overall 37% (13/35) cross amplification from potato, tomato and eggplant to silverleaf nightshade (Zhu et al. 2012).

Solanum is one of the largest genera of flowering plants. Compared to others, Solanaceae species might experienced a longer evolutionary process or higher speciation rate (Whalen and Caruso 1983). These factors may lead to great genetic divergence among *Solanum* species and therefore resulted in low transferability (Whalen and Caruso 1983; Torres et al. 2008).

Genetic Diversity among Silverleaf Nightshade

According to the UPGMA dendrogram, quena and silverleaf nightshade were clearly separated at similarity level of 0.13 based on Jaccard's coefficient (Fig 1), which indicated the great genetic divergence between the two species. Genetic similarity among silverleaf nightshade individuals ranged from 0.4 to 1.0, with an average genetic similarity of 0.79 (data not shown). Two main subgroups were observed among silverleaf nightshade individuals: one contained two silverleaf nightshade individuals (Lox 2 and Lox 3) from Loxton, South Australia and the other included all the left silverleaf nightshade samples. In this study, we found less diversity among silverleaf nightshade accessions as compared to previous genetic diversity study that was based on cross-species SSR markers (with an average genetic similarity of 0.37) (Zhu et al. 2012), This may be probably due to the conserved nature of EST-SSR marker (Cho et al. 2000).

Genetic diversity of silverleaf nightshade may be attribute to the propagate system and multiple introduction. Silverleaf nightshade propagate both sexually (self incompatibility) and asexually (through root fragments). Cross pollinating species usually have a higher level of genetic diversity than those reproduce clonally or self-pollinating (Ward and Jasieniuk 2009). Silverleaf nightshade samples used in this study were collected from geographically separated accession in NSW, SA and VIC. The possibility of multiple introductions in these states have been highlighted by many researches (Cuthbertson et al. 1976; Stanton et al. 2009), which will also add up genetic diversity of silverleaf nightshade in Australia.

In this study, five species-specific markers were identified in silverleaf nightshade (231, 328, 299, 185 and 216 bp from primer pair SLNZ 11, SLNZ 12, SLNZ 14, SLNZ 18 and SLNZ 19, respectively) and four in quena (223, 161, 220 and 285 bp from primer pair SLNZ 2, SLNZ 5, SLNZ 20 and SLNZ 21, respectively). Some diagnostic micro-morphological features were reported previously such as trichome intrusive base (Bean 2004; Zhu et al. 2011). However, currently only three unique alleles (85, 222 and 249 bp from EM 117, EM 135 and ESM 3, respectively) were available to distinguish these two species (Zhu et al. 2011). In conjunction with previously identified three genic markers (Zhu et al. 2011), these nine new species-specific SSR markers will enable to distinguish both quena and silver nightshade. Reliable identification of invasive species is required for selection of biocontrol agents (Nissen et al. 1995). In addition, correct identification also assist the

selection of herbicide and management strategies as quena is not a serious problem in Australian crop and pasture system and easier to control than silverleaf nightshade (Johnson et al. 2006).

In conclusion, we have developed the first set of species-specific SSR primers for silverleaf nightshade. These markers could be useful for determining natural population diversity and structure and to distinguish silverleaf nightshade from quena. EST-SSR markers are often preferable for comparative genomic analysis to other markers such as RFLP, AFLP and RAPD, as they show higher similarity across different species than intergenic chromosomal regions. Therefore, the marker set that we have developed, is anticipated to be directly applicable for comparative genomics and evolution studies on silverleaf nightshade and related species.

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Table1 Silverleaf nightshade and quena samples used in this study

Species	Location	Abbreviation	Samples	GPS (latitude/ longitude)
Silverleaf nightshade	Narrandera	Nar	5	-34°46′ / 146°25′
	Temora	Tem	4	-34°24′ / 147°36′
	Ungarie	Ung	5	-33°35′ / 146°55′
	Loxton	Lox	4	-34°38 ′ / 140°41′
	Wirrabara	Wir	4	-33°02 ′ / 138°16′
	Keith	Kei	4	-36°06 ′ / 140°16′
	Hopetoun	Hop	5	-35°36 ′ / 142°26′
	Serpentine	Ser	4	-36°24 ′ / 143°58′
	Jarklin	Jar	4	-36°14 ′ / 143°56′
Quena	Wagga Wagga	Q-W	1	-35°07 ′ / 147°20′
	Jarklin	Q-J	1	-36°14 ′ / 143°56′

Table 2 Characteristics of 26 SSR primers developed in *Solanum elaeagnifolium,* including locus name, forward (F) and reverse (R) primer sequence, repeat motif, annealing temperature (Ta), allele size range including 19 bp of M13-tail (–: no amplification) and GenBank accession number

Locus	Primer sequence (5'-3')	Repeat motif	Ta (°C)	Size range (bp)
SLNZ1	F: ACTAATACCTTACCCCGTTCATCT R: ATTCGTTCAAGAAGGGCTCC	$(TTC)_4$	55	308
SLNZ2	F: ATAGTACACTCAGCATCCATCATAAG R: ACAGGAGGAACAGCAAGGC	$(AT)_4…(TA)_4$	55	221-232
SLNZ3	F: TCACACCACTAAAGGGGGGAT R: ATCAACAGGAGGAACAGCAAGG	$(TTA)_3$	50-60	–
SLNZ4	F: ATGTAGGGACTAGTGCTCGAGTT R: AATAAAGCAAGGGCAATAGGTC	$(TCC)_3$	55	328-331
SLNZ5	F: TATGGGGCACATGGGAGAG R: AACCCCCATTCTAAATCCTTGT	$(CTTCT)_3$	58	196-204
SLNZ6	F: CTTTGTTCGGAGTTGTTGACC R: CCTCCATCGCAAAACCATC	$(GA)_5$	58	256-278
SLNZ7	F: AGAGTGGAGAGGAGAAGTAGAAGG R: GGTAAATTGAGGATCTTGGGTG	$(AAG)_3$	58	226-259
SLNZ8	F: GGAATTAAGGGTCCAAGGC R: CTCACAAGTTACTCGGGCTCT	$(ATG)_3…(TTA)_3$	58	195-202
SLNZ9	F: TTCATAAATGAGAACTTACACGGAC R: TCTTAGCAGCGAACTGGGAC	$(GTG)_3$	58	226-268
SLNZ10	F: CCAAGCGAGGAAATAGCACT R: GTGCTTCCGATTTCTCCAAC	$(ATG)_3$	58	213
SLNZ11	F: GGTGTTTGTTGGAGAAATCGG R: TCTTCTACGATTTCCTTGGTGC	$(CAA)_3$	60	231
SLNZ12	F: GAAATGAAAGTCCCATCTCC R: TGACTTCAGAACCAGTTACTCCT	$(TTTTAT)_4$	55	328
SLNZ13	F: CAATCACAGTAGAAAGGGTCGCT R: TTACCATTCCCTATGTTGATCCAG	$(TG)_4$	50-60	–
SLNZ 14	F: GCGAACGAATAATTGACCACC R: AGTCGCCAAACTCCACATCTC	$(TG)_4$	60	299
SLNZ15	F: TCATCACGCAAACGCTTACTC R: ATTTAACTATGTGCTAATTGTTATCGC	$(AAG)_4$	55	174-186
SLNZ16	F: CAAAGATACGGACCGCACCT R: GGTAAACGCCAGACGAACAAG	$(AT)_4$	50-60	–
SLNZ17	F: CCAAGGCTCGGAAGAACC R: CCACGAAAACACAACCTAACTAAC	$(AG)_4$	58	162-174

SLNZ18	F: GGCTAAGTGACTAAACAAAAATGG R: AGCAGTGGTATCAATTTGTGTCG	$(CA)_5$	55	185
SLNZ19	F: TGGTAGAGGCGAAGGCAT R: GCATCTTCAGGTCCCAACTT	$(AG)_4$	58	216
SLNZ20	F: CACTTGCCCCTATTCCTGTCAT R: CTTGTATCCTTCTCGCTACCTTTC	$(CA)_4$	58	218-242
SLNZ21	F: GCTGCTACTCCCAATCCTAACTG R: AAATCTCCGACGAAAGCTACTACT	$(TA)_4$	58	245-289
SLNZ22	F: GCAGAATCCCGTGAACCATC R: CGCCGAGAGAGTTGGGTTAC	$(CG)_5$	55	202-257
SLNZ23	F: ATTGGTTGGGCTGTGTTCCT R: TGGGCGGATTTAGCAACTG	$(TTA)_3$	55	294
SLNZ24	F: TTTAGCCTATTCCACAATGTCTCA R: TGGCGAATACAACCAACTATCAT	$(ATT)_3$	58	353
SLNZ25	F: TCACTATCTCTATGGGGTAAAAACG R: GCATAGTATTGTCCGATTCATAAGG	$(AAT)_3$	58	224
SLNZ26	F: GGCATTGGAAATACTTTTTATTAC R: CCTAAAAGCGGAGGAATGTC	$(TC)_4$	55	123-160

Table 3 Results of initial primer screening in *Solanum elaeagnifolium*, including the source of SSR for each primer pair, number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosity, and cross-species amplification in *S. esuriale* (+: amplified and –: not amplified)

Locus	Source	N_A	H_O	H_E	Cross transferability
SLNZ1	Nucleotide	1	0.00	0.00	+
SLNZ2	EST	2	0.00	0.10	+
SLNZ4	EST	2	0.00	0.23	–
SLNZ5	EST	3	0.31	0.30	–
SLNZ6	EST	3	0.10	0.50	–
SLNZ7[a]	EST	7	-	-	–
SLNZ8	EST	2	0.08	0.07	+
SLNZ9	EST	2	0.00	0.10	+
SLNZ10	EST	1	0.00	0.00	+
SLNZ11	EST	1	0.00	0.00	–
SLNZ12	EST	1	0.00	0.00	–
SLNZ14	EST	1	0.00	0.00	–
SLNZ15	EST	3	0.97	0.64	+
SLNZ17	EST	3	0.05	0.37	+
SLNZ18	EST	1	0.00	0.00	–
SLNZ19	EST	1	0.00	0.00	–
SLNZ20	EST	3	0.13	0.25	+
SLNZ21	EST	2	0.00	0.10	+
SLNZ22[a]	Nucleotide	10	-	-	+
SLNZ23	Nucleotide	1	0.00	0.00	+

SLNZ24	Nucleotide	1	0.00	0.00	+
SLNZ25	Nucleotide	1	0.00	0.00	+
SLNZ26	Nucleotide	4	0.03	0.17	+

[a] SLNZ marker with multiple bands amplifications.

Figures Legends

Fig. 1 UPGMA dendrogram calculated by Jaccard's coefficient showing three clusters. Sample details are included in Table 1