

DEVELOPMENT OF HIGHLY TRANSFERABLE MICROSATELLITES FOR *PANAX GINSENG* (ARALIACEAE) USING WHOLE-GENOME DATA¹

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- **Premise of the study:** Highly transferable expressed sequence tag (EST) microsatellites were developed for *Panax ginseng* (Araliaceae), one of the most celebrated traditional Chinese medicines and an endangered species in East Asia, using whole-genome data.
- **Methods and Results:** Twenty-one EST microsatellites were characterized from next-generation sequencing and were composed of di- and trinucleotide repeats. Polymorphisms and genetic diversity were evaluated for 45 accessions of three ginseng landraces. The number of alleles for each locus ranged from one to five among the landraces, and the polymorphism information content varied from 0.0000 to 0.6450. These microsatellites were also tested for congeneric amplification with *P. notoginseng*, *P. stipuleanatus*, *P. quinquefolius*, *P. bipinnatifidus*, and the closely related species *Aralia elata*.
- **Conclusions:** These novel EST-derived microsatellite markers will facilitate further population genetic studies of the genera *Panax* and *Aralia*.

Key words: Araliaceae; microsatellite; *Panax ginseng*; polyploidy; traditional Chinese medicine.

Panax L. (Araliaceae) is a medicinally important genus, which consists of seven well-recognized species and one species complex, and is widely distributed in East Asia and North America (Lee and Wen, 2004). *Panax ginseng* C. A. Mey. is very popular in traditional Chinese medicine and has been used as an herbal remedy in East Asia for thousands of years (Liu and Xiao, 1992). Although the pharmacology and medical effects of *P. ginseng* have been investigated extensively, only a few studies have focused on the genetic diversity and population structure of this species (Li et al., 2015), primarily owing to the limitations of molecular markers. In our recent studies, we employed single-copy nuclear genes to investigate the genetic diversity of cultivated and wild ginseng (Li et al., 2015). However, the relatively low mutation rate of single-copy nuclear genes and the recent domestication of cultivated ginseng largely limited the application of our selected nuclear genes in assessment of its population structure and domestication history. In addition, we have recently discovered multiple rounds of whole-genome duplication within the genus *Panax* (Shi et al., 2015). *Panax ginseng* is an allotetraploid species and has undergone two rounds of whole-genome duplication, which makes it difficult to obtain all alleles through

traditional Sanger sequencing. In this regard, as codominant markers with high mutation rates (Jarne and Lagoda, 1996), nuclear microsatellites might provide novel insights into the polyploidy of *Panax* and domestication of *P. ginseng*. In previous studies, other microsatellites have been developed (Kim et al., 2007; Yang et al., 2008; Ahn et al., 2009; Van Dan et al., 2010; Choi et al., 2011; Reunova et al., 2014). Nevertheless, most of these studies developed microsatellites based on traditional Sanger sequencing, and the transferability of most of these microsatellites to congeneric species remains unknown. We employed next-generation sequencing to develop microsatellites from whole-genome data and to test the transferability of these identified microsatellites in other *Panax* and *Aralia* L. species.

METHODS AND RESULTS

Plant material and DNA extraction—A total of 63 wild and cultivated ginseng accessions were collected from North Korea, eastern Russia, and northeastern China (Appendix 1). The related species *P. notoginseng* (Burkill) F. H. Chen ex C. Y. Wu & K. M. Feng, *P. stipuleanatus* H. T. Tsai & K. M. Feng, *P. bipinnatifidus* Seem., *P. quinquefolius* L., and *Aralia elata* (Miq.) Seem. were sampled from Yunnan, Jilin, and Sichuan provinces of China and from Wisconsin, USA (Appendix 1). We collected cultivated ginseng accessions from multiple geographic localities, while wild *P. ginseng* accessions and related species were generously provided by our collaborators. Of these *P. ginseng* accessions, 18 were subjected to whole-genome sequencing and 45 were used to test the polymorphisms of the identified microsatellites. Total genomic DNA was extracted from silica gel-dried leaves for each accession separately using the TIANGEN Plant Mini Kit (TIANGEN Biotech, Beijing, China).

Microsatellite development—The construction of DNA libraries of the 18 ginseng accessions was conducted by Novogene Corporation (Beijing, China),

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TABLE 1. Characteristics of 21 microsatellite loci developed in *Panax ginseng*.

Locus	Primer sequences (5'–3')	Repeat motif	Allele size (bp)	T_a (°C)	PIC	A	Putative function	GenBank accession no.
S2	F: CTTGCTGCTTCTACATCC R: GGTCTTGCTAATCCCAT	(CCA) ₄	470	56	0.0000	1	regulation of nuclear pre-mRNA domain protein	KU879256
S6	F: CCCAACCTACTAACATCC R: GGTTTAGCTGCTCTGTACT	(TCT) ₆	316	56	0.3749	2	subtilisin-like protease	KU879260
S11	F: AATTGTACCTCCATAAAACC R: AGAGCCCGAGATAACCTA	(TTC) ₆	370	56	0.0696	2	DEAD-box ATP-dependent RNA helicase	KU879265
S12	F: GCGACGAATTAGACGATG R: ATTGATTCTCCTGCTGA	(AG) ₅	358	48	0.0947	2	cyclic dof factor 2	KU879266
S13	F: TATTCCAATTCGGCAAAG R: GGAGTGTTGGGAGCATC	(GGA) ₆	301	56	0.4441	5	uncharacterized protein	KU879267
S16	F: ATGAAGCCGATGGTGGAG R: TTCTCCAATACTTCTCCC	(AAG) ₅	484	56	0.3748	2	translation initiation factor IF-2	KU879270
S15	F: TGAACTACTCCAGCTTCG R: ACGGTGATGGCTGGTGGT	(AGA) ₆	272	54	0.0000	1	C-type lectin receptor-like tyrosine-protein kinase	KU879269
S17	F: ATTCCCAGACAATAATGAG R: TTGAGGCAAGCAAGGTGA	(CT) ₅	329	54	0.3645	2	probable RNA helicase SDE3	KU879271
S19	F: GGGATGCCCTTACCCTTTG R: CGTGTGGCGTGTCTGTG	(GGC) ₄	429	56	0.3744	2	scarecrow-like protein 27	KU879273
S20	F: GTGCTTTATGGCATCTTT R: AACAGTGGTGCTTGAGT	(AAG) ₆	285	56	0.5943	4	septin and tuftelin-interacting protein 1	KU879274
S22	F: AAACCTTCTCCCTTATCT R: GGTTCGTTGGACCTTTT	(CTC) ₄	203	54	0.3738	2	uncharacterized protein	KU879276
S23	F: CTCAAATCTTACGCATCT R: GGTATTGTCCCATTGAGT	(TC) ₄	282	56	0.0000	1	receptor-like protein 12	KU879277
S24	F: GTAGAAGAAGAGCAGCACA R: CGGAGTAACTGAAGGGAG	(CGC) ₃	384	56	0.6447	4	uncharacterized protein	KU879278
S25	F: GCTGCTGTTCTGTACGC R: ATCTATCATCCACCTCCC	(GAT) ₃	376	56	0.0434	2	methionine-tRNA ligase	KU879279
S26	F: CTGTCCCAACTCCCAATA R: GGGTAGGCTAAATAACTGA	(CT) ₆	416	56	0.0000	1	D-xylose-proton symporter-like 3	KU879280
S27	F: AAAGACAATCCAGAAG R: CAAACTTGCTCTTCCCTCC	(AG) ₄	257	54	0.5480	4	uncharacterized protein	KU879281
S30	F: CTCACAGATGTTTCCACCCA R: TCCTACCCATTTCGCTCC	(ACC) ₄	450	56	0.0000	1	uncharacterized protein	KU879284
S31	F: TCAGGGTTCTCAGCATAA R: AACCATCAGTGAGCCAA	(TC) ₅	257	56	0.0000	1	uncharacterized protein	KU879285
S32	F: AGGAAAGCGAACACGAAC R: TAAATCCCAATCCAGCA	(TG) ₅	366	56	0.2150	2	4-coumarate-CoA ligase 2	KU879286
S33	F: AAGATTGAGCGTTATGTG R: CTTACTTATGGAAGCACC	(TGA) ₆	411	56	0.0000	1	ribosomal RNA processing protein 1 B	KU879287
S38	F: AACGGCTCCAGTGATGTA R: TGAACAGGTGGTTGAGTA	(CTG) ₅	283	56	0.6450	4	ENTH/VHS family protein	KU879292

Note: A = number of alleles; PIC = polymorphism information content; T_a = annealing temperature.

and then the libraries were sequenced using an Illumina HiSeq 2000 system (Illumina, San Diego, California, USA). We also downloaded the whole-genome data of one South Korean ginseng accession from GenBank (GenBank accession no.: SRR1181600). Because *P. ginseng* is an allotetraploid species with a large genome size (ca. 3 Gb), we were not able to perform de novo assembly in this study. Instead, we downloaded transcriptome data of the diploid species *P. notoginseng* (GenBank accession no.: SRX378873, SRX378878, and SRX378880) and performed de novo assembly using Trinity (Grabherr et al., 2011). These assembled transcripts were then used as references to perform the short read alignment for *P. ginseng* using Burrows-Wheeler Aligner's Smith-Waterman Alignment (BWA-SW; Li and Durbin, 2010). The insertions/deletions were reported using SAMtools (Li et al., 2009). Thereafter, we developed a series of Perl scripts to identify the polymorphic microsatellites from the obtained variant call format (VCF).

Microsatellite marker data analysis—A total of 60 multiallelic microsatellites were detected from the whole-genome data of the 19 ginseng accessions (including SRR1181600). To determine the function of microsatellite-associated unigenes, these assembled transcripts were searched against the GenBank nonredundant protein database using BLASTX (Altschul et al., 1997) with an expected value $<10^{-7}$. The putative functions of these microsatellite-associated genes are listed in Table 1. We chose the candidate microsatellites according to

the following criteria: (1) more than 20 bp at the flanking regions of the microsatellite repeat, and (2) no large intron (<500 bp in length) within the target region used to design primers. Forty-one microsatellites were selected to design the PCR primers, and 38 of them showed clean PCR amplifications in all five *P. ginseng* accessions tested. The PCR amplifications were conducted in a 25- μ L volume with 2.5 mM MgCl₂, 0.1 μ M forward and reverse primers, 400 μ M dNTPs, 1 unit *rTaq* (TaKaRa Biotechnology Co., Dalian, Liaoning, China), and 20–50 ng DNA. PCRs were performed for each microsatellite under the following conditions: an initial denaturation step of 5 min at 95°C; followed by 35 cycles of 94°C for 30 s, annealing temperature (Table 1) for 30 s, and 72°C for 30 s; and a final step of 8 min at 72°C. After amplification, the PCR products were sequenced on an ABI730 sequencer (Applied Biosystems, Foster City, California, USA). The obtained genomic DNA sequences of *P. ginseng* were then compared with the assembled transcripts of *P. notoginseng*. As expected, all microsatellites were confirmed in the genomic sequences of *P. ginseng*. All DNA sequences of *P. ginseng* obtained from this study were submitted to GenBank (accession no.: KU879255–KU879294; Table 1).

To further evaluate the polymorphisms in cultivated ginseng, we amplified these microsatellites with 45 accessions of three major ginseng landraces. Twenty-one microsatellites yielded abundant PCR products across the 45 ginseng accessions. Fluorescently labeled PCR products were resolved to genotype on an ABI 730 sequencer (Applied Biosystems). The number of alleles

TABLE 2. Allelic diversity in 21 microsatellites for three major ginseng landraces.^a

Locus	SHIZHU					BIANTIAO					COMMON				
	N	A	H _o	H _e	PIC	N	A	H _o	H _e	PIC	N	A	H _o	H _e	PIC
S2	15	1	0.000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000
S6	15	2	0.9375	0.4978	0.3741	15	2	1.0000	0.5000	0.3750	15	2	1.0000	0.5000	0.3750
S11	15	2	0.1667	0.1528	0.1364	15	2	0.0714	0.0689	0.0739	15	1	0.0000	0.0000	0.0000
S12	15	2	1.0000	0.3750	0.3047	15	2	0.1429	0.1327	0.1217	15	1	0.0000	0.0000	0.0000
S13	15	4	1.0000	0.5531	0.4528	15	4	1.0000	0.5272	0.4288	15	4	0.9091	0.5695	0.4794
S15	15	1	0.000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000
S16	15	2	0.9000	0.4950	0.3725	15	2	1.0000	0.5000	0.3750	15	2	1.0000	0.5000	0.3750
S17	15	2	0.9000	0.4550	0.3515	15	2	1.0000	0.5000	0.3685	15	2	1.0000	0.4888	0.3685
S19	15	2	0.8333	0.4861	0.3685	15	2	1.0000	0.4861	0.3701	15	2	1.0000	0.4988	0.3746
S20	15	4	1.0000	0.6693	0.5995	15	3	1.0000	0.6505	0.5764	15	4	1.0000	0.6632	0.6000
S22	15	2	0.8571	0.4841	0.3668	15	2	1.0000	0.5000	0.3750	15	2	1.0000	0.4994	0.3746
S23	15	1	0.000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000
S24	15	3	0.4000	0.5600	0.4992	15	3	0.7500	0.6563	0.5786	15	4	0.7500	0.6939	0.6388
S25	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	2	0.5000	0.1528	0.1364
S26	15	1	0.000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000
S27	15	4	0.4667	0.5450	0.4757	15	4	0.9333	0.6339	0.5880	15	4	0.9091	0.6301	0.5673
S30	15	1	0.000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000
S31	15	1	0.000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000
S32	15	2	0.2857	0.1327	0.1217	15	2	0.7857	0.3157	0.2688	15	2	0.8333	0.2698	0.2327
S33	15	1	0.000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000	15	1	0.0000	0.0000	0.0000
S38	15	4	1.0000	0.7188	0.6675	15	4	1.0000	0.6806	0.6205	15	4	1.0000	0.7071	0.6559

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals used; PIC = polymorphism information content.

^aLocality and voucher information are provided in Appendix 1.

of these microsatellites varied from one to five, and the polymorphism information content ranged from 0.0000 to 0.6450 (Table 1). We also evaluated polymorphisms of these microsatellites in each of the three ginseng landraces (Table 2). We noted that the polymorphic microsatellite S25 was monomorphic in the landraces SHIZHU and BIANTIAO. Similarly, the polymorphic microsatellites S11 and S12 were monomorphic in the landrace COMMON. The transferability of these primers was tested with *P. notoginseng*, *P. stipuleanatus*, *P. bipinnatifidus*, *P. quinquefolius*, and *A. elata*. All 21 primers amplified successfully in the four congeneric species except *P. bipinnatifidus* (Table 3). All of these microsatellites also yielded clear PCR products in the closely related species *A. elata*, suggesting the high transferability of these primers (Table 3). Notably, we found that seven microsatellites were monomorphic

across all 45 ginseng accessions, but five of them were polymorphic in related species (Table 3).

CONCLUSIONS

Development of molecular markers from nonmodel species has been increasing in recent years. In this study, we identified polymorphic microsatellite markers from the nonmodel species *P. ginseng* using whole-genome data. These polymorphic microsatellites provide useful molecular markers to assess the

TABLE 3. Cross-species amplification information for 21 microsatellite loci in closely related *Panax* and *Aralia* species.^a

Locus	<i>P. quinquefolius</i> (N = 7)			<i>P. notoginseng</i> (N = 7)			<i>P. stipuleanatus</i> (N = 6)			<i>P. bipinnatifidus</i> (N = 1)		<i>A. elata</i> (N = 3)
	A	H _e	H _o	A	H _e	H _o	A	H _e	H _o	A	A	
S2	1	0.0000	0.0000	3	0.5612	1.0000	2	0.5000	1.0000	1	1	
S6	2	0.5000	1.0000	2	0.1327	0.1429	2	0.3200	0.0000	3	3	
S11	4	0.6020	0.8571	2	0.5000	1.0000	2	0.2449	0.3333	2	1	
S12	1	0.0000	0.0000	2	0.5000	1.0000	1	0.0000	0.0000	NA	2	
S13	1	0.0000	0.0000	1	0.0000	0.0000	1	0.0000	0.0000	2	3	
S15	2	0.5000	1.0000	1	0.0000	0.0000	3	0.5694	0.6667	2	1	
S16	3	0.4965	0.6667	2	0.4082	0.5714	2	0.4688	0.7500	3	2	
S17	3	0.6607	1.0000	2	0.5000	1.0000	2	0.3750	0.5000	2	2	
S19	1	0.0000	0.0000	2	0.5000	1.0000	2	0.2188	0.2500	3	2	
S20	3	0.5612	1.0000	3	0.6633	1.0000	1	0.0000	0.0000	1	2	
S22	2	0.0689	0.1429	1	0.0000	0.0000	1	0.0000	0.0000	2	2	
S23	1	0.0000	0.0000	1	0.0000	0.0000	2	0.2778	0.0000	1	1	
S24	2	0.5000	1.0000	5	0.7245	1.0000	2	0.5000	1.0000	4	3	
S25	1	0.0000	0.0000	2	0.4898	0.5714	1	0.0000	0.0000	3	2	
S26	1	0.0000	0.0000	2	0.1327	0.1429	2	0.2778	0.0000	1	2	
S27	2	0.0689	0.1429	2	0.4592	0.7143	3	0.5000	1.0000	2	3	
S30	1	0.0000	0.0000	1	0.0000	0.0000	1	0.0000	0.0000	1	3	
S31	1	0.0000	0.0000	1	0.0000	0.0000	1	0.0000	0.0000	1	1	
S32	2	0.2934	0.5714	2	0.4592	0.7143	2	0.5000	1.0000	2	3	
S33	1	0.0000	0.0000	1	0.0000	0.0000	1	0.0000	0.0000	1	1	
S38	3	0.5332	1.0000	1	0.0000	0.0000	1	0.0000	0.0000	1	1	

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals used; NA = no PCR products.

^aLocality and voucher information are provided in Appendix 1.

germplasm resources of *P. ginseng*. In particular, the high transferability of these microsatellites provides reliable molecular markers to investigate the population genetics and polyploid evolution of *Panax* and *Aralia*.

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APPENDIX 1. Locality information for *Panax ginseng* and the other species sampled in this study.

Species	Accession	No. of accessions	Locality	Geographic coordinates	Voucher no. ^a
<i>Panax ginseng</i> C. A. Mey.	GL1	1	North Korea	NA	
	GL2	1	South Korea	NA	
	CB	1	Changbai, Jilin, China	41°39'442"N, 127°35'229"E	
	WH	1	Dunhua, Jilin, China	43°30'181"N, 127°54'193"E	
	TQ	1	Yanji, Jilin, China	43°36'129"N, 129°35'807"E	
	FS	1	Fusong, Jilin, China	42°24'216"N, 127°12'186"E	NENU20110718004
	SZ1	1	Kuandian, Liaoning, China	40°45'595"N, 125°20'863"E	
	SZ2	1	Kuandian, Liaoning, China	40°47'157"N, 125°23'036"E	
	BT1	1	Tonghua, Jilin, China	41°18'492"N, 125°49'954"E	
	BT2	1	Tonghua, Jilin, China	41°05'245"N, 125°55'337"E	
	SJ1	1	Songjiang, Jilin, China	NA	
	SJ2	1	Songjiang, Jilin, China	NA	
	SJ3	1	Songjiang, Jilin, China	NA	
	DP	1	Dapuchai, Jilin, China	NA	
	CY	1	Caiyuan, Jilin, China	NA	
	XL	1	Fusong, Jilin, China	NA	
	HR	1	Huanren, Liaoning, China	NA	
	KD	1	Kuandian, Liaoning, China	NA	
	EL	1	Russia	NA	
		BIANTIAO	15	Tonghua, Jilin, China	41°18'492"N, 125°49'954"E
	SHIZHU	15	Kuandian, Liaoning, China	40°45'595"N, 125°20'863"E	
	COMMON	15	Dunhua, Jilin, China	43°30'181"N, 127°54'193"E	
<i>Panax notoginseng</i> (Burkill) F. H. Chen ex C. Y. Wu & K. M. Feng	PN	7	Wenshan, Yunnan, China	NA	KUN0560433
<i>Panax quinquefolius</i> L.	PQ	7	Wisconsin, USA	NA	NENU20110713001
<i>Panax stipuleanatus</i> H. T. Tsai & K. M. Feng	PS	6	Pingbian, Yunnan, China	NA	
<i>Panax bipinnatifidus</i> Seem.	PB	1	Sichuan, China	27°31'839"N, 101°42'569"E	NENU20120801001
<i>Aralia elata</i> (Miq.) Seem.	MS	3	Changbai, Jilin, China	NA	

Note: NA = exact locations of these samples are unknown.

^aThe vouchers were deposited in the herbaria of Northeast Normal University (NENU), Changchun, Jilin, China, and Kunming Institute of Botany (KUN), Chinese Academy of Sciences, Kunming, Yunnan, China.