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Differentiation of wild accessions within the *Panax bipinnatifidus* complex using newly developed polymorphic microsatellite markers

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Abstract

Panax L., renowned as ginseng genus, is a famous medicinal group of family Araliaceae. Within this genus, the taxa of *Panax bipinnatifidus* complex are mainly distributed in Himalayas and Hengduan Mountain areas. Due to the complex evolutionary history and short-term rapid radiation, the relationships among species within the complex have not been clearly resolved, and the taxa identification is difficult due to the intermediate morphological traits. This study aimed to use the available restriction-site associated DNA sequence data from 29 individuals of *P. bipinnatifidus* complex to mine high-polymorphic simple sequence repeat (SSR) markers, with the goal of evaluating their utility in taxa identification. Eleven polymorphic SSR loci were ultimately selected and validated through polymerase chain reactions amplifying across 63 individuals of *P. bipinnatifidus* complex and 13 individuals of three outgroup species. The subsequent genetic diversity analysis uncovered 76 alleles in total, ranging from 5 to 15 per locus. Observed heterozygosity spanned 0.241–0.512, while expected heterozygosity ranged between 0.345 and 0.644. The genetic kinship analysis revealed a sister relationship between *Panax zingiberensis* and *Panax vietnamensis*. The analysis result also supported the classification of samples from Hunan and Hubei provinces into a single genetic unit within the *P. bipinnatifidus* complex. These newly developed SSR markers will facilitate the identification of wild ginseng plants.

Introduction

Panax L., a genus of family Araliaceae, is an important medicinal resource mainly distributed in Himalayas area and central and southwestern China. The pharmaceutical activities of *Panax* species attribute mainly to ginsenosides, which are salutary for cardiovascular health. Comparative genome analysis revealed the evolutionary trajectory of ginsenoside biosynthesis (Yang *et al.*, 2023). Four *Panax* taxa, *P. zingiberensis*, *P. vietnamensis*, *P. wangianus* and *P. bipinnatifidus* were treated as *P. bipinnatifidus* complex due to the difficulties in species delimitation and unresolved evolutionary relationships among them (Zuo *et al.*, 2011, 2015; Zhou *et al.*, 2020). This species complex, characterized by a spectrum of intermediates or transitional characters in root, leaf, flower and fruit morphology (Wen and Zimmer, 1996; Lee and Wen, 2004; Zhou *et al.*, 2020), underwent rapid demographic expansion during the Oligocene (Zuo *et al.*, 2017), and the taxonomic relationships among the species remain contentious (Lee and Wen, 2004; Zuo *et al.*, 2011, 2015; Wen *et al.*, 2014; Shi *et al.*, 2015; Ji *et al.*, 2019; Zhou *et al.*, 2020). This taxonomic uncertainty complicates the precise identification of species and may pose an obstacle to the conservation of these valuable wild resources.

Simple sequence repeats (SSRs), a form of Mendelian inheritance and codominant microsatellite markers, are renowned for their high levels of polymorphism and reproducibility (Varshney *et al.*, 2005; Kalia *et al.*, 2011). They have been extensively applied in studies of genetic diversity and species delimitation (Hauser *et al.*, 2021). Several researchers have focused on the development of SSR markers in the three most famous ginseng species, *Panax notoginseng*, *P. ginseng* and *P. quinquefolius* (Choi *et al.*, 2011; Jiang *et al.*, 2016; Jang *et al.*, 2020; Su *et al.*, 2023). Within the *P. bipinnatifidus* complex, only nine SSR markers were developed from the transcriptome data of *P. vietnamensis* (Vu *et al.*, 2020), and their transferability was not tested in the related species. The objective of this study was to develop highly polymorphic SSR markers from restriction-site associated DNA sequence (RAD-seq) data of *P. bipinnatifidus* complex, with the goal of ascertaining their efficacy in identifying the wild resources of this complex.

Experimental procedure

We selected RAD-seq datasets of 29 diploid individuals of the *P. bipinnatifidus* complex, from our previous study (Zhou *et al.*, 2020), to develop SSR markers utilizing Stacks v1.48 (Catchen

Table 1. Genetic properties of the 11 polymorphic SSR markers developed in *P. bipinnatifidus* complex

Locus	Repeat motif	Primer sequences (5′–3′)	T_m (°C)	Allele size (bp)	A	H_O	H_E	G_{is}
P1	ATC	F: TGGGTTGGGTATAGGCTGGA	60	98–128	10	0.288	0.465	0.380
		R: GGCTATGGGTTATGAAGGAGGAG						
P5	AG	F: GACCTCGTCTAAATTTACTCAGAAC	59	132–142	6	0.354	0.398	0.111
		R: CCGAGGTCAGGGAAACTAGC						
P6	ACC	F: CTCCGAGCTGGGTTCATTGA	60	84–111	15	0.512	0.644	0.201
		R: ACGTTCCTTTGAAGAATGAATAGGT						
P7	ACC	F: ATAGGACCACACCAAGCTGC	60	130–143	6	0.267	0.434	0.386
		R: TCAACATCAGTCGGTTCGGA						
P9	AAG	F: GTCCGTATGACAATTTGACAAATGT	58	130–142	5	0.424	0.537	0.210
		R: TAAGTCGACTGTACAGCTTCAA						
P10	AGC	F: TAATGCAGGAATCAGCCGCA	59	85–100	7	0.489	0.571	0.144
		R: GCAATTCCCAACTAGTGAGAAGA						
P21	AGC	F: CCGTATCCCAAAGAAATGGATCG	59	100–112	5	0.241	0.452	0.467
		R: CTCCACGGCTAACTTTCTTTGT						
P25	CCG	F: ACACCCATGATTCTTGTCTATTGA	60	113–125	5	0.308	0.434	0.290
		R: ACAACGGAAGGAGTAGATATTGT						
P46	AG	F: CCATCTCCCACATGGCTG	57	100–108	6	0.259	0.345	0.251
		R: TCTTGTTTCTTTCTCTTTCATCTCTT						
P55	AAG	F: TCCAGTCCATTTCCTTGACCA	59	100–120	6	0.278	0.430	0.354
		R: GCAAACAACTTCTGAGGTAGTCA						
P57	AG	F: CGCTATCATCAAGAGCATGCA	60	124–136	6	0.381	0.453	0.158
		R: ACTGTCGATTGACTGGCGTT						

T_m, annealing temperature; A, allele number; H_O, observed heterozygosity; H_E, expected heterozygosity; G_{is}, inbreeding coefficient.

et al., 2013) and QDD v3.1.2 (Meglécz *et al.*, 2014). The threshold for the minimum number of repeats was set at six for dinucleotides and trinucleotides, and five for tetra-, penta- and hexanucleotides. Subsequently, primer pairs were designed employing default parameters of Primer 3 (Untergasser *et al.*, 2012). Eighty-four primer pairs were successfully designed and their universality was ascertained through polymerase chain reactions (PCRs) with 10 individuals randomly chosen from *P. bipinnatifidus* complex. Details of the PCR mixture composition and protocol are provided in the online Supplementary file (Tables S1 and S2). Totally, 48 pairs of primers were successfully amplified, followed by linking with fluorescent adapters. Subsequent PCRs were performed to assess the polymorphisms within the *P. bipinnatifidus* complex (online Supplementary Table S3) using polyacrylamide gel electrophoresis. Allele scoring was conducted manually, by recording co-dominant values to generate a data matrix with GenALEx 6.5 (Peakall and Smouse, 2012) for subsequent analysis. Ultimately, 11 primer pairs with high polymorphisms were identified (Table 1), and the allele data from these loci were utilized to construct the phylogenetic trees encompassing 63 individuals from *P. bipinnatifidus* complex and 13 individuals from three outgroup taxa (*P. notoginseng*, *P. stipuleanatus* and *P. japonicus*) (online Supplementary Table S3). Total DNA extraction from leaf tissues was performed using a modified cetyltrimethylammonium bromide (CTAB) procedure (Doyle, 1991). The trees were constructed using the unweighted pair-group method with arithmetic average (UPGMA) in PowerMarker 3.25 (Liu and Muse, 2005), and a consensus tree (Fig. 1) was obtained using the program 'consensus' in the Phylip package (Felsenstein, 2005) after summarizing 2000 trees.

Discussion

Initially, our analysis detected 7458 loci containing perfect microsatellites in the 9,014,726 RAD reads, including the most abundant dinucleotide repeats (50.60%) and trinucleotide repeats (36.78%) as observed in *P. ginseng* (Choi *et al.*, 2011). After screening treatment following the criteria (online Supplementary Table S4), 11 SSR primer sets were retained and were successfully amplified across all the 63 individuals of the *P. bipinnatifidus* complex, demonstrating extremely high transferability in this taxonomic group. The features of these 11 SSR primer sets and the corresponding indices of genetic diversity within *P. bipinnatifidus* complex are detailed in Table 1. The analysis discovered 76 alleles in total across the 11 SSR loci, averaging 6.9 alleles per locus and ranging from 5 to 15. The observed heterozygosity (H_O) ranged from 0.241 to 0.512, with an overall mean of 0.346. The expected heterozygosity (H_E) ranged from 0.345 to 0.644, averaging 0.469 across loci. The maximum value of inbreeding coefficient (G_{is}) was 0.467 at locus P21, whereas the minimum value was 0.111 at locus P5. Cross-species amplification analysis within the three outgroups indicated high transferability in *P. japonicus* (97.7%), *P. stipuleanatus* (75%) and in *P. notoginseng*

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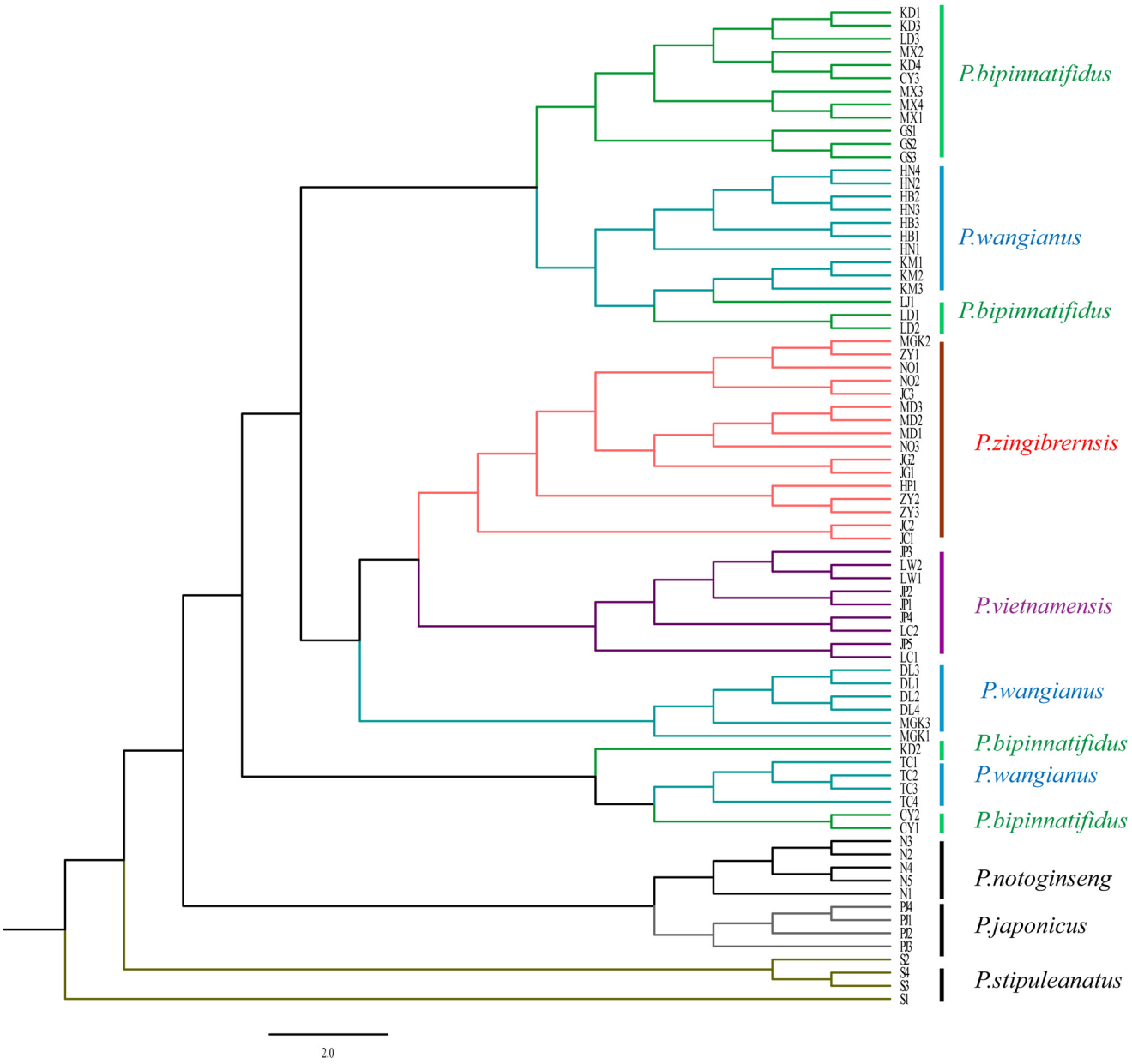

Figure 1. UPGMA tree of 76 individuals based on the data of 11 SSR markers. Branches with different colours represent different taxon clades.

(73.6%). Notably, locus P25 failed to be amplified in any individual of *P. stipuleanatus* and *P. notoginseng*, but succeeded in all *P. japonicus* individuals.

UPGMA tree (Fig. 1) revealed that 63 individuals of the *P. bipinnatifidus* complex were grouped into one big clade. The samples of *P. zingiberensis* and *P. vietnamensis* formed the monophyletic group, respectively, with their sister relationship corroborated by prior research utilizing the RAD-seq data (Zhou *et al.*, 2020). The remaining accessions of *P. bipinnatifidus* and *P. wangianus* formed several smaller clades and nested into each other. Additionally, the samples recently collected from Hunan and Hubei provinces, clustered together, exhibited a close genetic affinity with the samples identified as *P. wangianus* (KM) and *P. bipinnatifidus* (LJ and LD). This genetic concordance substantiated our initial taxonomic assignment of these samples as *P. wangianus*, primarily based on morphological characteristics such as the fleshy roots and lanceolate leaflets. These newly developed SSR markers will be important molecular resources for future investigation on conservation genetics of the wild ginseng plants.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S1479262124000480.

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Competing interests. None.

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