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SHORT REPORT

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Isolation and characterization of 18 microsatellites for the invasive native *Pedicularis kansuensis* (Orobanchaceae)

Wenjun Li[1] | **Zhihao Su**[1] | **Airong Li**[2] | **Kaiyun Guan**[1,2] | **Ying Feng**[1]

[1]CAS Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Urumqi, China

[2]Yunnan Key Laboratory for Research and Development of Wild Plant Resources, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China

Correspondence
Ying Feng, Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Urumqi, China.
Email: luckfy@ms.xjb.ac.cn

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Abstract

To quantify the population-level genetic characteristics of *Pedicularis kansuensis*, polymorphic microsatellite loci (simple sequence repeats) were identified and characterized. Methods and Results: Using Illumina HiSeq paired-end reads from genomic DNA, searches for repeat motifs identified 1594 sequences by four softwares simultaneously. We developed and characterized 18 polymorphic microsatellite primers for *P. kansuensis* using 45 individuals that were collected from three *P. kansuensis* populations. The number of alleles varied from two to 12. The observed heterozygosity and expected heterozygosity per locus ranged from 0.182 to 0.933 and 0.227 to 0.904 in the pooled population, respectively. All of the primers that were developed were also successfully applied in *Pedicularis cheilanthifolia and Pedicularis violascens*. These markers should be useful in probing the genetic diversity, genetic structure of *P. kansuensis*, which could provide information about assessment and prediction of potential risks of this species in its distribution areas.

KEYWORDS
invasive native species, *Pedicularis kansuensis*, polymorphic microsatellites

1 | INTRODUCTION

Rapid spreading of invasive native plants as well as invasive non-native plants has remarkably threatened to the ecosystem function and services. *Pedicularis kansuensis* Maxim. (2*n* = 16), an annual or facultative biennial hemiparasitic herb, is a highland plant species wide spread in western China and Nepal, showing a disjunctive distribution between the Qinghai-Tibetan Plateau (QTP) and the Tianshan Region (TSR) (Huang, Shen, & Lu, 1996; Li et al., 2016; Sui et al., 2016). And it can share markedly variation in morphology and chloroplast DNA, and can adapt to heterogenous habitats, and rapidly expand until the habitats suited. In nearly twenty years, *P. kansuensis* has caused great loss of herbage yield and threatened the local livestock industry because its rapidly expand in population sizes and became weedy in Bayanbulak Grassland of the TSR (Sui, Huang, Li, Guan, & Li,2015). And now, the terrible situation are not only been found in Bayanbulak Grassland, but also in north and east of QTP, such as Menyuan county and Xunhua county in Qinghai province (Bao et al., 2015). Therefore, it is a typical invasive native plant (Alpert, Bone, & Holzapfel, 2000; Li et al., 2016; Sui et al., 2016). Some invasive native plant can adapt the environment under genetic differentiation by changing genetic diversity and genetic structure in the population level (Sultan, 1987). Simple sequence repeat (SSR) markers are highly polymorphic and useful as molecular markers in population genetic studies due to their codominance, hypervariability, and reliable scorability (Kindiger, Conley, Keith-Stanley, & Cai, 2011; Zhao, Yu, Li, Wang, & Dou, 2015). However, there are not suitable microsatellite markers for *P. kansuensis*, though some microsatellite markers have been published in this genus (Cho, Choi, & Choi, 2015; Huang, Yu, Yang, Wang, & Lu, 2010; Huang et al., 2008). In the current study, to facilitate population genetic analyses of effective population size and population structure of the rapidly expand invasive native plant, we developed and characterized 18 genomic microsatellite (SSR) markers for *P. kansuensis* using Illumina next-generation sequencing and a bioinformatics pipeline.

2 | MATERIALS AND METHODS

Genomic DNA was extracted from leaf material of a single plant collected in Bayanbulak, Xinjiang province, China (43°21′N/93°42′E) using

TABLE 1 Characterization of 18 polymorphic microsatellite loci developed in *Pedicularis kansuensis*

Locus	Primer	Repeat motif	Size (bp)	Ta (°C)	GenBank accession no.
PK2	GCATAAAATCGCCTGAAAGC GCGGAAGGACAAGATTCAAG	$(GAA)_6$	255–260	61	MG832824
PK7	TCTCCGCTGATCATGTATGG TTATTGGATTCGGCCTTGAC	$(TCTT)_5$	245–363	61	MG832825
PK9	GGTGTCCGAAAACAGAATGG TCGTTTTAACACTAGCAGAAAGTCC	$(CTT)_{12}$	260–360	61	MG832826
PK10	TGCGAAAGTTGTCAATTTAATCC ATATCCGCACGTGGCTTTAG	$(AAT)_{21}$	214–273	61	MG832827
PK15	CGATAACCCTAAAACTTCGCC TCTCAAAATTTACTAACTTCCAAACA	$(ATT)_6$	215–254	61	MG832828
PK17	TGACTCATGACATGGGGTAAG TGAGTAAAACGGACACCCAA	$(TA)_{17}$	230–319	58	MG832829
PK18	CCCCATTCCATGAAATTCTTT CAAATCAGCAATGTGAACCC	$(AT)_{13}$	138–200	61	MG832830
PK19	GGGTTTCGCTGGTGAATAAA TGGAATTGCTCCAACACTCC	$(AATG)_5$	269–317	61	MG832831
PK20	TATTGTTCTCCGCAAAACCC AGCCCGAAGACGACATAGAG	$(TCT)_{14}$	234–258	61	MG832832
PK21	TTGAACACATAAACAAGATTTCCTT TACGTGGATTCCGTGAGGTT	$(ATAA)_{12}$	202–265	61	MG832833
PK23	CGGATTATAATTATCGACGATCC TCGTTCGATAGAACAATCAACTAAT	$(ATT)_{10}$	241–282	61	MG832834
PK30	CTAGTCGCCAGGTCCATGTT AGAGCCGTTATGTTGCCTTG	$(GTT)_7$	371–443	61	MG832835
PK33	CAGCGATCGCATATGAATTT AAAGCTTGCGCGAGAATTTA	$(GAA)_8$	258–315	61	MG832836
PK34	CAAACCACACCAAACCAAAC CCATAAATTTTCCACGAGTTTCA	$(AT)_{11}$	180–262	58	MG832837
PK35	TTTGCTGCCTACTTCCCTTC TATTGCAAAATGCGGCATAA	$(CA)_{14}$	235–270	61	MG832838
PK37	TGATGTTTCAGTCCCACCAA ACCTGCCATTTTTACTTGCATT	$(CA)_{20}$	171–211	61	MG832839
PK39	TCTTTTGCCTCTCTTCAGCG ATTCGGATTATTGTGGCTGC	$(TTA)_{19}$	110–202	61	MG832840
PK42	CGTGAGTTTTCTTTAACCTATCATC GAAGTTTGGGTGTGCGTAGC	$(AATT)_6$	276–307	61	MG832841

a Plant Genomic DNA Isolation kit (Tiangen, Beijing, China) following the manufacturer's instructions. DNA sequencing libraries were constructed following the manufacturer's protocol (NEBNext® Ultra™ DNA Library Prep Kit for Illumina®, https://www.neb.com/-/media/catalog/datacards-or-manuals/manuale7370.pdf). For each sample, 2 μg genomic DNA was randomly fragmented to <500 bp by sonication (Covaris S220, Covaris, Woburn, MA, USA). Size selection of Adaptor-ligated DNA was then performed using AxyPrep Mag PCR Clean-up (Axygen, New York, NY, USA), and fragments of ~410 bp (with the approximate insert size of 350 bp) were recovered. Each sample was then amplified by polymerase chain reaction (PCR) for 8 cycles using P5 and P7 primers, with both primers carrying sequences which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by Qubit2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Then libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2 × 150 paired-end (PE) configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPipeline-1.8 (Illumina) on the HiSeq instrument. Sequencing resulted in >5.7 million reads, with a modal read length of 300 bp.

We used the Trimmomatic (Bolger, Lohse, & Usadel, 2014) to screen the raw sequences, remove adapter and other illumina-specific sequences and get a clean data with >4.2 million reads that longer than 75 bp. And then, those 4.2 million reads was used

TABLE 2 Genetic properties of 18 newly developed polymorphic microsatellites of *Pedicularis kansuensis*

	XH (*N* = 16)			BLK (*N* = 14)			BY (*N* = 15)			Pooled locations (*N* = 45)			
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e	PIC
PK2	2	0.813	0.514*	4	0.786	0.616**	3	0.933	0.570**	4	0.844	0.558	0.451
PK7	11	0.813	0.780	8	1.000	0.847	8	1.000	0.779*	16	0.933	0.810	0.780
PK9	4	0.375	0.337	5	0.571	0.561	4	0.667	0.641	6	0.533	0.527	0.479
PK10	10	0.500	0.825***	11	0.357	0.876***	12	0.667	0.908**	16	0.511	0.883	0.862
PK15	3	0.313	0.284	3	0.214	0.204	3	0.200	0.191	5	0.244	0.227	0.217
PK17	3	0.188	0.567**	4	0.429	0.635*	5	0.400	0.618*	8	0.333	0.614	0.533
PK18	6	0.500	0.736***	10	0.286	0.889***	9	0.200	0.871***	16	0.333	0.893	0.872
PK19	8	0.688	0.722	7	0.714	0.825*	9	0.733	0.825	12	0.711	0.792	0.761
PK20	7	0.750	0.750	7	0.643	0.780	5	0.400	0.664**	8	0.600	0.781	0.741
PK21	11	0.750	0.871	9	0.857	0.852	10	0.933	0.880	15	0.844	0.904	0.884
PK23	8	0.375	0.766***	4	0.429	0.550	6	0.733	0.763	8	0.511	0.758	0.710
PK30	11	0.625	0.853*	10	0.929	0.876	10	0.667	0.862	18	0.733	0.890	0.870
PK33	5	0.438	0.718*	9	0.571	0.791*	8	0.467	0.821*	10	0.489	0.780	0.744
PK34	2	0.875	0.508**	5	0.786	0.643	7	0.600	0.582	7	0.756	0.579	0.510
PK35	3	0.200	0.301	3	0.143	0.265	5	0.200	0.253	6	0.182	0.270	0.257
PK37	7	0.313	0.774***	4	0.286	0.690**	8	0.333	0.844***	10	0.311	0.797	0.757
PK39	10	0.625	0.679	7	0.714	0.712	9	0.600	0.841*	13	0.644	0.755	0.726
PK42	6	0.438	0.677*	7	0.571	0.799	6	0.667	0.818	9	0.556	0.769	0.727

Notes. *A*, number of alleles; BLK, balikun; BY, bayanbula; *F*, fixation index; *He*, expected heterozygosity under random mating; *Ho*, observed heterozygosity; *N*, number of individuals sampled; PIC, polymorphism information content; XH, Xunhua.
Significant deviation from Hardy-Weinberg expected genotype frequencies: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

to assemble to 1.7 million contigs by Pandaseq (Masella, Bartram, Truszkowski, Brown, & Neufeld, 2012). Among them, 1.3 million sequences (>300 bp) were used to detect SSR by MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html), MREP (http://mreps.univ-mlv.fr/), SSRIT (http://archive.gramene.org/db/markers/ssrtool) and TRF (http://code.google.com/p/highssr/). And, 85295, 137696, 13083 and 528460 sequences were detected by MISA, MREP, SSRIT and TRF, respectively. At last, 1594 sequences were detected by four softwares simultaneously, and were used to design primers with PCR product lengths of 100–280 bp using Primer 3. Among them, 43 pairs of the designed primer were tested for consistency in PCR amplification and polymorphisms.

DNA was extracted for genotyping using a method similar to that described above for leaf samples from 45 individuals collected from three populations in the 2014. Among those three populations, Bayanbulak (42°84′N/83°72′E) and Balikun (43°21′N/93°42′E) locate in Xinjiang, and Xunhua (35°36′N/102°41′E) locate in Qinghai. The PCR amplification was performed using a SimpliAmpTM Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) in a 20 μl reaction mixture containing 0.5 U TransStart Taq Polymerase (TransGen, Biotech, Suzhou, China), 2 μl 10 × PCR Buffer (TransGen, Biotech, Suzhou, China), 0.5 mmol L^{-1} dNTPs, 10 pmol of each primer and (1 μl) 30 ng of template DNA. The forward SSR-specific primer with the M13 universal primer at 5′-end-labeled with 6-FAM according to the method of Markus (2000). The PCR reactions were run for 5 min at 95°C followed by 10 cycles of 30 s at 95°C, 30 s at 61/58°C, and 30 s at 72°C, followed by 32 cycles of 30 s at 95°C, 30 s at 58/55°C, and 30 s at 72°C, with a 5 min final extension at 72°C. The PCR amplification was considered to be successful based on the presence of a visible band after running 1 μl of the PCR product on a 1.5% denaturing agarose gel. The GeneRuler 100 bp plus DNA Ladder molecular weight marker (Thermo Fisher Scientific, Waltham, MA, USA) was used as a standard to assess the product size. If no amplification was detected, that primer set was excluded from further analysis. For the remaining loci, genetic variation was examined in all collected samples.

The PCR products were submitted for genotyping on an ABI3730 automated sequencer (Applied Biosystems, Waltham, MA, USA) using a mixed molecular size marker (10ul LIZ500: 1000 μl Hi-Di, Applied Biosystems). The genotypes were scored using GeneMapper 4.0 (Applied Biosystems). The number of alleles per locus (*A*), observed heterozygosity (H_o), expected heterozygosity (H_e) and polymorphism information content (PIC) for all individuals and three populations were calculated using CERVUS v3.0.7 (Kalinowski, Taper, & Marshall, 2007). Hardy–Weinberg equilibrium at each locus of all populations was evaluated by 1000 randomizations, with adjustment of *p* values by the sequential Bonferroni correction in the web version of GENEPOP (Raymond & Rousset, 1995; Rousset, 2008). Finally, all of the newly developed polymorphic microsatellite loci in *P. kansuensis*

were assessed for cross-amplification in another sympatric species in *P. cheilanthifolia* and *Pedicularis violascens*.

3 | RESULT AND DISCUSSION

In total, 18 of the 43 primer pairs showed clear amplification and polymorphism (Table 1). The number of alleles observed for the 18 polymorphic loci ranged from 2 to 12 for each sampled location, with almost all alleles common to each location. And in the pooled population, the observed and expected heterozygosities ranged from 0.182 to 0.933 and 0.227 to 0.904, respectively (Table 2). Four were moderately polymorphic (0.2 < PIC < 0.5) and 12 loci were highly polymorphic (PIC > 0.5; Table 2). Four loci showed significant departure from Hardy–Weinberg equilibrium with deficits of heterozygotes after Bonferroni corrections ($p < 0.001$). These 18 primer pairs successfully amplified fragments in *P. cheilanthifolia* and *P. violascens*, showing microsatellite polymorphisms.

These 18 microsatellite loci showed a high level of polymorphism. These loci will be used to population genetic studies. This study could provide information for further studies on population level to assess and predict the potential risks of this species in its distribution areas and, guide for long-term and effective management of this invasive native plant.

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ORCID

Wenjun Li http://orcid.org/0000-0002-2932-0783

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