Isolation and characterization of microsatellite loci from *Oxytropis diversifolia* (Fabaceae)

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PREMISE OF THE STUDY: Microsatellite primers were developed for a perennial legume from northern China, *Oxytropis diversifolia* (Fabaceae), to investigate population genetic structure of this taxon, as well as potential hybridization events with closely related taxa in this genus.

METHODS AND RESULTS: One hundred and five primer pairs were designed from Illumina sequence data and screened for suitability. Fifteen of these primer pairs were polymorphic, and these primers amplified tri-, tetra-, and pentanucleotide repeats with 10–56 alleles per locus. Cross-amplification tests in three other *Oxytropis* species from northern China ( *O. leptophylla*, *O. neimonggolica*, and *O. squammulosa*) revealed that all of these loci can be amplified successfully and show polymorphism.

CONCLUSIONS: These primer pairs can be used to assess the genetic diversity and population structure in future studies of *O. diversifolia*, as well as studies of potential hybridization events with closely related taxa in this genus.

KEY WORDS: Fabaceae; next-generation sequencing; nuclear microsatellites; *Oxytropis diversifolia*.

*Oxytropis diversifolia* E. Peter (Fabaceae) is a perennial herb occurring in dry *Stipa* L. grasslands/semi-desert regions of northern China and Mongolia (Zhu et al., 2010). In the Nei Mongol region of China, populations of this species show morphological variation for leaf shape: individuals may have leaves with only one leaflet, three leaflets, or one to three leaflets. Because of the essential role that leaves play in photosynthesis, it is broadly accepted that variation in leaf shape has major ecological and evolutionary consequences, and such a character is expected to experience different selection depending on environmental conditions (Nicotra et al., 2011). However, direct evidence to demonstrate that leaf shape is actually adaptive is comparatively rare (Kidner and Umbreen, 2010). Intraspecific phenotypic variation in leaf shape could simply be the result of either random genetic drift or indirect selection on genetically correlated characters. A classical approach to assess the roles of purely neutral processes and natural selection in phenotypic differentiation is to compare the geographic pattern for the trait of interest to a set of putatively neutral loci (e.g., allozymes, microsatellites, amplified fragment length polymorphism [AFLP] markers, and single-nucleotide polymorphism [SNP] markers). Currently, random-amplified polymorphic DNA (RAPD) and AFLP markers have been developed and used in *Oxytropis* DC. species (e.g., Chung et al., 2004), but microsatellite markers are lacking.

Here, we describe the development of microsatellite markers that will facilitate future research on leaf shape variation in *O. diversifolia*. In addition, the degree of congeneric cross-transferability of the markers was also assessed in three related *Oxytropis* species from northern China: *O. leptophylla* (Pall.) DC., *O. neimonggolica* C. W. Chang & Y. Z. Zhao, and *O. squammulosa* DC. We are particularly interested to test for potential hybridization of *O. leptophylla* with *O. diversifolia* (H. Wang, Northwest A&F University, Yangling, Shaanxi, China, personal observation).

METHODS AND RESULTS

Total genomic DNA was extracted from a dry leaf sample collected in Urad Zhongqi, Nei Mongol, China (Pop8, Appendix I; BioSample accession SAMN08408037), using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1990). A DNA library was constructed with the KAPA Hyper Prep Kit (catalog no. KK8500; Kapa Biosystems, Wilmington, Massachusetts, USA), and 2 × 150-bp paired-end sequencing was performed on an Illumina HiSeq 2500 system (Illumina, San Diego, California, USA) at the Sequencing and Genotyping Facility of Beijing Micoread Gene Technology Co. Ltd. (Beijing, China). A total of 3,421,900 raw sequence reads (2.52 Gbp, GenBank Short Read Archive accession...
SRP131738, BioProject ID PRJNA431827) were obtained. The paired-end reads were then processed using Trimmomatic version 0.35 (Bolger et al., 2014) and merged into ~240-bp sequences using FLASH version 1.2.11 (Magócs and Salzberg, 2011). In total, 3,079,710 clean reads assembled into 2,949,319 contigs.

SSR_pipeline software (Miller et al., 2013) was used to detect tri-, tetra-, and pentanucleotide repeats on the sequence set, and Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA) was used to develop primers for 105 loci, prioritizing motif diversity and melting temperature difference ≤1°C. An M13 tag (5′-TGTAAAACGACGGCCAGT-3′) was added to the 5′ end of the shorter primer of each locus. These primer pairs were tested on seven O. diversifolia individuals from different populations (Appendix 1). Each locus was initially amplified individually in 15-μL PCR reactions that contained 1.5 μL of 10× Buffer I, 200 μM of dNTPs, 0.27 μM of M13 primer (labeled with HEX), 0.1 μL of 1× TaKaRa HS Taq (TaKaRa Biotechnology, Dalian, Liaoning, China), 0.67 μM of reverse primer, and 1 μL of diluted template DNA. PCR thermocycling conditions were an initial denaturation of 95°C for 5 min; 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s; and a final extension at 60°C for 30 s, 56°C for 30 s, and 72°C for 30 s; followed by 10 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s; and a final extension at 60°C for 30 min. The PCR products were examined on a 2% agarose gel.

TABLE 1. Characteristics of 15 microsatellite loci developed for Oxytropis diversifolia.*

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | Fluorescent dye (Group) | GenBank accession no. |
|-------|-------------------------|--------------|-----------------------|-------------------------|----------------------|
| N754892 | F: CGGTGATATTTCAATTTGCG | (ATAG)12 | 156–274 | 6-FAM (1) | MG693777 |
| R: TGGGTCCCACTTATGGTTATCC | | | | | |
| N145635 | F: CTGGGTGAGAAGAGGAGAAGA | (GAG)12 | 102–207 | HEX (1) | MG693767 |
| R: TTCTCAAGCTTACTATTTTGGAC | | | | | |
| N2724893 | F: ACTAAGGGACACCATATATCA | (AAC)10 | 108–138 | ROX (1) | MG693774 |
| R: TACCTGATAATCATTTGGGA | | | | | |
| N876535 | F: GAGGAAAGGGGAAGTGGAGA | (TCT)10 | 126–322 | ROX (1) | MG693779 |
| R: CGATGCGTGATTACCTTTAGCA | | | | | |
| N2720763 | F: GCCGTTTATGATGATTACCTT | (TTT)10 | 119–215 | 6-FAM (2) | MG693773 |
| R: GAAAGACTGAGGGGTAATCCCA | | | | | |
| N2717495 | F: CCAACAATCAAAATTTGGCGG | (TCTA)10 | 144–199 | HEX (2) | MG693772 |
| R: GGAGTGTGTTTGTGATGAAAGT | | | | | |
| N178451 | F: TCAGTACTTCTCCACATCA | (ATA)12 | 97–183 | ROX (2) | MG693769 |
| R: GGGAAATAGAGAGATATCCACTGC | | | | | |
| N161850 | F: CTGCACTACACCTTCTTGTGTT | (AAT)12 | 105–180 | 6-FAM (3) | MG693768 |
| R: CCAACAACCTTCCTTCTGCG | | | | | |
| N49251 | F: CCAATGCGACGACCTCTTCAAA | (TCT)11 | 103–136 | HEX (3) | MG693776 |
| R: GGAGTACGCAATCCAGTCTTCAAAA | | | | | |
| N350553 | F: TCAATTCCATCTCCTGGAACC | (TTT)12 | 130–280 | HEX (3) | MG693775 |
| R: TGGAGCTACATCCATCTACGA | | | | | |
| N935993 | F: GATCACCTGGTGATGATTGGG | (ATG)12 | 90–117 | ROX (3) | MG693780 |
| R: CGCACTACACCCCTTGGAAT | | | | | |
| N1172223 | F: TGGGATATGGAGGAGTCTGAG | (ATA)12 | 107–197 | ROX (3) | MG693766 |
| R: GACACCCCCGCATCAAT | | | | | |
| N803014 | F: CTGGATGAGATTGGCTCTGAG | (AAT)12 | 125–197 | 6-FAM (4) | MG693778 |
| R: TGGATTTTCTATGCAGAAG | | | | | |
| N2582349 | F: TCCTCTAATGAGGATCTCAGGA | (ATCT)10 | 136–224 | HEX (4) | MG693770 |
| R: TGGAGATGAGAAGACCAAA | | | | | |
| N2697375 | F: TGGCCTATGTTTGGGTTA | (TTATG)13 | 141–238 | ROX (4) | MG693771 |
| R: TCAAGAAGGAAATACCTGGGA | | | | | |

*All values are based on 114 samples representing four populations (Baotoubei, Pop8, Hu, Dian1) located in dry grassland-semi-desert regions of northern China. For details of voucher and locality information, see Appendix 1.

**Annealing temperature was 56°C for all loci.**
in *O. diversifolia*. Tests of pairwise linkage disequilibrium were performed using GENEPOP 4.7 (Rousset, 2008). Only two genotypic disequilibria out of 420 (N876535 and N2720763, N876535 and N35053 in Pop8) were significant at the 5% level after Benjamini–Hochberg correction (Benjamini and Hochberg, 1995). We also used exact tests implemented by GENEPOP software to test for departure from Hardy–Weinberg equilibrium (HWE). A significant departure from HWE was recorded for almost all loci across the four populations (Table 2). Nine of the 15 loci failed to meet HWE expectations in at least one population. MICRO-CHECKER (van Oosterhout et al., 2004) identified the possibility of null alleles in some loci. Three loci (N2724893, N350553, N803014) showed evidence of stuttering in some populations, but not consistently across populations, indicated as a deficit of heterozygote genotypes with alleles of one repeat unit difference. No large allele dropouts were identified. These departures from HWE are likely due to inbreeding or genetic drift.

Cross-amplification of the 15 primer pairs was conducted on three related species from northern China: *O. leptophylla*, *O. neimonggolica*, and *O. squammulosa* (Appendix 1). All of the loci were successfully amplified and polymorphic. The number of alleles per locus varied from two to 22 in *O. leptophylla*, three to 21 in *O. neimonggolica*, and two to 18 in *O. squammulosa* (Table 3).

### CONCLUSIONS

The 15 polymorphic microsatellites developed here will be used for population genetic studies on *O. diversifolia*. Cross-amplification experiments confirmed that these markers should be applicable in *O. neimonggolica*, *O. leptophylla*, and *O. squammulosa*, thus providing a novel population genetic tool in *Oxytropis*. The low-genomic-coverage Illumina sequencing reads generated in the present study could potentially be used to assemble high-copy-number gene regions, such as complete or partial chloroplast and mitochondrial genomes, as well as nuclear ribosomal RNA genes. Such gene sequences can be informative in phylogenetic

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**Table 2.** Genetic properties of the 15 polymorphic microsatellite loci in *Oxytropis diversifolia*.

| Locus     | Baotoubei (n = 20) | Pop8 (n = 30) | Hu (n = 32) | Dian1 (n = 32) | Total (n = 114) | A_s |
|-----------|-------------------|---------------|-------------|----------------|-----------------|-----|
| N745892   | 11                | 0.550 0.864***| 17 0.600 0.889***| 17 0.563 0.921***| 21 0.710 0.937***| 29  |
| N145635   | 8                 | 0.111 0.832***| 16 0.517 0.877***| 19 0.690 0.928***| 18 0.645 0.906***| 29  |
| N2724893  | 6                 | 0.750 0.728   | 7 0.433 0.699***| 8 0.452 0.759***| 8 0.406 0.584***| 11  |
| N876535   | 10                | 0.316 0.868***| 27 0.724 0.946***| 28 0.781 0.968***| 28 0.531 0.960***| 56  |
| N2720763  | 8                 | 0.421 0.852***| 12 0.567 0.866***| 10 0.438 0.880***| 10 0.400 0.871***| 13  |
| N2717495  | 9                 | 0.278 0.876***| 14 0.241 0.859***| 18 0.531 0.925***| 18 0.367 0.936***| 25  |
| N178451   | 9                 | 0.368 0.836***| 14 0.800 0.873* | 12 0.688 0.850*  | 12 0.563 0.900***| 18  |
| N161850   | 8                 | 0.850 0.837***| 18 0.897 0.907***| 21 0.844 0.924** | 16 0.839 0.901*  | 25  |
| N49251    | 8                 | 0.750 0.791***| 9 0.733 0.815***| 14 0.469 0.764***| 16 0.467 0.823***| 35  |
| N350553   | 7                 | 0.313 0.788***| 21 0.500 0.930***| 14 0.781 0.785***| 8 0.581 0.717*  | 10  |
| N935993   | 5                 | 0.600 0.601***| 8 0.567 0.767*  | 10 0.781 0.785*  | 8 0.581 0.717*  | 10  |
| N1172223  | 12                | 0.700 0.885***| 17 0.444 0.924***| 19 0.533 0.925***| 18 0.483 0.947***| 26  |
| N803014   | 3                 | 0.412 0.597*  | 9 0.048 0.817***| 10 0.240 0.857***| 10 0.160 0.829***| 16  |
| N2528349  | 7                 | 0.368 0.812***| 14 0.600 0.788***| 6 0.469 0.567*  | 7 0.531 0.589*  | 18  |
| N2697375  | 8                 | 0.750 0.835***| 17 0.552 0.898***| 18 0.677 0.921***| 16 0.633 0.900***| 31  |

Note: A_s = number of alleles detected across all individuals; A = total number of alleles; H_e = unbiased expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

aVoucher and locality information are provided in Appendix 1.

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**Table 3.** Cross-amplification of the 15 microsatellites developed for *Oxytropis diversifolia* in *O. neimonggolica*, *O. leptophylla*, and *O. squammulosa*.

| Locus     | O. leptophylla (n = 19) | O. neimonggolica (n = 20) | O. squammulosa (n = 16) |
|-----------|-------------------------|---------------------------|-------------------------|
| N745892   | 13 188–250              | 17 154–262                | 10 160–234              |
| N145635   | 6 114–129               | 18 99–228                 | 4 99–117                |
| N2724893  | 6 120–135               | 7 120–138                 | 7 117–138               |
| N876535   | 16 154–316              | 20 135–226                | 18 194–310              |
| N2720763  | 5 183–215               | 9 141–207                 | 2 167–171               |
| N2717495  | 11 144–210              | 15 160–202                | 4 164–170               |
| N178451   | 11 103–158              | 10 103–168                | 7 103–158               |
| N161850   | 22 102–171              | 19 102–192                | 7 105–177               |
| N49251    | 4 106–118               | 6 112–130                 | 8 112–133               |
| N350553   | 3 172, 178              | 6 96–111                  | 6 172–187               |
| N935993   | 2 102, 105, 108         | 21 136–262                | 3 99, 105, 111          |
| N1172223  | 6 125–179               | 16 131–203                | 14 137–203              |
| N803014   | 4 134–215               | 3 134, 137, 140           | 6 134–149               |
| N2528349  | 10 150–244              | 6 149–184                 | 3 154, 158, 162         |
| N2697375  | 10 157–205              | 17 161–209                | 3 161, 167, 205         |

Note: A = number of alleles detected across all individuals; n = number of individuals sampled.

aVoucher and locality information are provided in Appendix 1.
reconstruction of the genus *Oxytropis*, or even in a much broader phylogenetic scope.

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**AUTHOR CONTRIBUTIONS**

H.W. designed the experiment, collected samples in the field, analyzed the data, and wrote the manuscript. H.Y. did the molecular work under the supervision of H.W. P.-L.L. was responsible for the field work, collected the voucher specimens, and participated in writing the manuscript. C.S. and L.X. participated in the field work under the supervision of H.W. P.-L.L. was responsible for the field work, collected the voucher specimens, and participated in writing the manuscript. Z.-Y.C. supervised the entire study. All authors approved the final version of the manuscript.

**DATA ACCESSIBILITY**

Next-generation sequencing data: (1) BioSample accession SAMN08408037, (2) BioProject ID PRJNA431827, (3) GenBank Short Read Archive accession SRP131738. Sequence data for the 15 microsatellite loci were submitted to GenBank, and accession numbers are listed in Table 1.

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**APPENDIX 1**

Voucher and locality information for *Oxytropis* species used in this study.

| Species | Population | n   | Voucher no.* | Collection locality | Geographic coordinates |
|---------|------------|-----|--------------|---------------------|------------------------|
| *Oxytropis diversifolia* E. Peter | Baotoubei | 20 | Chang2016005 | Baotou, Nei Mongol, China | 40°42′53.9"N, 110°6′15.9"E |
| *O. diversifolia* | Pop8 | 30 | Chang2016024 | Urad Zhongqi, Nei Mongol, China | 41°24′06.3"N, 109°21′8.65"E |
| *O. diversifolia* | Hu | 32 | Chang2017034 | Urad Zhongqi, Nei Mongol, China | 41°34′16.3"N, 108°18′31.1"E |
| *O. diversifolia* | Dian1 | 32 | Chang2017030 | Urad Zhongqi, Nei Mongol, China | 41°34′16.3"N, 108°18′31.1"E |
| *O. diversifolia* | Pop6 | 1 | Chang2016022 | Urad Zhongqi, Nei Mongol, China | 41°34′16.3"N, 108°18′31.1"E |
| *O. diversifolia* | Damaonan | 1 | Chang2016015 | Urad Zhongqi, Nei Mongol, China | 41°25′58.13"N, 109°58′13.5"E |
| *O. diversifolia* | Changewenduoer | 1 | Chang2016076 | Urad houqi, Nei Mongol, China | 41°28′64.7"N, 106°57′05.4"E |
| *O. leptophylla* (Pall.) DC. | Bop2 | 19 | Chang2016088 | Guyang County, Nei Mongol, China | 41°4′15.7"N, 110°6′25.2"E |
| *O. neimongolica* C. W. Chang & Y. Z. Zhao | Yangcigoukou | 20 | Chang2017005 | Aba Zhuqi, Nei Mongol, China | 39°1′89.7"N, 106°7′26.56"E |
| *O. squammulosus* DC. | Line | 16 | Chang2017043 | Urad Zhongqi, Nei Mongol, China | 41°24′06.3"N, 109°21′8.65"E |

Note: n = number of individuals sampled; Chang = Zhao-Yang Chang, group collection indicator.

*All voucher specimens are deposited in the Northwest A&amp;F University Herbarium (WKU), Yangling, Shaanxi, China.

Sample used for initial library construction.