Characterization of Polymorphic Microsatellite Markers for Primula sikkimensis (Primulaceae) Using a 454 Sequencing Approach

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CHARACTERIZATION OF POLYMORPHIC MICROSATTELITE MARKERS FOR *PRIMULA SIKKIMENSI S* (PRIMULACEAE) USING A 454 SEQUENCING APPROACH

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**Premise of the study:** Microsatellite markers from *Primula sikkimensis* (Primulaceae) were developed for testing deep lineage divergence and speciation events.

**Methods and Results:** A total of 3112 microsatellites were identified from 61,755 unique reads though 454 pyrosequencing technology. Twenty-nine microsatellite loci were selected for PCR amplification and polymorphic analyses. Among the 29 tested markers, 17 microsatellite loci were further used for genotyping in three wild *P. sikkimensis* populations. The number of alleles varied from one to eight, and the observed heterozygosity ranged from 0.111 to 1.000. Ten simple sequence repeat loci could be successfully cross-amplified in two *Primula* species. The transferability values were 76.5% in *P. florindae* and 58.8% in *P. alpicola*, respectively.

**Conclusions:** These microsatellite markers will be valuable for testing the hypothesis of lineage divergence, genetic introgression, and cryptic speciation events between *P. sikkimensis* and its closely related taxa.

**Key words:** cross-amplification; deep lineage divergence; genetic introgression; microsatellites; *Primula sikkimensis*; Primulaceae.

The Himalayan region and the adjacent Hengduan Mountains of southwestern China, known as the Himalaya–Hengduan Mountains (HHM) region, have been designated as two of the world’s 34 most important biodiversity hotspots (Myers et al., 2000). The HHM region is considered to be the cradle of many endemic plant groups (Li and Li, 1993) and the center for rapid radiation of several large alpine genera, such as *Primula* L., *Pedicularis* L., and *Rhododendron* L., as well as the center of the Sino-Himalayan floristic subkingdom (Wu and Wang, 1983). Its high species endemism is a likely product of high net diversification rates in the region, as seen in páramo hotspots evaluated by Madriñán et al. (2013). A number of studies have been devoted to the differences between the two parts of the HHM region (the Himalayas and the Hengduan Mountains), such as the direction of the mountain ranges, the time scale of the Qinghai–Tibet plateau uplift process, and the effects of climate oscillations during the Quaternary (Favre et al., 2015). Correspondingly, the Sino-Himalayan floristic subkingdom in the HHM region has been recognized as including at least four subregions (Wu et al., 2011). However, it is not clear whether these differences between the Hengduan Mountains and the Himalayan regions have resulted in deep intraspecific lineage divergences and/or cryptic speciation in plant groups.

*Primula sikkimensis* Hook. (Primulaceae) is an endemic species in the HHM region (Hu and Kelso, 1996) and is the only species in *Primula sect. Sikkimensis* that is widely distributed in the region. It therefore provides a good example to examine the hypothesis of deep lineage divergence between the Himalaya and Hengduan mountains (Gao et al., 2007). Here, we developed a set of variable microsatellite markers using 454 pyrosequencing technology and further tested its cross-amplification in closely related taxa. These microsatellite markers will be important tools for surveying genetic divergence and cryptic speciation events in *P. sikkimensis* and its relatives.

**METHODS AND RESULTS**

Leaf samples of 62 individuals were collected in three populations from Chayu, Galongla, and Luding in China (Appendix 1). One individual of *P. sikkimensis* (sampled from Julong, China; Appendix 1) was used to isolate the microsatellite loci. Voucher specimens have been deposited at the herbarium of the South China Botanical Garden (IBSC), Guangzhou, Guangdong, China. Total DNA extraction of all samples was performed using a modified version of the cetyltrimethylammonium bromide (CTAB) protocol of Doyle and Doyle (1987). Microsatellite markers were isolated using a high-throughput genomic sequencing method as described by Wang et al. (2015). A shotgun library shearing 1 μg of genomic DNA was built using the DNA Library Preparation Kit (Roche Applied Science, Indianapolis, Indiana, USA) following the GS FLX+ library preparation protocol. The library was further enriched by hybridization with biotinylated primer probes. Leaf DNA libraries were sequenced on a Roche 454 GS FLX+ platform.

A total of 3112 microsatellites were identified from 61,755 unique reads though 454 pyrosequencing technology. Twenty-nine microsatellite loci were selected for PCR amplification and polymorphic analyses. Among the 29 tested markers, 17 microsatellite loci were further used for genotyping in three wild *P. sikkimensis* populations. The number of alleles varied from one to eight, and the observed heterozygosity ranged from 0.111 to 1.000. Ten simple sequence repeat loci could be successfully cross-amplified in two *Primula* species. The transferability values were 76.5% in *P. florindae* and 58.8% in *P. alpicola*, respectively.

**Conclusions:** These microsatellite markers will be valuable for testing the hypothesis of lineage divergence, genetic introgression, and cryptic speciation events between *P. sikkimensis* and its closely related taxa.

**Key words:** cross-amplification; deep lineage divergence; genetic introgression; microsatellites; *Primula sikkimensis*; Primulaceae.

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Table 1. Characteristics of 17 microsatellite loci developed in *Primula sikkimensis*.

| Locus | Primer sequences (5′–3′) | Repeat motif | Fluorescent dye | Allele size range (bp) | $T_e$ (°C) | GenBank accession no. |
|-------|--------------------------|--------------|----------------|------------------------|-----------|----------------------|
| S1    | F: CCCCCTGTCGCAAGATTGCTT | (AG)$_{12}$  | HEX            | 148–168                | 54        | KU697616             |
|       | R: AAGATCGACCCACGATCAAT  | (CT)$_{14}$  | FAM            | 228–288                | 60        | KU697617             |
| S2    | F: CCAACACACAAACACCC     | (AAC)$_{13}$ | HEX            | 148–191                | 60        | KU697618             |
| S3    | F: CCACTTGATGGATTAGGCGG  | (AG)$_{18}$  | HEX            | 106–124                | 59.8      | KU697619             |
| S4    | F: CCCTAGATCTCCAGCGAGTG  | (AG)$_{16}$  | HEX            | 124–131                | 60        | KU697620             |
| S5    | F: GATTTGCAATAGCGAGAGCC  | (AG)$_{16}$  | HEX            | 98–122                 | 60        | KU697623             |
| S6    | F: GGGTGTTCCAAGATTTGGTG  | (AG)$_{15}$  | HEX            | 118–130                | 64.9      | KU697624             |
| S7    | F: GGGCAGCGATGGATTAGGCGG | (CT)$_{13}$  | HEX            | 160–182                | 60        | KU697627             |
| S8    | F: GAGAGACCGATGGATTAGGCGG| (AG)$_{13}$  | HEX            | 234–300                | 59.8      | KU697622             |
| S9    | F: GGGTAGCCGTCTCTCTCC    | (GT)$_{15}$  | HEX            | 272–290                | 62.3      | KU697624             |
| S10   | F: AAACGCTATTCTTGGTCTGAG| (AG)$_{16}$  | HEX            | 260–328                | 60        | KU697625             |
| S11   | F: CGATGAAAGAAACTGAGACGA| (AG)$_{16}$  | HEX            | 198–218                | 59.8      | KU697630             |
| S12   | F: CAAAACACACAAACACCC    | (AAC)$_{13}$ | HEX            | 148–191                | 60        | KU697618             |
| S13   | F: ATGTTACCGACTCTTTCTCA  | (AG)$_{16}$  | HEX            | 234–300                | 59.8      | KU697622             |
| S14   | F: GGAATTGAGAGGAGACGAGA  | (AG)$_{16}$  | HEX            | 98–122                 | 60        | KU697623             |
| S15   | F: ATGTTACCGACTCTTTCTCA  | (AG)$_{16}$  | HEX            | 118–130                | 64.9      | KU697624             |
| S16   | F: GGTACCGGCTTATCCTTTTA | (TC)$_{14}$  | FAM            | 234–300                | 59.8      | KU697622             |
| S17   | F: GGTACCGGCTTATCCTTTTA | (AG)$_{15}$  | HEX            | 118–130                | 64.9      | KU697626             |
| S18   | F: GGTACCGGCTTATCCTTTTA | (AG)$_{15}$  | HEX            | 118–130                | 64.9      | KU697626             |
| S19   | F: GGTACCGGCTTATCCTTTTA | (AG)$_{15}$  | HEX            | 118–130                | 64.9      | KU697626             |

**Note:** $T_e$ = annealing temperature.
genetic studies. Cross-amplification of these microsatellite loci in two related Primula species (P. alpica and P. florinidae) was successful, which enables further studies to clarify underlying genetic introgression and cryptic speciation events between P. sikkimensis and its closely related taxa.

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APPENDIX 1. Locality and voucher information for *Primula* individuals used in this study. Voucher specimens are deposited at the herbarium of the South China Botanical Garden (IBSC), Guangzhou, Guangdong, China.

| Species       | Population code | Collection locality   | Geographic coordinates    | Voucher no. |
|---------------|-----------------|-----------------------|---------------------------|-------------|
| *Primula sikkimensis* | XZCY            | Chayu, Tibet          | 27°00′N, 100°10′E         | Hao 934     |
|               | SCLD            | Luding, Sichuan       | 29°55′N, 102°3′E          | Hao 456     |
|               | XZGLL           | Galongla, Tibet       | 29°16′N, 95°05′E          | Wuxing s.n. |
|               |                 | Juulong, Sichuan      | 29°0′N, 101°30′E          | Y2014163    |
| *Primula alpicola* | —               | Paizhen, Tibet        | 29°19′N, 95°19′E          | Hao & Xu 120195 |
| *Primula florindae* | —               | Lulang, Tibet         | 29°42′N, 94°43′E          | Hao & Xu 120281 |