Development of microsatellite markers for Viscum coloratum (Santalaceae) and their application to wild populations

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Premise of the study: Microsatellite primers were developed for Viscum coloratum (Santalaceae), a semiparasitic medicinal plant that is known for its anticancer properties. Due to excessive human harvesting and loss of suitable habitat of its populations, it has become a potentially threatened species requiring immediate conservation efforts.

Methods and Results: Based on transcriptome data for V. coloratum, 124 primer pairs were randomly selected for initial validation, of which 19 yielded polymorphic microsatellite loci, with two to six alleles per locus. The usefulness of these markers was assessed for 60 individuals representing three populations of V. coloratum. Observed and expected heterozygosity values ranged from 0.033 to 0.833 and 0.032 to 0.672, respectively. Cross-species amplification for 19 loci in the related species V. album was conducted.

Conclusions: The 19 newly developed loci are expected to be useful for studying the population genetics and ecological conservation of V. coloratum.

Key words: genetic diversity; medicinal plant; microsatellite; mistletoe; Santalaceae; Viscum coloratum.

Mistletoes have been proposed to be a keystone resource influencing biodiversity in forest ecosystems globally (Cooney and Watson, 2008). The Korean mistletoe, Viscum coloratum (Kom.) Nakai (Santalaceae), is distributed in many countries, including Korea, Japan, China, and Russia (Qi and Gilbert, 2003). Viscum L. species have lectins that are known for their potential therapeutic, immunomodulatory, and anticancer properties (Lavastre et al., 2002; Lyu and Park, 2007). According to previous studies, V. coloratum possesses similar cytotoxic and immunological activities as seen in European mistletoe, V. album L. (Lee et al., 2009; Lyu and Park, 2010). Such uses have led to a great demand for these plants, resulting in the large-scale harvesting of wild populations of V. coloratum. The increasing demand has raised concerns about its status as a potentially threatened species. Recently, the environmental management of mistletoes for conservation has become an international focus. For example, the International Union for Conservation of Nature (IUCN) has listed 19 species of mistletoe on the official IUCN Red List of Threatened Species (International Union for Conservation of Nature, 2006). For this reason, the genetic diversity and population structure of V. coloratum should be immediately investigated for resource conservation. Despite the ecological and medical importance of V. coloratum, no studies have evaluated the genetic diversity in wild populations of this species.

Expressed sequence tags–simple sequence repeats (EST-SSRs) have proven valuable for their cross-transferability, facilitating studies of population genetic diversity in many plant species (Dikshit et al., 2015; Zhou et al., 2016). In this study, 19 polymorphic microsatellite loci for V. coloratum were developed based on EST data obtained from Illumina paired-end sequencing. The usefulness of these markers was assessed for 60 individuals representing three populations of V. coloratum in Korea, Japan, and China. Cross-species amplification was tested using 20 individuals of V. album, a close relative of V. coloratum.

METHODS AND RESULTS

We collected 60 individuals of V. coloratum from natural populations from three countries (Korea, Japan, and China), and the voucher specimens representing each population were deposited in the Herbarium of the National Institute of Biological Resources (KB) and the Herbarium of Hallym University (HHU), Republic of Korea (Appendix 1). To test cross-species amplification, we collected 20 individuals of V. album from a single population in Japan (Appendix 1). Whole genomic DNA was extracted from silica gel–dried leaf tissue using the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). DNA concentrations were estimated using the NanoDrop 2000c (Thermo Fisher Scientific, Waltham, Massachusetts, USA), and samples were stored at −20°C.

For RNA library construction, total RNA was extracted from the leaf of a single individual plant collected from Korea (voucher no.: GEIBGR0000298682;
Vi-06
F: ATCATGCGCAATACAAACCTCTAC
R: GAGAATCTGACACCCAAGGAA
CAT6
362–365 bp
58
FAM
Pr032816424
Vi-13
F: ATCTATCTAACAACATTCGG
R: TATTTGAGTCTTCTCCTACTCG
TCT7
391–397 bp
58
FAM
Pr032816426
Vi-14
F: TAGCACTCTCTCTGAGGCTTT
R: GGTTGTGATGGATCATTAAA
TCT7
160–166 bp
58
FAM
Pr032816431
Vi-22
F: CCAATTTCCTGATGACCTCTA
R: TTCTATGATTCCTCCCTGGAT
GAA6
320–344 bp
58
FAM
Pr032816420
Vi-25
F: ATTCATCACCTCTAACACCAC
R: GTAGATGCGGATGCTATCC
GAA6
290–296 bp
57
FAM
Pr032816428
Vi-26
F: TTGTGAGAATCTCCTACTCTCTA
R: TATTTGGGTTTTCTCCATAACG
GAA6
250–259 bp
58
FAM
Pr032816429
Vi-31
F: CCCCATTTCCTCTCCCTCTAG
R: CCTCTTAACACTGCTCTCCCG
CTC7
341–347 bp
58
HEX
Pr032816430
Vi-32
F: CTTAGAGAGGGCGACAGAG
R: GATCATAGTTCCGAAATACC
GAC7
143–151 bp
58
HEX
Pr032816422
Vi-54
F: TGAGAAGGATGACCTCTTTTT
R: AGACACCCTCTCTCTCTCTCT
GGC6
248–254 bp
58
HEX
Pr032816416
Vi-60
F: GTTTAAATCCCAGATCCAGAT
R: CACATGCGGAGGACTAATTT
GAC6
230–233 bp
58
FAM
Pr032816425
Vi-63
F: CCAAAATGATACAGGACACAG
R: ATTACCATCCCACAGGACAT
AAG6
435–441 bp
58
HEX
Pr032816415
Vi-71
F: GCGAATTACAAGGCTCGG
R: CATCCTCCTCTCTCTCTCT
CAT6
349–364 bp
58
FAM
Pr032816419
Vi-77
F: GAGAAGGATGACAGGCT
R: CATGACCTGACGTTGGAGGA
AGA6
131–134 bp
58
HEX
Pr032816414
Vi-83
F: AATGACCTCTCTGATGATGGCT
R: CCTTTTGCTGCTCTCTGG
TTA6
170–176 bp
58
FAM
Pr032816427
Vi-87
F: ACCCTCTTGCGAAGAATAAG
R: ATCCACCACCTCTCTCTCT
AGC6
185–191 bp
58
FAM
Pr032816421
Vi-88
F: GCCCTCAGGCGCTCTCTCTCT
R: AAGAAGGATGACCCGGGAT
AGC6
289–298 bp
58
FAM
Pr032816423
Vi-96
F: GGGTTTCTCTTTCTCCGGAT
R: GAGAATCTGACCCGACGAC
GA7A
318–321 bp
58
FAM
Pr032816417
Vi-97
F: GTCCTGAAGATGGACAGAGC
R: GAAGCTCCTTAAAGGACGAT
GA7A
306–318 bp
58
HEX
Pr032816418
Vi-108
F: TGGCTCTGAGAATGTTGCT
R: TGCTCTGAGAAATCTCTCTC
GGC6
349–364 bp
57
FAM
Pr032816413

Note: $T_a$ = annealing temperature.

http://www.bioone.org/loi/apps
countries, these markers exhibited favorable stability and high degrees of polymorphism, with an average of 3.26 per marker. The observed and expected heterozygosity ranged from 0.033 to 0.833 and 0.032 to 0.672, respectively (Table 2). Thirteen loci significantly deviated from Hardy–Weinberg equilibrium after Bonferroni correction (√0 < 0.05) within the populations. Additional tests of cross-amplification in *V. album* were successful across all 19 markers (Table 3).

### CONCLUSIONS

In this study, we developed 19 novel polymorphic microsatellite markers for the medicinal plant *V. coloratum*. The results of cross-species amplification testing indicate that these markers can also be applicable for the genetic investigation of the related species *V. album*. These markers will be useful for estimating the genetic structure and diversity among and within populations of these species, and will further help in the development of effective strategies for their conservation.

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**APPENDIX 1.** Locality and voucher information for *Viscum coloratum* and *V. album* populations sampled in this study. Voucher specimens were deposited in the Herbarium of the National Institute of Biological Resources (KB) and the Herbarium of Hallym University (HHU), Republic of Korea.

| Species                  | Population | Locality          | n   | Geographic coordinates | Voucher no. |
|--------------------------|------------|-------------------|-----|------------------------|-------------|
| *Viscum coloratum* (Kom.) Nakai | Korea      | Hapcheon, Gyeongnam | 20  | 35°47′59.9″N, 128°05′00.1″E | GEIBGR0000298682 |
|                          | Japan      | Higashiomi, Shiga | 20  | 35°06′29.4″N, 136°13′43.6″E | GEIBGR0000298782 |
|                          | China      | Yanbian, Jilin    | 20  | 42°25′09.3″N, 128°02′60.1″E | GEIBGR0000298761 |
| *Viscum album* L.        | Japan      | Higashi, Fukuoka  | 20  | 33°37′52.2″N, 130°26′26.8″E | KNR2015086   |

Note: n = number of individuals sampled.