Development of Microsatellite Markers for Viscum coloratum (Santalaceae) and Their Application to Wild Populations

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Primer Note

Development of microsatellite markers for *Viscum coloratum* (Santalaceae) and their application to wild populations[1]

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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 (Qiu 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;

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Appendix 1). Total RNA quality and quantity were verified using the NanoDrop 2000c (Thermo Fisher Scientific) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California, USA). We constructed Illumina-compatible transcriptome libraries using a TruSeq RNA Library Preparation Kit version 2 (Illumina, San Diego, California, USA), according to the manufacturer's instructions. In brief, mRNA was purified from total RNA by polyA selection, and was then chemically fragmented and converted into single-stranded cDNA with random hexamer-primed reverse transcription. A second cDNA strand was generated to create double-stranded cDNA for TruSeq library construction. The short double-stranded cDNA fragments were then connected using sequencing adapters. Finally, RNA libraries were built by PCR amplification. The RNA libraries were quantified using real-time PCR (qPCR), according to the qPCR Quantification Protocol Guide (Illumina), and qualified using an Agilent 2200 Bioanalyzer.

Paired-end 150-bp sequencing of *V. coloratum* was conducted on the Illumina HiSeq 2000 platform. All sequence information has been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (Bioproject no. SRP092226). Adapter/quality trimming was performed using Trimmomatic 0.32 (Bolger et al., 2014) with the following parameters: seed mismatch of 2, palindrome clip threshold of 30, simple clip threshold of 10, a minimum adapter length of 2, headcrop of 7, leading and trailing quality of 3, sliding window size of 4 with an average quality of 20 and a minimum sequence length of 50 bases. After trimming, there were 39,226,078 reads for a total length of 6,216,400,383 bp. The de novo transcriptome assembly of these reads was performed using the short-read assembling program Trinity (Haas et al., 2013) with default settings: seqType fq, min contig length 200, group pair distance 500, path reinforcement distance 75, min kmer cov 1, SS lib type FR.

Microsatellites were detected using the Perl script MIcroSAtellite (MISA) identification tool (Thiel et al., 2003) with thresholds of 10 repeat units for mononucleotides, six for dinucleotides, and five for tri-, tetra-, penta-, and hexanucleotides. MISA identified 15,562 microsatellite sequences, of which 124 loci were selected for further testing (based on the above criteria) in 60 individuals of *V. coloratum* from three countries (Appendix 1). Primers were designed using Primer3 (Rozen and Skaletsky, 1999) to flank the microsatellite-rich regions with a minimum of six repeats.

PCRs were performed in a total volume of 25 μL containing 10× Ex Taq buffer (TaKaRa Bio Inc., Otsu, Shiga, Japan) 2.5 μL, 2.5 mM dNTPs 2 μL, 0.01 μM forward primers, 0.01 μM reverse primers, 5 units TaKaRa Ex Taq (TaKaRa Bio Inc.) 0.1 μL, 5–10 ng template DNA, and distilled water up to the final volume. Reactions were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Carlsbad, California, USA) programmed with an initial denaturation step at 98°C for 5 min; followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min; and a final extension step at 72°C for 10 min. Fluorescently labeled PCR products were analyzed using an ABI 3730XL sequencer with the GeneScan 500 LIZ Size Standard (Applied Biosystems). The resulting microsatellite profiles were examined using GeneMapper 3.7 (Applied Biosystems), and peaks were scored manually by visual inspection. Population genetic parameters, including number of alleles per locus, observed heterozygosity, and expected heterozygosity, were estimated using GeneAlEx 6.5 (Peakall and Smouse, 2012). Deviation from Hardy–Weinberg equilibrium was estimated with GENEPOP 4.0 (Rousset, 2008).

Of the 124 microsatellite primer pairs screened, 19 yielded polymorphic SSR loci in *V. coloratum* (Table 1), with the number of alleles ranging from two to six per locus. Through the prescreening of 60 different individuals from three

TABLE 1. Characteristics of the 19 microsatellite loci developed for *Viscum coloratum* in this study.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	Fluorescent dye	GenBank accession no.
Vi-06	F: ATCATGGCCAAATCAACTTAAC	$(CAT)_6$	362–365	58	FAM	Pr032816424
	R: GAGAATCTGAACACCAAGGAA					
Vi-13	F: ATCCTATCCAACCAAATCTCG	$(TCT)_7$	391–397	58	FAM	Pr032816426
	R: TATTTGGGTTTTCTCCATAACG					
Vi-14	F: TAACATCTTCTTGGATGGCTTT	$(TCT)_6$	160–166	58	FAM	Pr032816431
	R: GTGGTTGTGATCTGCATTAAAA					
Vi-22	F: CCAATTTTCCTGGATACTTTCA	$(GAA)_6$	320–344	58	FAM	Pr032816420
	R: TTCTAGGTATTCCCCTGTGATG					
Vi-25	F: ATTCATTCACCTTCAAACCAAC	$(GAA)_6$	290–296	57	FAM	Pr032816428
	R: GTAGTAGGCGTGAGTCTGATCC					
Vi-26	F: TTGTTGAAGCTTCCCACTTAAT	$(TGA)_6$	250–259	58	FAM	Pr032816429
	R: TCATTGTTCCCTCGCTTC					
Vi-31	F: CCCAATTTTCTCATCTCTTACG	$(CTC)_7$	341–347	58	HEX	Pr032816430
	R: CTTTCTAATCACATCCTCTCGG					
Vi-32	F: CTTGAAAGACGACCAAGAAGAC	$(GAC)_7$	143–151	58	HEX	Pr032816422
	R: GATCATAGTCCCGAAATCACC					
Vi-54	F: TGAGGACCTACGCACTTTATTT	$(CGG)_6$	248–254	58	HEX	Pr032816416
	R: AGCAACCTTCTTCTCCTCTCTC					
Vi-60	F: GTTGAATTCCGACATCCAGTAT	$(CCG)_6$	230–233	58	FAM	Pr032816425
	R: CCACATCGTGAAGGACTAATTT					
Vi-63	F: CCCAAAGATACAGAAAGACAGC	$(AAG)_6$	435–441	58	HEX	Pr032816415
	R: ATATCAATCCCAATGGACACAT					
Vi-71	F: CGCACTTTTAGCTTACCTGAGT	$(CAT)_6$	349–364	58	FAM	Pr032816419
	R: CATCGTCTTCCTTTTGATCTTC					
Vi-77	F: GACGAGCAGATGACGTGG	$(AGA)_6$	131–134	58	HEX	Pr032816414
	R: CATTATCTGACTGGTTCGGAAG					
Vi-83	F: AATGATCTTCTTGGATGGCTTT	$(TTA)_6$	170–176	58	FAM	Pr032816427
	R: CTTATGTTGTTTCAACTCGCAA					
Vi-87	F: ACCTTCTGTCGCAAGAAATAGA	$(AGC)_6$	185–191	58	FAM	Pr032816421
	R: ACTCAGCTTCCATGTCAACTCT					
Vi-88	F: GGCTCAGGGACTTCTTGTTATT	$(AGC)_6$	289–298	58	FAM	Pr032816423
	R: AAGAACGTTTTCTTCCGCAT					
Vi-96	F: CCTGTTCCCACTTCTGAAGATA	$(GAA)_7$	318–321	58	FAM	Pr032816417
	R: GAAGTCCTCTTAAGGCAGCTAAG					
Vi-97	F: GCTTCTGAAGATAAAGCAGAGC	$(GAA)_7$	306–318	58	HEX	Pr032816418
	R: TGAATCTGCAGTTTATGCTCAC					
Vi-108	F: TGATTCTCGTAAACACTCCCTC	$(GGA)_8$	349–364	57	FAM	Pr032816413
	R: TTGTCTCGAGAATAGTTTGCCT					

Note: T_a = annealing temperature.

TABLE 2. Genetic diversity in three *Viscum coloratum* populations[a] based on the 19 newly developed polymorphic microsatellite markers.

	Korea (N = 20)			Japan (N = 20)			China (N = 20)			Total (N = 60)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
Vi-06	1	0.000	0.000	1	0.000	0.000	2	0.100	0.095	2	0.033	0.032
Vi-13	3	0.333	0.558	3	0.579	0.522	1	0.000	0.000	3	0.304	0.360
Vi-14	1	0.000	0.000	2	0.333	0.475	2	0.111	0.105	3	0.148	0.193
Vi-22	3	0.353	0.547	1	0.000	0.000	2	0.400	0.320	4	0.251	0.289
Vi-25	3	0.188	0.174	1	0.000	0.000	2	0.150	0.139	3	0.113	0.104
Vi-26	2	0.188	0.264	3	0.100	0.096	3	0.250	0.629*	4	0.179	0.330
Vi-31	2	0.056	0.054	2	0.200	0.375*	2	0.150	0.139	3	0.135	0.189
Vi-32	2	0.158	0.145	3	0.050	0.386*	3	0.100	0.184*	4	0.103	0.238
Vi-54	1	0.000	0.000	2	0.053	0.051	3	0.400	0.464*	3	0.151	0.172
Vi-60	2	0.889	0.494*	2	1.000	0.500*	2	0.150	0.139	2	0.680	0.378
Vi-63	1	0.000	0.000	2	0.895	0.494*	3	0.462	0.370	3	0.452	0.288
Vi-71	2	0.867	0.491*	1	0.000	0.000	2	1.000	0.500*	4	0.622	0.330
Vi-77	2	0.650	0.439*	2	1.000	0.500*	2	0.850	0.499*	2	0.833	0.479
Vi-83	3	0.700	0.471	3	0.850	0.571*	3	0.833	0.573*	3	0.794	0.538
Vi-87	3	0.632	0.447	1	0.000	0.000	2	0.200	0.180	3	0.277	0.209
Vi-88	3	0.143	0.135	2	0.053	0.145*	2	0.056	0.054	3	0.084	0.112
Vi-96	2	0.556	0.401	2	0.550	0.439	2	1.000	0.500*	2	0.702	0.447
Vi-97	5	0.667	0.778	5	0.350	0.610*	3	0.667	0.628	5	0.561	0.672
Vi-108	4	0.471	0.649*	4	0.250	0.606*	3	0.059	0.112*	6	0.260	0.456
Mean	2.37	0.342	0.302	2.21	0.313	0.289	2.32	0.347	0.281	3.26	0.334	0.291

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals.

[a] Locality and voucher information are provided in Appendix 1.

* Significant deviation from Hardy–Weinberg equilibrium after correction for multiple tests ($P < 0.05$).

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 ($P < 0.05$) 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.

TABLE 3. Genetic properties of a single population of 20 individuals of *Viscum album*[a] for the 19 microsatellite loci developed for this study.

Locus	A	Allele size range (bp)
Vi-06	3	359–365
Vi-13	4	391–400
Vi-14	2	163–166
Vi-22	4	323–344
Vi-25	2	293–296
Vi-26	1	256
Vi-31	2	344–347
Vi-32	2	149–151
Vi-54	1	254
Vi-60	2	230–233
Vi-63	1	438
Vi-71	3	346–352
Vi-77	2	131–134
Vi-83	3	170–176
Vi-87	1	191
Vi-88	3	289–298
Vi-96	3	315–321
Vi-97	4	306–315
Vi-108	3	352–361

Note: A = number of alleles.

[a] Locality and voucher information are provided in Appendix 1.

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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.