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Source: Current Herpetology, 38(2) : 169-172

Published By: The Herpetological Society of Japan

URL: https://doi.org/10.5358/hsj.38.169

Current Herpetology 38(2): 169–172, August 2019 doi 10.5358/hsj.38.169

Development of Polymorphic Microsatellite Markers for the Okinawa Pit Viper, *Ovophis okinavensis*

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Abstract: We isolated and characterized 11 microsatellite markers for *Ovophis okinavensis*, a viperid species endemic to the Ryukyu Archipelago. These markers showed polymorphism among 25 individuals from Okinawajima Island. The number of alleles per locus was 3–11, and observed and expected heterozygosity were 0.240–0.960 and 0.218–0.853, respectively. Cross-amplification confirmed that at least seven out of the 11 microsatellite markers were applicable to a putative relative, *Trimeresurus gracilis*. The markers provided here are expected to be valuable for population- and/or individual-level genetic studies of not only *O. okinavenesis*, but also its relatives.

Key words: *Ovophis okinavensis*; Primer sequence; Short tandem repeat; *Trimeresurus gracilis*; Viperidae

Introduction

Ovophis okinavensis is a short, stout-bodied viperid snake that inhabits forest areas, especially near streams, ponds, and marshes, on many subtropical islands of the Okinawa and Amami groups, Ryukyu Archipelago, Japan. This snake is a typical ambush forager and exhibits a foraging strategy that is adjusted to spatial and temporal fluctuations of the emergence of two frog species with different breeding periods (Kadota, 2011). The specialized foraging behavior together with its broad geographic range suggests that this snake has a heterogeneous population structure. However, there are currently no useful genetic markers (including those of the relatives) to obtain fine-scale genetic information on this species. Here, we report newly developed 11 microsatellite primers of *O. okinavensis* and examine the suitability of these markers for *Trimeresurus gracilis*, which is the putative sister taxon to *O. okinavensis* (Castoe and Parkinson, 2006; Malhotra et al., 2010).

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Materials and Methods

We isolated microsatellite loci from ventral

TABLE 1. Characteristics of 11 microsatellite loci developed for *Ovophis okinavensis* and the results of cross-amplification in *Trimeresurus gracilis*.

Locus (Accession no.)	Primer sequence (5'–3')	Repeat motif	*T*a	*Ovophis okinavensis* (n=25)				*Trimeresurus gracilis* (n=4)			
				Size range (bp)	*A*	H_O	H_E	Size range (bp)	*A*	H_O	H_E
OvoP1	F: GCCCATCGACTTTGTTTTGC	$(AC)_{23}$	64	168–192	10	0.96	0.85	168	1	0.00	0.00
(LC414977)	R: GGTTTTCTTGCCTTCAGCAC										
OvoP7	F: GGTGCATACAACCATCAACAGG	$(AC)_{12}$	64	243–249	4	0.68	0.56	240–252	3	0.50	0.41
(LC414978)	R: GAACAACGGAGGCAAAGAAG										
OvoP11	F: TCTGAGACATAGTGGAGGATGG	$(GT)_{13}$	62	198–204	4	0.24	0.22	191–204	4	0.75	0.72
(LC414979)	R: GTGCCACAGGCAATGTTTTC										
OvoP12	F: AAAGAGGCAGTGGAGCATGT	$(TG)_{12}$	64	248–264	4	0.64	0.7	256–268	5	1.00	0.78
(LC414980)	R: GATGAGAGACTGAGAGGATAGGG										
OvoP17	F: GTGGAAAGAAAGGTAAGCATTG	$(GT)_8$	62	305–321	5	0.52	0.43	318–328	4	0.25	0.72
(LC414981)	R: ATGAAAACCCATGGAAGGTG										
OvoP24	F: AAGGGACATGTGTGTGTGTG	$(TG)_{13}$	62	208–212	3	0.56	0.65	190–208	3	0.25	0.59
(LC414982)	R: CGAGTGCGGTATCTTCTTCC										
OvoP29	F: CACATACACACACGCACAGG	$(AC)_9$	64	200–210	5	0.56	0.71	191–217	4	1.00	0.69
(LC414983)	R: TGGGGCAAAGCTGACTTAAC										
OvoP30	F: GGTTGTCCAATTCCTTCTGG	$(GT)_{18}$	64	258–266	5	0.76	0.66	—	—	—	—
(LC414984)	R: GTTTGGCCCACAAATAGCC										
OvoP38	F: GTTACACATTGCTCGCTTGC	$(CA)_{22}$	62	265–383	11	0.88	0.83	—	—	—	—
(LC414985)	R: CTTCCTGGTGGTCACGTTTT										
OvoP40	F: CCTCTTCACTCCACCCAGTC	$(TG)_{16}$	62	306–320	8	0.56	0.66	311–312	2	—*	—*
(LC414986)	R: CGCTTAGCGACCAAAGTTAC										
OvoP45	F: AAAAACAGTCGGGTTTGTGG	$(TG)_{12}$	62	248–266	4	0.36	0.33	250–260	5	0.5	0.75
(LC414987)	R: CCAGTCTCCCTTTATCTCTTTGA										

*T*a, annealing temperature (°C); *A*, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *only one individual succesfully amplyfied.

scale tissue samples from Okinawajima Island snakes using the method slightly modified from Glenn and Schable (2005). The detailed methods for library preparation and primer design are described in Kurita et al. (2013). For amplification trials, 25 individuals of *O. okinavensis* from the northern part of Okinawajima Island were used (DNA identification numbers by Y. Kadota: 4, 100–102, 106, 107, 111, 114, 117, 128, 129, 133, 135, 138, 139, 145, 149, 151, 152, 154, 158, 164, 170, 175, 177). In addition, we tested cross-species amplification of developed markers on four individuals of *T. gracilis* from Taiwan (temporal specimen numbers by Ming-Chung Tu: 79804–805 and 79807–808). PCR amplification was conducted using fluorescently (FAM, HEX, NED) labeled M13(-21) universal primers (Schuelke, 2000) with the TaKaRa Ex Taq kit (Takara Bio, Otsu, Japan). Fragment sizes were measured with GeneScan 400HD ROX size standard (Applied Biosystems, Foster City, CA, USA) on ABI 3130xl Genetic Analyzer (Applied Biosystems) and analyzed by the Peak Scanner software (Applied Biosystems). Number of alleles (A), and observed (H_O) and expected heterozygosities (H_E) were calculated using GenAlEx 6.503 (Peakall and Smouse, 2006, 2012). Deviation from Hardy–Weinberg equilibrium and linkage disequilibrium between loci were tested using GENEPOP 4.7.0 (Rousset, 2008). Null allele frequency was estimated using CERVUS 3.0.7 (Kalinowski et al., 2007).

Results and Discussion

The characteristics of the 11 isolated microsatellite loci are summarized in Table 1. These loci exhibited high or moderate allelic polymorphism in *O. okinavensis*. The number of alleles per locus ranged from 3 to 11. The observed and expected heterozygosity ranged from 0.240 to 0.960 and from 0.218 to 0.853, respectively. There was no evidence of deviations from the Hardy-Weinberg equilibrium at any locus and significant linkage disequilibrium at any pair of loci after the sequential Bonferroni correction (Rice, 1989). The estimated frequency of null alleles ranged from –0.13 to 0.12. In *T. gracilis*, eight of 11 loci were successfully amplified for all individuals examined, and seven loci showed polymorphism. As such, the microsatellite markers developed here will be useful for population- and/or individual-level genetic studies of *O. okinavensis* and its relatives, in the fields of ecology and population genetics (e.g. population structure, genetic variation, demography, and relatedness analyses including paternity).

Acknowledgments

We would like to thank M.-C. Tu and C.-F. Lin for collecting and providing valuable tissue samples of *T. gracilis*. The samples were from protected animals in Taiwan. They were permitted to collect by Dr. Tu who obtained his permission from Council of Agriculture, Executive Yuan; and T. Hikida and T. Okamoto for their technical support. We also thank anonymous reviewers for their comments in improving our manuscript. This research was financially supported in part by the Global COE Program A06 to Kyoto University.

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Accepted: 7 May 2019