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No Genetic Differentiation of *Elaphe schrenckii* Subspecies in Korea Based on 9 Microsatellite Loci

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ABSTRACT

The Russian ratsnake, *Elaphe schrenckii*, is found in Russia, China, and Korea, and is considered to be an endangered species by the Ministry of Environment in South Korea. Due to habitat loss and use in oriental medicine, their population has been severely decimated. In South Korea, two subspecies of *E. schrenckii* has been defined according to body color: *E. s. schrenckii* (blackish) and *E. s. anomala* (yellow-brownish). Molecular genetic studies on *Elaphe schrenckii* are very scarce and the taxonomy of *Elaphe schrenckii* subspecies is uncertain. From the present study, we attempted to identify the genetic differences of these two subspecies using species-specific microsatellites developed from the genomic library of *E. schrenckii*. Nine polymorphic loci were tested on 19 individuals from *E. s. schrenckii* (n=10) and *E. s. anomala* (n=9) in South Korea. The mean number of alleles was 3.78 in *E. s. schrenckii* and 4.11 in *E. s. anomala*. The average expected heterozygosity was 0.542 and 0.511 in *E. s. schrenckii* and *E. s. anomala*, respectively. We found a lack of genetic structure between two subspecies (F_{ST}=0.016) and no genetic discrimination between two subspecies was found. Based on the present findings by microsatellites, two subspecies can be considered as one species, *E. schrenckii*. However, further investigations on taxonomical status using mitochondrial and nuclear DNA sequences need to be performed and morphological & ecological data should be revised. The genetic markers should benefit future studies of the endangered species of other *Elaphe* species for the study of genetic diversity and potential conservation management.

Keywords: Colubridae, *Elaphe schrenckii schrenckii*, *Elaphe schrenckii anomala*, microsatellite, genetic diversity

INTRODUCTION

The family Colubridae includes 12 subfamilies. Three of the 40-55 ratsnake species in the genus *Elaphe* of subfamily Colubrinae, are in South Korea: *E. schrenckii*, *E. dione*, and *E. rufodorsata* (National Institute of Environmental Research, 2006). The distribution of *E. schrenckii* habitat includes China, Russia, and Korea. Based on body color and scales on the cheek, two subspecies of *E. schrenckii* are recognized in South Korea: *E. s. schrenckii* Strauch, 1873 and *E. s. anomala* Boulenger, 1916. *E. s. schrenckii* is more blackish and *E. s. anomala* is brighter such as yellowish and brownish color. Although the distribution of two subspecies overlaps in regions in South Korea except for Jejudo Island, *E. s. schrenckii* is more dominant in the northern part of the Korean peninsula, whereas *E. s. anomala* is so in the southern part. Among 14 known terrestrial snakes in Korea, *E. schrenckii*, is the largest non-venomous snake and found in barnyards and woodlands near human habituation in Korea. They mainly feed on rodents (rat, squirrel, chipmunk etc) and rarely attack bird nests. Due to their use in oriental medicine and habitat loss the *E. schrenckii* population has declined and has become endangered. Therefore, *E. schrenckii* was designated as an endangered species grade I by Ministry of Environment in South Korea and is also classified as a protected species in Russia (Zoological society of London, 2006).

Neutral genetic markers such as microsatellite have been used for studying the population genetics for various taxa (timber rattlesnake- Clark et al., 2008; western corn rootworm- Kim et al., 2008; grasshopper sparrow- Mylecraine et al., 2008; european beaver- Pelz-Serrano et al., 2009). Microsatellites contain a number of advantages making them a popular genetic marker, widely used to test ecological and evolutionary hypotheses (Zhang and Hewitt, 2003; Selkoe and Toonen, 2006). They are highly polymorphic, codomi-

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nant, relatively easy to multiplexing, and high-throughput genotyping although developing microsatellites involves considerable time and cost (Zane et al., 2002).

Several molecular studies have investigated the phylogeny of the genus *Elaphe* using mitochondrial markers (cytochrome *b* and control region- Burbrink et al., 2000; cytochrome *b*- Burbrink, 2002; 12S and 16S rRNA- Heise et al., 1995; 12S rRNA and COI- Utiger et al., 2002; complete mitochondrial genome- Woo et al., 2009). Heise et al. (1995) represented the higher-level phylogeny of snakes, including Colubrids, using partial sequences from 12S and 16S rRNA. Colubrids were found to be paraphyletic and the associations of the genera among the Colubrids were not fully resolved with high bootstrap values. Nevertheless, sister group relationship between the genera *Elaphe* and *Boiga* was found with moderate supportive values. However, all these genetic studies have not included *E. schrenckii* except for the most recent study by Woo et al. (2009). Up to now, no investigations on taxonomic status and phylogeographic relationships of *E. schrenckii* and their subspecies have been completed.

Although two subspecies of *E. schrenckii* are acknowledged in South Korea, no genetic studies on their phylogenetic relationship have been accomplished. In the most recent article by An et al. (in press), 9 microsatellite loci were developed and characterized for *E. schrenckii* from South Korea, Russia and China, and *E. anomala* from China. Microsatellite markers are available for evaluating the extent of genetic variation. And also these markers have been extensively used to investigate genetic diversity and population structure in snakes (Manier and Arnold, 2005; Clark et al., 2008; Tzika et al., 2008). Several literatures cover the assessment of genetic diversity and differences among subspecies in various taxa using microsatellites (elk- Meredith et al., 2007; mosquitoe- Kothra et al., 2009; massasauga rattlesnake- Murphy, 2009). In this study, we sought to evaluate the genetic diversity and to determine the genetic discrimination between *E. s. schrenckii* and *E. s. anomala* using microsatellites developed from the genomic library of *E. schrenckii* in South Korea.

MATERIALS AND METHODS

Sampling

Nineteen tissue samples (10 *E. s. schrenckii* and 9 *E. s. anomala*) were collected from the wild all over South Korea and registered into CGRB (Conservation Genome Resource Bank for Korean Wildlife). All samples were genotyped for nine microsatellite loci. Table 1 contains the information of ID number and localities.

Table 1. Localities and ID number for two *E. schrenckii* subspecies in South Korea.

Taxon	ID number	Collection locality
Elaphe schrenckii schrenckii	mms1059	Jaecheon-si, Chungcheongbuk-do
	mms1073	Incheon
	mms1074	Incheon
	mms1076	Incheon
	mms1179	Gwangju
	mms1184	Busan
	mms1788	Pocheon-si, Gyeonggi-do
	mms1789	Pocheon-si, Gyeonggi-do
	mms1845	Pocheon-si, Gyeonggi-do
	mms1868	Pocheon-si, Gyeonggi-do
Elaphe schrenckii anomala	mms1052	Jecheon-si, Chungcheongbuk-do
	mms1053	Jecheon-si, Chungcheongbuk-do
	mms1056	Jecheon-si, Chungcheongbuk-do
	mms1057	Jecheon-si, Chungcheongbuk-do
	mms1062	Jecheon-si, Chungcheongbuk-do
	mms1064	Jecheon-si, Chungcheongbuk-do
	mms1186	Yanggu-gun, Gangwon-do
	mms1842	Yeongweol-gun, Gangwon-do
	mms1844	Pocheon-si, Gyeonggi-do

Methods

DNA extraction, PCR amplification, and Genotyping

DNA from tissue samples including nine *E. s. anomala* and ten *E. s. schrenckii* was extracted using DNeasy blood and tissue kit (Qiagen, USA). Genotypes of 19 individuals were determined using 9 microsatellites (*Es*01, *Es*02, *Es*03, *Es*04, *Es*06, *Es*07, *Es*09, *Es*11 and *Es*12) developed previously (An et al., in press).

PCR genotyping was performed in a 20-μL reaction volume containing 40-100 ng of genomic DNA, 1 × PCR buffer, 0.2 μM of each primer, 2.5 mM dNTPs, 50 mM $MgCl_2$ and 1 U of *Taq* polymerase. Samples were amplified in a DNA thermal cycler (Takara, Japan) programmed for denaturation at 94˚C for 5 min, followed by 20 cycles of 94˚C for 40 sec, 60˚C for 40 sec with decrease by 0.5˚C per cycle, 72˚C for 40 sec, followed by 20 cycles at 94˚C for 40 sec, 50˚C for 40 sec, 72˚C for 40 sec, and a final extension at 72˚C for 10 min. PCR products were sized on ABI3730xl DNA Analyser (ABI, USA) alongside GeneScan 500 Rox size standard using GeneMapper software v.4.0.

Data analysis

The number of alleles, range of product size, observed heterozygosity (H_o), expected heterozygosity (H_e), polymorphic information content (PIC), and *P*-value were calculated for each locus and each subspecies using CERVUS v.3.0 (Kalinowski et al., 2007). Departures from Hardy-Weinberg equilibrium at each locus and linkage disequilibrium between loci were tested using Genepop v.3.4 (Raymond and Rousset,

Table 2. Characteristics of the nine microsatellite loci optimized for *E. schrenckii*. Locus name, clone ID, sample size (N), number of alleles per locus (k), observed (H_o) and expected (H_e) heterozygosities, PIC (polymorphic information content), and *P*-value are reported.

Locus	Clone ID	Taxon	N	k	Size	H_o	H_e	PIC	*P*-value
*Es*01	AF2	*E. s. schrenckii*	9	6	203-235	0.222	0.753	0.763	0.586
		E. s. anomala	8	4	225-239	0.375	0.484	0.544	0.000*
*Es*02	AC2	*E. s. schrenckii*	10	5	114-132	0.700	0.660	0.611	0.205
		E. s. anomala	9	5	110-130	0.556	0.735	0.690	0.206
*Es*03	AF8	*E. s. schrenckii*	9	5	223-267	0.333	0.698	0.710	0.289
		E. s. anomala	8	7	223-263	0.875	0.766	0.779	0.010
*Es*04	AA2	*E. s. schrenckii*	10	4	198-222	0.700	0.575	0.526	0.155
		E. s. anomala	9	4	198-222	0.444	0.512	0.473	0.433
*Es*06	AF9	*E. s. schrenckii*	10	3	206-220	0.200	0.540	0.466	0.002
		E. s. anomala	9	3	206-220	0.556	0.660	0.586	0.002
*Es*07	BC11	*E. s. schrenckii*	9	3	165-174	0.111	0.438	0.498	1.000
		E. s. anomala	9	3	165-174	0.222	0.204	0.194	0.012
*Es*09	BH6	*E. s. schrenckii*	10	2	245-247	0.400	0.420	0.332	1.000
		E. s. anomala	9	2	245-247	0.333	0.401	0.321	1.000
*Es*11	CC2	*E. s. schrenckii*	9	1	164	0.000	0.000	0.164	–
		E. s. anomala	9	1	164	0.000	0.000	0.000	–
*Es*12	CC5	*E. s. schrenckii*	10	5	178-200	0.600	0.790	0.756	0.019
		E. s. anomala	8	8	170-194	0.500	0.836	0.908	0.018

*Locus not in Hardy-Weinberg equilibrium ($P < 0.05$).

1995). Genetic differentiation between two *E. schrenckii* subspecies based on pairwise F_{ST} was estimated using ARLEQUIN v.3.1 (Excoffier et al., 2005).

RESULTS AND DISCUSSION

Molecular genetic markers can provide valuable insights into the geographical distribution of genetic diversity of species. Most of wildlife including mammals, birds, amphibians, reptiles, and so on is considered to be endangered or threatened because of illegal hunting, oriental medicine, habitat destruction. Especially, natural population of snakes in reptiles constantly declined, mainly because of predation by introduced species, human persecution, and habitat destruction (Tzika et al., 2008). A comprehensive assessment of population structure is one critical component for their effective conservation. To establish long term conservation strategy, it is worthwhile to take account for genetic approach.

Here, we used nuclear microsatellites developed from genomic DNA library of *E. schrenckii* from South Korea. Among the newly developed 10 loci, nine were selected for this study. Characteristics of the nine microsatellite loci for each subspecies assessed are shown in Table 2. In total, 49 alleles for the nine loci were identified in both subspecies. The number of alleles ranged from one to eight per locus in *E. s. anomala* and one to six per locus in *E. s. schrenckii*. Average expected heterozygosities were 0.511 (0.000-0.836) in *E. s. anomala* and 0.542 (0.000-0.790) in *E. s. schrenckii*. H_o was rather low compared to H_e, an average of 0.429 (0.000-0.875) in *E. s. anomala* and a mean of 0.363 (0.000-0.700) in *E. s. schrenckii*. No significant differentiation in genetic diversity was found between the two subspecies. Mean H_e calculated from *E. goydi* was 0.53 which is compatible with the values of *E. shcrenckii* subspecies but is rather small than other snakes (0.59 in timber rattlesnakes, Clark et al., 2008; 0.64 in jamaican boa, Tzika et al., 2008).

In addition, we tested for deviation from Hardy-Weinberg equilibrium using the HW exact test and the Markov chain method for *P*-value estimation (Guo and Thompson, 1992). Only one locus in *E. s. anomala* population, *Es*01, was significantly deviated from HWE after a sequential Bonferroni correction (α=0.05, $P > 0.05$; Rice, 1989) because of a deficiency of heterozygotes, which might be due to the Wahlund effect (Templeton, 2006). No significant linkage disequilibrium between loci across two subspecies was observed.

F_{ST} between the two subspecies was 0.016 and genetic differentiation was not significant ($P > 0.05$) between two subspecies at all loci. Within the species, genetic differentiation on the basis of F_{ST} value could be identified. Tzika et al. (2008) reported that the F_{ST} value of three populations for jamaican boa (*Epicrates subflavus*) was 0.093 ($P < 0.001$). Among 5 populations of timber rattlesnake (*Crotalus horridus*), F_{ST} values were ranged from 0.000 to 0.005 (Clark et

al., 2008). Compared to other results, the value of F_{ST} between the two subspecies, *E. s. schrenckii* and *E. s. anomala*, was generally lower than others and indicating no genetic discrimination (F_{ST}=0.016, $P > 0.005$).

In addition, no distinctive clusters by the STRUCTURE analysis were detected. Both F_{ST} statistics and STRUCTURE analysis indicate no genetic discrimination between *E. s. schrenckii* and *E. s. anomala* in South Korea. *E. s. schrenckii* and *E. s. anomala* have either sympatric or parapatric distribution in South Korea, indicating that they might be considered as the same species with different individual color variation (Park, 2010). Similar findings of low genetic differentiation among subspecies were shown in two literatures. Palo et al. (2001) found that low differentiation was detected between Baltic seal (*Phoca hispida botnica*) and Arctic seal (*P. h. hispida*) at eight microsatellite loci, implying the possibility of the same species. In addition, no significant differentiation of morphological characters between two subspecies was identified (Park, 2010). Further investigations of the taxonomic status of *E. schrenckii* from Russia, China, and Korea need to be confirmed using mitochondrial and nuclear DNA sequences, as well as morphological taxonomy including *E. schrenckii* from Russia and ecological data. To conclude, genetic markers including species-specific microsatellites, DNA sequences will be helpful tools for population genetic and ecological studies of the endangered ratsnakes and related other *Elaphe* species.

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