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Cross-amplification of microsatellite loci reveals multiple paternity in Halys pit viper (*Gloydius halys*)

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Abstract. For Halys pit viper (*Gloydius halys*) species-specific microsatellite primers are not available. We tested a set of twenty primer pairs, originally developed for various Crotalinae species, for cross-amplification with *Gloydius halys*. The level of allelic polymorphism was assessed for eight successfully amplified loci via genotyping of a population sample. Between three to 24 alleles per locus were recorded. We examined a female and seven of its embryos for multiple paternity using seven microsatellite loci. More than two paternal alleles were detected in two loci indicating that two or more fathers were involved. This is the first report of multiple paternity in the wild population of Crotalinae. The life history characteristics of Halys pit-viper that can be associated with multiple paternity are discussed.

Keywords. Microsatellites, multiple paternity, *Gloydius halys*, pit viper, Crotalinae.

During the last two decades the introduction of molecular techniques has greatly facilitated kinship analysis in wild populations of animals. Multiple paternity has been documented for various taxonomic groups of vertebrates (Laurila and Seppa, 1998; Wink and Dyrcz, 1999; Avise et al., 2002; Westneat and Stewart, 2003; Gottelli et al. 2007; Uller and Olsson, 2008). In scaled reptiles (Squamata) multiple paternity seems to be a very common phenomenon and reaches the highest levels known in vertebrates (Uller and Olsson, 2008). However, cases of multiple fecundation have only been reported for 36 out of more than 8900 squamate species and they are limited to nine out of 52 squamate families (Uetz et al., 2007; Uller and Olsson, 2008; Voris et al., 2008). Recently, several authors have recognized the need to consider this phenomenon in scaled reptiles in the broader phylogenetic context (Voris et al., 2008; Wusterbarth et al., 2010) because our knowledge on the occurrence of multiple paternity in diverse squamate taxa is still insufficient.

Microsatellites are very useful for paternity studies due to their co-dominance, biparental inheritance and high variability (Webster and Reichart, 2005). The development of these

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markers is still relatively expensive and a time consuming task, while recent achievements in application of 454 pyrosequencing for microsatellite development have greatly facilitated this process (e.g. Castoe et al., 2010; Malausa et al., 2011). Given that the flanking regions of microsatellite loci may be quite conserved in related taxa (Moore et al., 1991; Primmer et al., 1996), cross-species amplification is a widely used way to avoid the process of the microsatellite development for each newly studied species (Bushar et al., 2001; Anderson, 2006). Because specific microsatellite primers were not available for Halys pit viper (*Gloydius halys*) a set of microsatellite primers from other snakes were tested for cross-species amplification. The primer set found was subsequently utilized in a paternity analysis of a female with seven embryos that had been found dead on a roadside.

We performed cross-species microsatellite amplification on 156 individuals of adult *G. halys* that were sampled during 2008 - 2010 in the Novosibirsk region (West Siberia, Russia). Buccal swabs and scale clippings were used to obtain the tissue samples which were stored in 95% ethanol at 4 °C. After sampling and examination all snakes were released at the site of capture. In attempt to detect multiple paternity in *G. halys* we used a road-killed gravid female, collected in August 2009 at the same study area. The dissection of the female revealed seven well-developed embryos that were measured and sexed. Intercostal muscle tissue of the female and tail tips of the embryos were used for DNA extraction.

Total genomic DNA was isolated using standard proteinase K and phenol-chloroform protocols (Sambrook et al., 1989). A panel of 20 microsatellite loci which had been developed for Crotalinae species (genera *Crotalus* and *Sistrurus*) was tested for cross-species amplification with *G. halys* (Table 1). Initially we performed PCR with each primer pair in a set of six samples using the following conditions – initial denaturation at 94 °C for 5 min followed by 45 cycles of 60 s at 94 °C, annealing at 53 °C for 60 s, 60 s extension at 72 °C, and a final extension of 5 min at 72 °C. At a second step all primer pairs which showed positive results were employed in gradient PCR with Tgradient TermoCycler (Biometra). For this step we used three samples out of previous set and the same PCR conditions, but the annealing temperature ranged from 50 to 65 °C. After that we rejected all primers which had yielded ambiguous patterns (i.e. a lot of non-specific amplifications) at all thermal regimes. Finally, we selected the optimal annealing temperature for each locus (Table 1) and performed PCR with the six samples. The loci which exhibited more than two alleles were applied for further genotyping of the whole data set. The female and its embryos were genotyped twice to be sure that an observed pattern is not a result of an amplification/electrophoresis error.

All PCRs were performed in 25 µl reaction mix containing 15-60 ng DNA, 0.1 mM each of dGTP, dCTP and dTTP, 0.045 mM dATP, 0.1 µl ^{33}P-α-dATP (Amersham Biosciences), 1.5 U of Top-Taq DNA polymerase (BIORON), 2.5 µL of 10 × amplification buffer (10 mM Tris-HCl pH 8.5, 50 mM KCl and 2.5 mM $MgCl_2$), and 10 pmol of forward and reverse primers. Amplification products of all PCRs were separated by high-resolution electrophoresis in 6% denaturing polyacrylamide gels at 65 W for 1.5 h using a Base Acer Sequencer (Stratagene). After the gels were vacuum dried they were exposed for 24-96 h to X-ray film (BioMax MR Film, Kodak). PCR products were sized with reference to a known sequencing reaction of the pBlueScript SK+ plasmid. We checked for possible scoring errors due to the production of stutter bands, allele dropout and the presence of null alleles using Micro-Checker v. 2.2.3 software (Oosterhout et al., 2004). Test for link-

age disequilibrium was performed in Genepop web version 4.0.10 (Rousset, 2008) using exact probability test with a Markov Chain approximation. The critical P-values were corrected for multiple tests by the Benjamini and Yekutieli (B–Y) method (Benjamini and Yekutieli, 2001).

Table 1. Microsatellite loci and results of their amplification with *Gloydius halys*.
Reference and used species: [a]Munguia-Vega et al. (2009), *Crotalus tigris*; [b]Goldberg et al. (2003), *C. tigris*; [c]Villarreal et al. (1996), *C. horridus*; [d]Holycross et al. (2002), *C. willardi*; [e]Oyler-McCance et al. (2005), *C. viridis*; [f]Gibbs et al. (1998), *Sistrurus catenatus*. N_A, number of observed alleles; T_a, annealing temperature. *Allele sizes are based on the size of the clone sequenced for each locus (Gibbs et al. 1998).

Locus	Repeat motif	Allelic size, bp	N_A (n)	Amplification with *G. halys*			
				Yes/No/ Ambiguous	Allelic size, bp	N_A (n)	T_a (°C)
*Crti*14[a]	$(GT)_{19}$	274-314	14 (25)	No	-	-	-
*Crti*19[a]	$(CA)_{18}$	212-235	6 (25)	Ambiguous	-	-	-
*Crti*12A[a]	$(CA)_{22}$	219-240	6 (25)	Yes	206-220	7 (164)	60
*Crti*37[a]	$(GT)_{12}(GA)_{26}$	274-312	14 (25)	Yes	264-292	7 (164)	55
*Crti*95[a]	$(CA)_{22}$	174-211	10 (25)	Yes	172-202	16 (164)	56
*Crti*10[b]	$(GAA)_{48}$	219-300	22 (149)	Yes	240-312	23 (164)	55
*Crti*12[b]	$(CA)_{14}$	217-225	5 (149)	Yes	216-232	3 (164)	57
*Ch*5A[c]	$(CA)_{17}$	164-142	8 (29)	Yes	144-150	2 (6)	56
*Ch*7-150[c]	$(CA)_{13}$	146-144	2 (32)	Yes	122-130	2 (6)	56
Ch 5-183[c]	$(CA)_{11}$	136-124	7 (26)	No	-	-	-
Ch 7-144[c]	$(CA)_{16}(GA)_{12}$	116-100	5 (18)	No	-	-	-
Ch 7-87[c]	$(CA)_{12}$	159-145	3 (16)	Yes	138-180	12 (164)	55
Ch 3-155[c]	$(CA)_{13}$	146-122	4 (22)	No	-	-	-
*Cw*A14[d]	$(AC)_{24}$	147-175	7 (54)	Yes	144-174	8 (164)	57
*Cw*A29[d]	$(AC)_{13}$	160-190	5 (54)	No	-	-	-
*Cw*B6[d]	$(GA)_{19}$	122-130	5 (54)	Ambiguous	-	-	-
MFRD5[e]	$(TG)_{23}$	172-194	9 (192)	Yes	160-186	10 (157)	56
*Scu*01[f]	$(AG)_{24}$	149*	12 (73)	Ambiguous	-	-	-
*Scu*16[f]	$(AC)_{17}$	167*	4 (74)	No	-	-	-
*Scu*26[f]	$(AC)_{24}$	173*	5 (74)	Ambiguous	-	-	-

Results of cross-amplification testing are summarized in Table 1. Unambiguous results were obtained for ten out of twenty tested microsatellite loci initially amplified with six samples of *G. halys*. Most of them (50%) were originally developed for *Crotalus tigris*, 30% for *C. horridus* and 10% for *C. willardi* and *C. viridis*. No STR locus of *Sistrurus catenatus* could be successfully amplified with *G. halys.* Eight of cross-amplified loci were used to screen with the whole data set (n = 164). Polymorphism varied between three (*Crti*12) to 24 (*Crti*10) alleles per locus. Number of alleles per locus and range of allelic sizes in Halys pit viper was generally similar to that of the original species (Table 1). There was no evi-

dence for scoring errors resulting from stuttering or large allele dropout. However, MicroChecker detected the signs of presence of a null allele for locus *Crti*12A with an estimated frequency of 0.08. No linkage disequilibrium tests were significant after B-Y correction.

We examined the female and its embryos for multiple paternity using seven microsatellite loci. More than two paternal alleles were detected for the locus *Crti*95 (three paternal alleles) and *Crti*10 (four paternal alleles). This is good evidence for multiple fertilization of the litter by two or more males (Table 2).

Table 2. Microsatellite DNA genotypes of *Gloydius halys* female and its embryos.

Locus	Maternal genotype	Offspring genotypes							Inferred paternal alleles
		1 (f)	2 (f)	3 (f)	4 (f)	5 (m)	6 (m)	7 (m)	
*Crti*12A	212/206	212/206	212/212	212/212	212/208	212/212	212/206	212/206	212, 208
*Crti*37	288/264	292/288	292/288	292/288	288/264	292/288	292/264	292/264	292, 288 or 264
*Crti*95	172/196	194/196	198/196	172/196	198/196	172/196	172/196	172/196	194,198, 196
*Crti*10	279/258	309/258	306/258	258/258	279/279	258/258	309/258	309/258	258, 279, 306, 309
*Crti*12	230/216	230/216	232/216	216/216	232/216	232/230	216/216	232/216	216, 232
Ch 7-87	156/156	156/156	156/156	156/156	156/156	156/152	156/152	156/156	152, 156
*Cw*A14	174/172	172/144	174/174	172/144	174/172	172/144	172/144	172/144	144, 174

Successful cross-species amplification of microsatellites in snakes is known for a number of loci and was used both in population genetics and paternity testing studies (e.g., Clark et al., 2008; Wusterbarth et al., 2010). The three loci tested in the present work (*Scu01*, *Scu16* and *Scu26*) have been previously cross-amplified with other pit vipers, as well as with representatives of Colubridae (Gibbs et al., 1998; Anderson, 2006). Surprisingly, we could not achieve satisfactory results with them in our analysis. However, PCR products belonging to microsatellites were produced for loci *Scu*01 and *Scu*26, but their interpretation was severely complicated by the numerous non-specific bands. Further improvements of PCR conditions via selection of various PCR buffers may solve this problem, as it was shown in Anderson (2006). In general, the 50% rate of successful amplifications reached in our study confirms that cross-species utilization of microsatellites may be considered as preferred convenient way to avoid the development of the specific loci for each newly studied species.

To our best knowledge, only two documented cases of multiple paternity in vipers (Viperidae) have been reported. Occurrence of multiple paternity in the common adder (*Vipera berus*) is a well established fact (e.g., Stille et al., 1986; Ursenbacher et al., 2009). In the pit viper (Crotalinae) subfamily there is one report of multiple paternity in captive Copperhead (*Agkistrodon contortrix*) caused by sperm storage and revealed by the phenotypes of the offspring (Schuett and Gillingham, 1986). It is notable that attempts to

detect multiple paternity in other pit vipers had failed. Villarreal et al. (1996) had tested two litters of timber rattlesnake (*Crotalus horridus*) using six loci, whereas Gibbs et al. (1998) analysed two litters of massasauga (*Sistrurus catenatus*). Hence, we documented here the first case of multiple paternity in a free-living Crotalinae, and *G. halys* is the second Viperidae species for which this phenomenon has been documented in the wild. We encourage more intense sampling and paternity testing both for Old World and New World pit vipers, considering that the prevalence of genetic monandry (instead of multiple paternity) may be a likely phenomenon in some snake lineages, as has been recently been shown by Lukoschek and Avice (2011) for true sea snakes of genus *Hydrophis*.

Several life history traits of the Halys pit viper suggest that multiple paternity may occur in this species. First, although there are no known recordings of multiple mating in this species, *G. halys* occurs at high population densities, at least at our study site (267 individual/hectare; Simonov, 2007), with apparent breeding aggregations. Importantly, it has been hypothesised that population density and male-biased operational sex ratio are linked to the occurrence (and frequency) of multiple paternity in snakes and other squamates (Uller and Olsson, 2008; Voris et al., 2008). However, some recent studies fail to find evidence of such a link (Blouin-Demers et al., 2005; Ursenbacher et al., 2009). Next, mating has been observed in the middle and end of the activity season in *G. halys* (Paraskiv, 1956; Yakovleva, 1964; Yakovlev, 1985), a behaviour known to be associated with sperm storage in snakes (e.g., Halpert et al., 1982). Thus, under the assumption that females multiple mate and store sperm, two common features in snake species (Olsson and Madsen, 1998), it is likely that multiple paternity is promoted in this species. In this study, we show that multiple paternity does occur, and now we recommend further studies to be undertaken in order to understand the extent and evolutionary consequences of multiple paternity in this species.

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