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Two Naturally Occurring Intergeneric Hybrid Snakes (*Pituophis catenifer sayi* × *Pantherophis vulpinus;* Lampropeltini, Squamata) from the Midwestern United States

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ABSTRACT.—**Two intergeneric hybrid snakes (*Pituophis catenifer sayi* × *Pantherophis vulpinus*) are described from the midwestern United States; one collected in south central Iowa and the other from southeastern Minnesota. Both specimens are morphologically intermediate between the putative parental species *P. c. sayi* and *P. vulpinus*. Hybrid origin was verified by comparing DNA sequence data from the hybrids to the putative parental species. Both hybrid specimens possessed *P. c. sayi* mitochondrial DNA haplotypes. Examination of the nuclear gene Vimentin (intron 5) showed both specimens were heterozygous at most variable sites confirming their hybrid origin. These snakes represent only the second and third confirmed instances of naturally occurring intergeneric hybridization among squamate reptile species.**

Naturally occurring hybrids among squamate species have been reported with some regularity, but are generally considered uncommon (Murphy and Crabtree, 1988; Campbell et al., 1989; Leaché and Cole, 2007; Mebert, 2008; Kearney et al., 2009). Intergeneric hybridization among squamate species is even more rare, with only three cases reported in the literature. Two of these involved snakes. The first, a cross between *Crotalus horridus* and *Sistrurus catenatus*, was based on detailed morphological evidence with the putative hybrid possessing characteristics intermediate to the parental species (Bailey, 1942). The second case involved a putative *Lampropeltis californiae* × *Pituophis catenifer* cross and featured no formal analysis, simply a photo (Hubbs, 2009). Both instances were based solely on morphology with no genetic data to confirm the morphological hypotheses. The third example involved the hybridization between a Marine Iguana (*Amblyrhynchus cristatus*) and Galapagos Land Iguana (*Conolophus subcristatus*) (Rassmann et al., 1997). Hybrid origin was confirmed by analyzing genetic data with both mitochondrial DNA and restriction fragment length polymorphism (RFLP) analysis of nuclear ribosomal DNA. Examining both morphological and genetic data is the most accurate means to identify putative hybrids in a variety of taxa (Murphy and Crabtree, 1988; Delsuc et al., 2007). Morphological data are essential for making an initial hypothesis of hybridization, as F1 hybrids are generally morphologically intermediate between the two parental species (Bailey, 1942; Hubbs, 1955; Dowling and Secor, 1997). Molecular genetic data can identify parentage and are necessary to verify hybrid origin and confirm the initial morphological identifications. Here we use both morphological and molecular data to describe two instances of naturally occurring intergeneric hybridization in squamates.

MATERIALS AND METHODS

We collected putative *Pituophis catenifer sayi* × *Pantherophis vulpinus* hybrids at two different sites in the midwestern United States, one in south central Iowa and the other in southeastern Minnesota. Both *P. c. sayi* and *P. vulpinus* occur sympatrically at the localities where the putative hybrids were collected (JBL, JS, CES, and EPH, pers. obs.). We (JBL and JS) collected the first specimen on 16 May 2009 at 1815 hours beneath a large rock on a rock strewn grassy hillside in Madison County, Iowa. The Iowa specimen was captured alive and is currently maintained in captivity (JBL). We (CES and EPH) collected the Minnesota specimen on 18 September 2009 at 2000 hours in Wabasha County, Minnesota. The specimen was found recently hit and dead on the road. We took several photographs in situ and then placed the specimen on ice until tissue could be removed and the specimen could be properly preserved and deposited in the Bell Museum of Natural History, University of Minnesota (JFBM).

We extracted DNA from tissues with the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. We amplified a fragment of the mitochondrial *ND4* gene with primers ND4–F (5′-TGA CTA CCA AAA GCT CAT GTA GAA GC-3′) (Forstner et al., 1995) and Leu–R (5′-CAT TAC TTT TAC TTG GAT TTG CAC CA-3′) (Arevalo et al., 1994). We also amplified intron 5 of the nuclear gene Vimentin with primers VimExon5F (5′-AAC AAT GAT GCC CTG CGC CA-3′) and VimExon6R (5′-CAA TAT CAA GAG CCA TCT TTA CAT T-3′) (Pyron and Burbrink, 2009). PCR products were purified with the use of Exonuclease I and Shrimp Alkaline Phosphatase (Hanke and Wink, 1994) and sequenced in both directions with the use of Big Dye Terminator 3.1 chemistry on an ABI 3730xl at the Biomedical Genomics Center at the University of Minnesota. Sequences were assembled and checked for accuracy with Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI).

Newly sequenced material, consisting of one *P. c. sayi*, one *P. vulpinus*, and the two putative hybrids, were combined with sequences from GenBank for all analyses (see Appendix 1). Sequences were aligned with the use of ClustalW (Thompson et al., 1994) and, for the *ND4* data, nucleotides were translated into amino acids with the use of MacClade 4.0.8 (Maddison and Maddison, 1992) to confirm alignment and ensure there were no premature stop codons.

We conducted phylogenetic analysis of the *ND4* data with the use of partitioned maximum likelihood, implemented in

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TABLE 1. Morphometric and meristic measurements. Data summaries (mean and range) are presented for parental species. All measurements are in millimeters and from the right side unless otherwise noted.

	Pituophis catenifer sayi ($N = 12$)		*P. c. sayi* × *Pantherophis vulpinus*, Iowa specimen	*P. c. sayi* × *P. vulpinus*, Minnesota specimen	*P. vulpinus* ($N = 12$)	
	Mean	Range			Mean	Range
Ventrals	222.8	213–235	209	206	201.4	189–208
Subcaudals	50.9	37–61	51	55	55.2	48–63
Supralabials	8.3	8–9	8	8	8.1	8–9
Infralabials	10.9	9–12	9	8	10.2	8–11
Prefontals	4.1	3–6	2	3	2.1	2–3
Dorsal scales (row 50)	29.5	28–32	28	27	24.6	22–26
Dorsal blotches	52	45–59	48	52	42.8	37–57
Rostral width	5.2	3.0–8.0	7.35	6.44	7.1	3.5–8.5
Rostral height	6.9	3.8–10.1	6.15	6.22	4.7	2.6–6.4
Prefrontal length	4.8	2.9–6.6	7.07	5.04	5.2	2.8–6.8
Snout length	12.1	6.2–17.3	14.73	12.03	10.3	5.4–13.0
Head length	36.9	20.9–50.3	35.64	38.7	31.2	16.5–39.4
Head width	21.8	11.3–35.3	19.79	19.5	18.1	9.3–22.2
Snout–vent length	1,054	410.0–1,605.0	1,203	1,010	894.2	298–1,210

RAxML 7.0.4 (Stamatakis, 2006). Data were partitioned by codon with a fourth partition for the tRNA. All partitions were assigned the GTR + G model of sequence evolution. We assessed nodal support using 100 nonparametric bootstrap replicates (Felsenstein, 1985). We included additional sequences from *P. c. sayi* and *P. vulpinus* specimens along with sequences from several other *Pituophis* and *Pantherophis* species. *Lampropeltis triangulum* was used as an outgroup.

Phylogenetic analysis of the mitochondrial *ND4* gene allowed us to determine the maternal component of the putative hybrids but could not confirm that they were indeed hybrids. We verified the hybrid origin of the two specimens by examining an independent nuclear locus, intron 5 of Vimentin. We considered heterozygosity in the putative hybrids at the majority of segregating sites between "pure" *P. c. sayi* and *P. vulpinus* as strong evidence for a hybrid origin. We determined heterozygosity by examining chromatograms in Sequencher 4.8 (Gene Codes Corp.). Heterozygous sites were identified easily by the possession of two overlapping peaks at a single locus.

We compared external morphology of the presumed hybrids to putative parental species, *P. c. sayi* and *P. vulpinus*. We collected morphometric and meristic data from 12 *P. c. sayi* and 12 *P. vulpinus* as well as the two putative hybrids (*P. c. sayi* × *P. vulpinus*) (Appendix 1). Measurements were made with the use of Mitutoyo calipers, and all measurements were made to the nearest 0.1 mm. Character descriptions follow Burbrink (2001), and a full list of characters can be found in Table 1. We explored morphological differences among *P. c. sayi*, *P. vulpinus*, and the putative hybrids with the use of principal-components analysis (PCA) with JMP 8 (SAS, 2007). We removed the effect of covariation with snout–vent length (SVL) by using the residuals of the linear regressions between SVL and morphometric measurements.

RESULTS

Maximum-likelihood analysis of 800 base pairs of the mitochondrial *ND4* gene resulted in a well-supported tree (Fig. 1) consistent with recently published molecular phylogenies (Burbrink and Lawson, 2007; Pyron and Burbrink, 2009). Both putative hybrids formed a well-supported clade with other *P. c. sayi* specimens.

We confirmed the hybrid origin of the two snakes by sequencing an approximately 600 base-pair fragment of Vimentin intron 5. We excluded 40 base pairs of low-quality sequence data from the 3′ end of the Iowa hybrid specimen to ensure base call accuracy. Examination of Vimentin intron 5 recovered 13 variable sites, 9 of which were fixed synapomorphies for the *P. c. sayi* and *P. vulpinus* specimens we examined

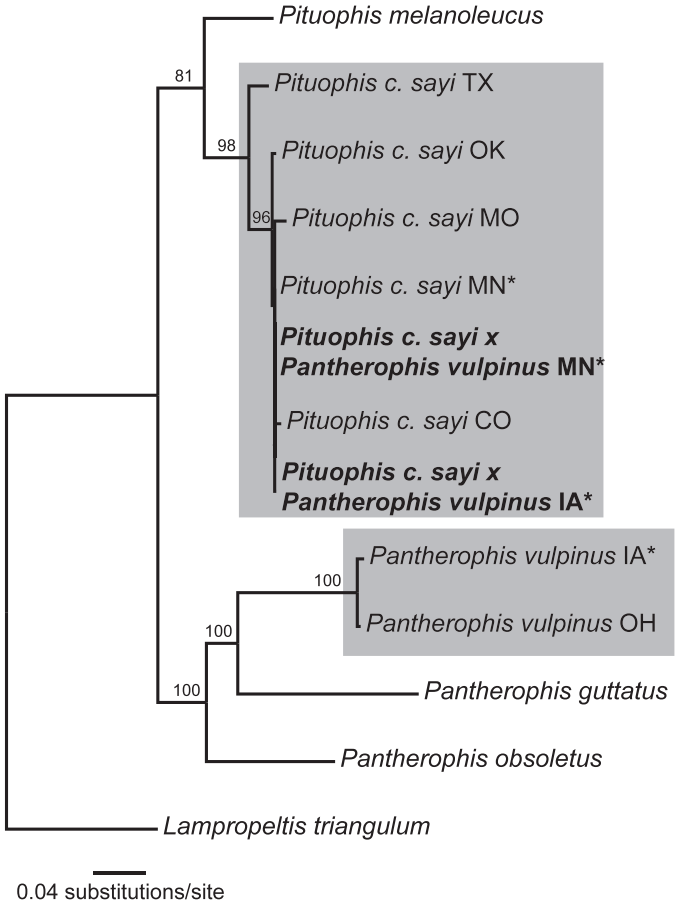

FIG. 1. Phylogenetic relationships among mitochondrial *ND4* haplotypes, constructed with the use of partitioned maximum likelihood (−ln L 2347.270395). *Pituophis catenifer sayi* and *Pantherophis vulpinus* haplotypes are highlighted. Putative hybrid (*P. c. sayi* × *P. vulpinus*) specimens are in bold. Newly sequenced individuals are indicated by an asterisk. Numbers above nodes represent maximum likelihood bootstrap values.

TABLE 2. Variable sites from the Vimentin intron 5 data. Sites representing species-specific synapomorphies are indicated with an asterisk (*).

Sample	30*	42*	69*	149	156*	256*	281*	321	325	408	412*	430*	563*
Pituophis catenifer sayi, Cochise County, Arizona (FJ627902)	G	C	A	T	A	A	T	A	A	A	T	A	C
P. c. sayi Sherburne County, Minnesota (JFBM 16740)	G	C	A	T	A	A	T	T	C	C	T	A	C
Pantherophis vulpinus Ottawa County, Ohio (FJ627910)	A	G	G	G	T	G	A	T	C	A	C	G	G
P. vulpinus Bremer County, Iowa (TG 00132)	A	G	G	T	T	G	A	T	C	A	C	G	G
P. c. sayi × *P. vulpinus* Madison County, Iowa	A/G	G/C	A/G	T	A/T	A/G	A/T	A/T	A/C	A	C/T	A/G	–
P. c. sayi × *P. vulpinus* Wabasha County, Minnesota (JFBM 16865)	A/G	C	A/G	T	A/T	A/G	A	A/T	A/C	A	C/T	A/G	G/C

(Table 2). The putative hybrids were heterozygous at most variable sites, e.g., the Minnesota specimen was heterozygous at 69% of variable sites and the Iowa specimen was heterozygous at 83% of variable sites, confirming their hybrid origins.

The two hybrid specimens were morphologically intermediate between *P. c. sayi* and *P. vulpinus*, which was confirmed by the PCA (Figs. 2–4). The first principal component accounted for 41.4% of between-group variability, and the second principal component accounted for 16.1%. Principal component 1 was most strongly loaded among rostral height and rostral width, the number of ventral and prefrontal scales, and the number of dorsal scales. Principal component 2 was most strongly loaded by head length and width and snout length (Table 3).

DISCUSSION

We used both external morphology and molecular genetic data to identify two intergeneric hybrid snakes, *P. c. sayi* × *P. vulpinus*. Both hybrid specimens were intermediate between the parental species in overall appearance. This was particularly evident in cranial morphology, where *P. c. sayi* and *P. vulpinus* are quite different from each other (Fig. 3). Other characteristics were intermediate between the normal ranges for the parental species. The hissing capabilities of the Iowa hybrid, for example, were midway between the two parental species: longer in duration than the short, forced hiss of *P. vulpinus*, but not nearly as prolonged or raspy as *P. c. sayi* (JBL, pers. obs.). The number of ventral scales and dorsal scale rows in the hybrids were intermediate between the scale counts of the parental species. Not all traits were intermediate, though, and several traits were more closely aligned with one or the other of the parental species. The two hybrids, for example, had divided anal plates as in *P. vulpinus*, compared to the single anal plate of *P. c. sayi*.

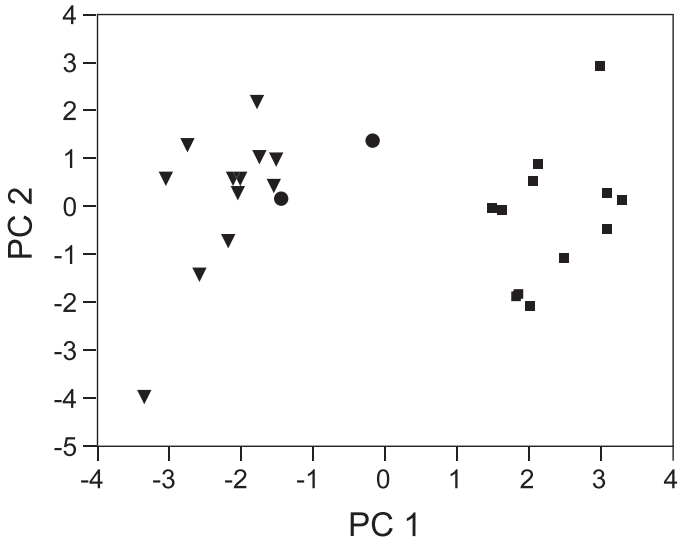

FIG. 2. Principal-component scores for morphological data. Squares represent *Pituophis catenifer sayi*, triangles represent *Pantherophis vulpinus* and circles represent the two *Pituophis c. sayi* × *Pantherophis vulpinus* hybrids.

The occurrence of *P. c. sayi* × *P. vulpinus* hybrids from two different sites is evidence that isolating mechanisms between *P. c. sayi* and *P. vulpinus* occasionally break down. There are two general categories of isolating mechanisms to prevent hybridization among individuals of different species: prezygotic isolating mechanisms that attempt to prevent mating between different species and postzygotic isolating mechanisms that negatively impact hybrid survival and fertility (Dobzhansky, 1951). Although there is not much information on isolating mechanisms in snakes, there is little evidence to suggest an important role for postzygotic isolating mechanisms. Indeed, many snake species will readily hybridize and produce healthy offspring in captivity, and many of these hybrid offspring are fertile. This even includes captive hybrids among different genera: e.g., *Morelia spilota* × *Liasis mackloti* (Banks and Schwaner, 1984); *Lampropeltis californiae* × *Pantherophis guttatus*; *Pituophis catenifer* × *P. guttatus*; *L. triangulum* × *P. guttatus*; *P. catenifer* × *Pantherophis obsoletus*; *P. catenifer* × *P. vulpinus* (JBL, JS, CES, TG, pers. obs.). Prezygotic isolating mechanisms, on the other hand, likely play a more important role in preventing hybridization among different snake species, including courtship rituals, pheromones, body size, and temporal shifts in

TABLE 3. Character loadings (eigenvectors) for the principal-component (PC) analysis of morphometric and meristic data.

Character	PC 1	PC 2
Ventrals	0.37858	−0.2327
Subcaudals	−0.18868	0.07512
Supralabials	0.13925	−0.04199
Infralabials	0.14064	−0.26451
Prefrontals	0.38481	0.03101
Dorsal scales (50)	0.38755	−0.04305
Dorsal blotches	0.28128	−0.12858
Rostral width (residual)	−0.37863	0.14264
Rostral height (residual)	0.34654	0.2546
Prefrontal length (residual)	−0.25039	0.3013
Snout length (residual)	0.13987	0.50462
Head length (residual)	0.17228	0.466
Head width (residual)	0.17066	0.44876

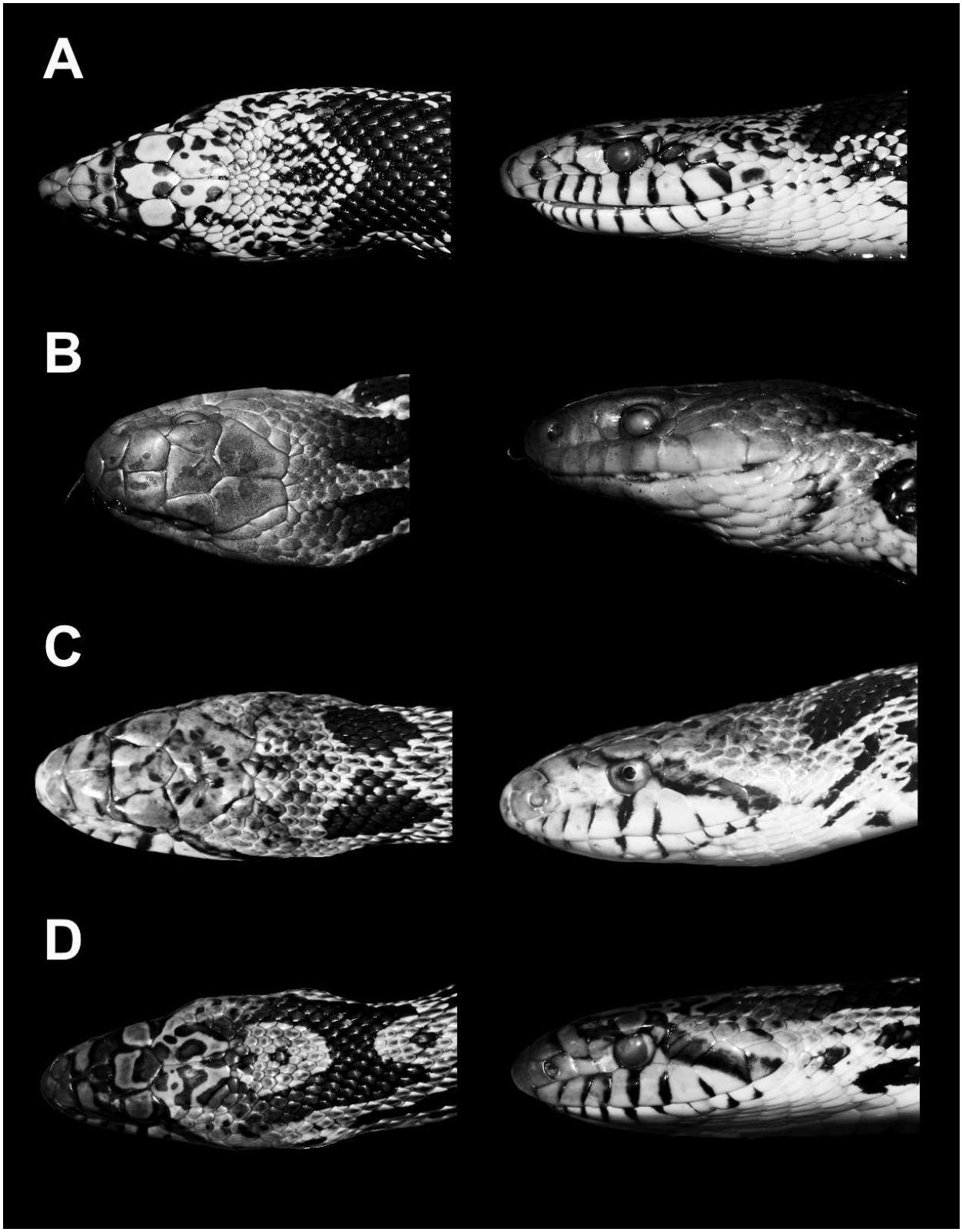

FIG. 3. Dorsal and lateral views of the heads of (A) *Pituophis catenifer sayi*, JFBM 16649, Sioux County, North Dakota; (B) *Pantherophis vulpinus*, JFBM 14521, Renville County, Minnesota; (C) *P. c. sayi* × *Pantherophis vulpinus*, Madison County, Iowa; (D) *Pituophis c. sayi* × *Pantherophis vulpinus* JFBM 16865, Wabasha County, Minnesota. Photos are not to scale.

breeding activity (Shine et al., 2002, 2004). Similarities in mating behavior between *P. c. sayi* and *P. vulpinus* may also explain how such a pairing could occur. Coital biting behavior occurs in both *Pituophis melanoleucus* and *P. vulpinus*, for example, but not in other studied *Pantherophis* species such as *P. obsoletus* and *P. guttatus* (Shaw, 1951; Gillingham, 1974; Gillingham, 1979; Lewke, 1979). Although we cannot say specifically which of these isolating mechanisms failed, resulting in these two hybrid

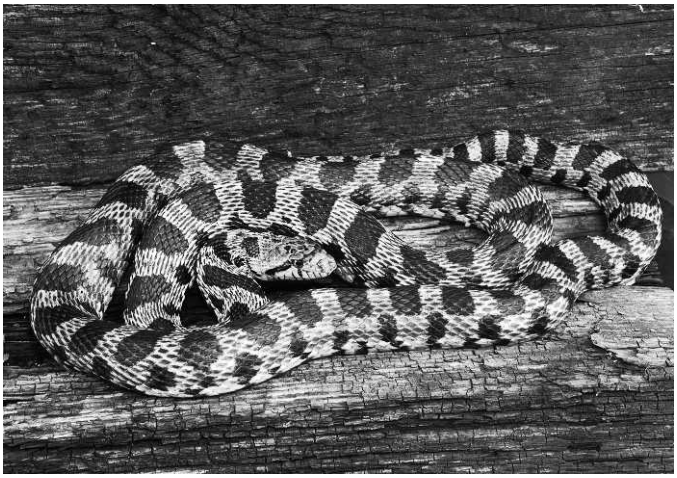

FIG. 4. *Pituophis catenifer sayi* × *Pantherophis vulpinus* hybrid from Madison County, Iowa.

individuals, it is interesting to note that both instances involved a male *P. vulpinus* breeding with a female *P. c. sayi*. Male choice appears to be important in preventing hybridization in snakes (Shine et al., 2002, 2004) and these mechanisms apparently broke down on at least two occasions in the midwestern United States.

Hybridization appears to be uncommon among snake species in the wild and is even rarer between snakes in separate genera. Indeed, these are the first instances of intergeneric hybridizations among wild snakes that have been confirmed with genetic data and only the second and third confirmed instances of intergeneric hybridization in a squamate reptile (Rassmann et al., 1997). Despite the broad sympatry and phylogenetic proximity of *P. c. sayi* and *P. vulpinus* (Conant and Collins, 1998; Burbrink and Lawson, 2007), there are no previous reports of natural hybridization between these two species. We are aware of only one successful *P. c. sayi* × *P. vulpinus* breeding in captivity, the result of an accidental pairing in a zoo (M. Edgar, St. Louis Zoo, pers. comm.). Intergeneric hybrids are known among turtles (Karl et al., 1995; Harding and Davis, 1999; Parham et al., 2001); birds (Graves and Zusi, 1990; Grant and Grant, 1992; Graves, 2007); mammals (Jolly et al., 1997; Caballero and Baker, 2010); and are widespread in fish (Hubbs et al., 1988; Burkhead and Williams, 1991; Garrett, 2005). Why intergeneric hybrids appear to be so uncommon in squamates is difficult to determine. This could be partly due to the unequal evolutionary distances between genera in different vertebrate clades (Avise, 2008). Described squamate genera may simply be older and/or more divergent than teleost, mammalian, or avian genera, making successful intergeneric hybridization among squamates less likely. Given the apparent rarity of intergeneric hybrids in the wild, understanding the exact cause(s) of intergeneric hybridization among squamate species will likely remain elusive for some time.

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Appendix 1. Specimens Examined

Pituophis catenifer sayi: Morphology—Chisago County, Minnesota: JFBM 13234; Hennepin County, Minnesota: JFBM R1038, JFBM 14536; Houston County, Minnesota: JFBM R1332; Pierce County, Minnesota: JFBM 13235; Sherburne County, Minnesota: JFBM 16740; Goodhue County, Minnesota: JFBM 12712, JFBM 12865, JFBM 12870; Wabasha County, Minnesota: JFBM 16866, JFBM 12181; Sioux County, North Dakota: JFBM 16649. Genetics—Sherburne County, Minnesota: JFBM 16740 (GenBank *ND4* JF750662; *Vim* JF750666).

Pantherophis vulpinus: Morphology—Olmsted County, Minnesota: JFBM 12868; Ramsey County, Minnesota: JFBM R528, JFBM R992; Renville County, Minnesota: JFBM 14521; Rice County, Minnesota: JFBM R367, JFBM R1138; Houston County, Minnesota: JFBM 13530; Sibley County, Minnesota: JFBM 14599; Wabasha County, Minnesota: JFBM R559, JFBM R1170, JFBM 12936, JFBM 16366. Genetics—Bremer County, Iowa TG00132 (GenBank *ND4* JF750661, *Vim* JF750665).

Pituophis c. sayi × *P. vulpinus:* Madison County, Iowa: JBL, live (will be deposited to Drake University upon its demise; GenBank *ND4* JF750664; *Vim* JF750664); Wabasha County, Minnesota: JFBM 16865 (GenBank *ND4* JF750663; *Vim* JF750667).

GenBank Material: *ND4—Pituophis c. sayi* (AF141125, AF141123, AF141124, AF141122); *Pituophis melanoleucus* (AF14111); *Pantherophis vulpinus* (DQ902306); *Pantherophis guttatus* (AM236349); *Pantherophis obsoletus* (DQ902296); *Lampropeltis triangulum* (AY739638). Vimentin (intron 5)—*Pituophis c. sayi* (FJ627902); *Pantherophis vulpinus* (FJ627910).