

Karyotypes of Six Species of Colubrid Snakes from the Western Hemisphere, and the 140-Million-Year-Old Ancestral Karyotype of Serpentes

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ABSTRACT

Karyotypes are described for six species of snakes from the Western Hemisphere, and comparisons are made with all species of snakes from around the world that have been karyotyped with modern methods. Although there is significant karyotypic variation in snakes, there is one basic karyotype that is shared by members of all families of snakes, representing widely divergent lineages, extending from today back through the evolutionary history of the Serpentes. Long-term survival of the ancestral snake karyotype may be a result of canalization, similar to some ancient chromosomes of turtles.

INTRODUCTION

During the 1970s–1980s we made chromosome preparations of selected specimens of amphibians and reptiles for which the karyotype might be useful in studying evolutionary history. Preparations were made serendipitously as specimens became available. *Cemophora coccinea* appeared to be of special interest because it has morphological similarities to *Lampropeltis* and *Rhinocheilus* (although it is unique in some details), it has ecological and behavioral similarities with *Rhinocheilus*, and the overall geographic ranges of *Cemophora* and *Rhinocheilus* in North America appear, as one might expect, in a scenario of ecological replacement, competi-

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tive exclusion, or niche occupation (compare field guides by Stebbins, 2003: map 155, and Conant and Collins, 1991: map 151). *Oxybelis aeneus* was of interest because of its distinct morphology, behavior, and range, its distribution broader than that of any other snake in the Western Hemisphere. Here we present the karyotypes of *Cemophora coccinea* and *Oxybelis aeneus* and compare them with karyotypes of representatives of the other genera mentioned above and with additional species representing diverse families of snakes from around the world.

MATERIALS AND METHODS

Chromosomes were prepared from vertebral bone marrow, testes, and in vitro blood culture. For bone marrow and testes we followed Hardy (1976), usually using colchicine instead of velban. For blood culture we followed Cole and Dowling (1970), and for chromosome morphology we followed Cole (1970). The specimens examined are listed in appendix 1.

RESULTS

Cemophora coccinea: Examination of 66 cells from six specimens (5 males, 1 female) revealed a diploid number of 34 ($2n = 34$), with 16 macrochromosomes and 18 microchromosomes. Individual pairs of macrochromosomes are designated with numbers according to decreasing size in the karyotype, illustrated for a male in figure 1A. Chromosome number 1 is metacentric; 2, submetacentric; 3 metacentric; 4 metacentric; 5 metacentric; 6 submetacentric to subtelocentric; and 7 and 8 are submetacentric. Pairs 1 and 2 stand out as the longest, and among the smaller macrochromosomes, pairs 5 and 6 are similar in length while 7 and 8 are shorter but similar to each other in length. In general, the microchromosomes are too small to be resolved clearly, but in many cells four were clearly biarmed and in the clearest cells, up to eight appeared biarmed. Neither secondary constrictions nor satellites were observed. The female is the heterogametic sex (ZZ:ZW sex chromosome system), as in many species of advanced colubroids. Chromosome pair number 4 includes the sex chromosomes; the Z is metacentric and the W is clearly submetacentric (fig. 1B).

Lampropeltis triangulum: Examination of 19 cells from three specimens (males) revealed a diploid number of 36 ($2n = 36$), with 16 macrochromosomes and 20 microchromosomes. Description of the karyotype (fig. 1C) is precisely as for *Cemophora coccinea* except for the following: chromosome number 6 is telocentric; number 8 is subtelocentric; there is one more pair of microchromosomes; and in the clearest cell, 10 microchromosomes appeared to be biarmed. As no females were examined, shape of the W was not determined.

Lampropeltis pyromelana: Examination of 10 cells from one specimen (male) revealed a diploid number of 36 ($2n = 36$), with 16 macrochromosomes and 20 microchromosomes. Description of the karyotype is precisely as for *Lampropeltis triangulum* except for the following: in the clearest cell, four microchromosomes appeared to be biarmed. As no females were examined, shape of the W was not determined.

Lampropeltis splendida × *Lampropeltis californiae*: Examination of 11 cells from one specimen (male) and one cell each from two siblings (one male, one female) revealed a diploid number of 36 ($2n = 36$), with 16 macrochromosomes and 20 microchromosomes. Description of the karyotype is precisely as for *Lampropeltis triangulum* except for the following: chromosome number 5 is submetacentric and, in the clearest cell, seven microchromosomes appeared to be biarmed. The cell from the female showed that chromosome 4 is the heteromorphic pair with the W being clearly submetacentric, as in *Cemophora coccinea*. This is consistent with the karyotype reported by Bury et al. (1970) and Baker et al. (1972) under the name *Lampropeltis getulus*, but those authors did not note the sex chromosomes.

Rhinocheilus lecontei: Examination of 13 cells from one specimen (male) revealed a diploid number of 36 ($2n = 36$), with 16 macrochromosomes and 20 microchromosomes. Description of the karyotype is precisely as for *Lampropeltis triangulum* except for the following: chromosome number 5 is submetacentric, number 8 is submetacentric, and in the clearest cell, four microchromosomes appeared to be biarmed. As no females were examined, shape of the W was not determined. This is consistent with the karyotype of this species reported by Bury et al. (1970).

Oxybelis aeneus: Examination of 40 cells from four specimens (1 male, 3 females) revealed a diploid number of 34 ($2n = 34$), with 16 macrochromosomes and 18 microchromosomes. The karyotype (fig. 2A, from a female), including the sex chromosomes, is similar to that of *Cemophora coccinea* (fig. 1A, B), except for the following: chromosome number 5 is subtelo-centric and has a secondary constriction beside the centromere, and number 6 is metacentric to submetacentric. In *O. aeneus* the W chromosome (fig. 2A) is somewhat shorter than in *C. coccinea* (fig. 1B), being approximately the size of the chromosomes of pair number 6. In the clearest cells, up to 6 microchromosomes appeared to be biarmed. It is unusual to see secondary constrictions on snake chromosomes without using Ag-NOR staining. In other snakes, for example, they have been found on chromosome 2 (Itoh et al., 1970; Moreno et al., 1987), 3 (García and Hernando, 2007), 5 (Becak and Becak, 1969), and on microchromosomes (Wynn et al., 1987; Mezzasalma et al., 2014).

DISCUSSION

We reviewed the global literature on snake karyotypes, including taxa of all families for which karyotypic data are available, beginning with publications dating from 1959. With one exception, the papers cited used current methods of chromosome preparation (use of colchicine or velban, suspension of cells in hypotonic citrate, flame- or air-drying of slides, and use of Giemsa or acetic acid-orcein stain). The exception is Werner (1959: 197) who used “acetic-orcein squash-preparations...preceded by ‘pretreatment’ with hypotonic saline.” C.J.C. has used this method to get perfectly clear chromosomes of anurans, so we have confidence in Werner’s results, although microchromosomes can be difficult to resolve with this method, and Werner did not present photomicrographs. Earlier studies using histological sections of wax-embedded tissues did not provide clear images of individual chromosomes.

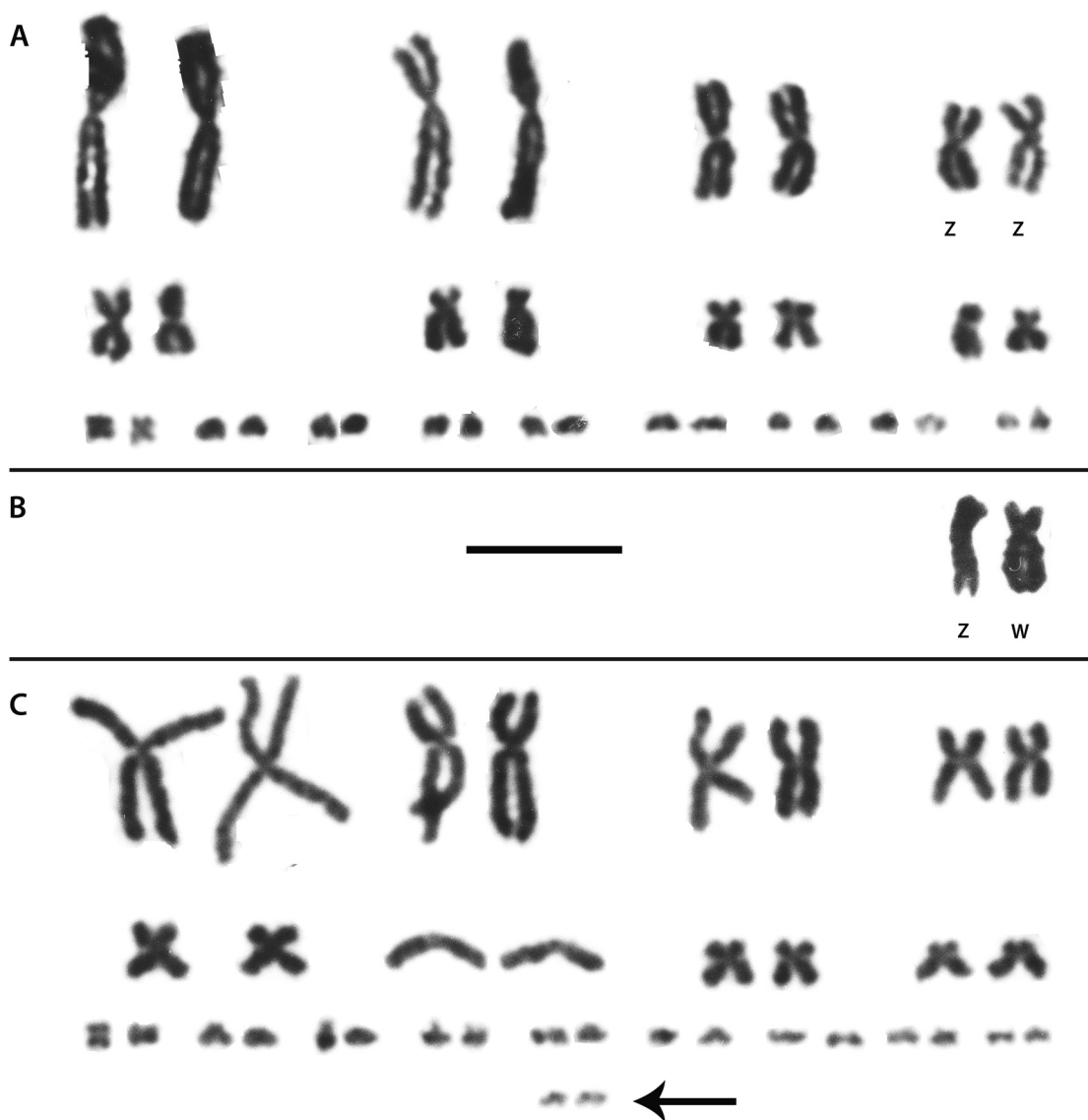


FIGURE 1. Snake chromosomes. **A.** Karyotype of *Cemophora coccinea* ($2n = 34$, with 16 macrochromosomes and 18 microchromosomes), AMNH R-107321, male, with homomorphic sex chromosomes, ZZ. **B.** Heteromorphic pair of sex chromosomes (ZW) from a female *Cemophora coccinea*, AMNH R-110750. Scale bar = 10 μm . **C.** Karyotype of *Lampropeltis triangulum* ($2n = 36$, with 16 macrochromosomes and 20 microchromosomes), AMNH R-109512, male, with one more pair of microchromosomes (arrow) than in A, with C representing the ancestral karyotype of colubroids.

Instead of relying on review papers in which chromosomes were briefly described by other authors with possibly different terminology for centromere position, we personally examined the published photographs throughout the global snake literature in order to make our own direct comparisons. In the early years, several authors suggested that a karyotype similar to that of *L. triangulum* (fig. 1C) might have been ancestral to all or nearly all the snakes (Wer-



FIGURE 2. **A.** Karyotype of *Oxybelis aeneus* ($2n = 34$, with 16 macrochromosomes and 18 microchromosomes), AMNH R-107584, female, with heteromorphic sex chromosomes, Z and W. **B.** Karyotype of *Xenopeltis unicolor* ($2n = 36$, with 16 macrochromosomes and 20 microchromosomes), AMNH R-104370, male, representing an ancient karyotype of Serpentes (reproduced, reformatted, from Cole and Dowling, 1970). Scale bar = 10 μm .

ner, 1959; Bianchi et al., 1969; Baker et al., 1971, 1972; Gorman, 1973). As that suggestion decades ago was based on fewer species examined and some sketchy karyotype descriptions, we review that hypothesis here.

COMPARISONS WITHIN LAMPROPELTINI AND OTHER COLUBRIDS

Representatives of nearly all genera of the Lampropeltini have been karyotyped, including *Arizona*, *Bogertophis*, *Cemophora*, *Lampropeltis*, *Pantherophis*, and *Pituophis*. This clade diverged from other Colubrids about 24.5 mybp (million years before the present; Pyron and Burbrink, 2009; note that dates for nodes within phylogenies are estimates). Nearly all these snakes share a basic karyotype extremely similar to that of *Lampropeltis triangulum* (fig. 1C), but with variation in centromere position on one or a few of the smaller macrochromosomes. The similarities include the following details: diploid chromosome number; number of macro- and microchromosomes; and relative sizes and centromere positions on nearly all the macrochromosomes, including chromosome 6 being telocentric. This condition is shared by a great number of species of colubroids from around the world that have been karyotyped, with minor variation (e.g., see karyotypes in Kobel, 1967; Becak and Becak, 1969; Bianchi et al., 1969; Bury et al., 1970; Dutt, 1970; Itoh et al., 1970; Baker et al., 1972; Singh, 1972, 1974; Ivanov, 1975; Hardy, 1976; Van Devender and Cole, 1977; De Smet, 1978; Mengden and Stock, 1980; Cole and Hardy, 1981; Gutiérrez et al., 1984; Yang et al., 1986; Moreno et al., 1987; Tan et al., 1987; Wei et al., 1992; Aprea et al., 2003; Matsubara et al., 2006; Mezzasalma et al., 2014). Nevertheless, some species have strikingly different karyotypes (e.g., *Bogertophis subocularis*; see Baker et al., 1971). Even so, the basic shared karyotype occurs in the species representing the ancestral form (see below), most of the recently derived forms, and the vast majority of the species. In the phylogeny of the Lampropeltini (Pyron and Burbrink, 2009; Pyron et al., 2013), the ancestor of *Arizona* and *Rhinocheilus* apparently split off prior to the common ancestor of *Cemophora* and *Lampropeltis*, so it appears as if the shift in centromere position on chromosome 6 and apparent loss of a pair of chromosomes in *Cemophora* occurred after the clade to *Cemophora* diverged. In fact, Kobel (1967) and Aprea et al. (2003) reported that the basic karyotype of the Lampropeltini occurs in the European *Coronella austriaca*, including the detail of chromosome 6 being telocentric. This species was used as the outgroup by Pyron and Burbrink (2009), which, if correct, extends existence of the same ancestral karyotype back to approximately 24 mybp or more.

Looking further back throughout the phylogeny of Colubroidea to the common ancestor with the Viperidae and Preatidae, to more than approximately 75 mybp (Pyron and Burbrink, 2012), the majority of the known karyotypes remain similar again, although with some differences in centromere positions on chromosomes 5–8 and more frequent divergence from having 20 microchromosomes (e.g., Yang et al., 1989 and other references above). Nevertheless, Ota (1999) found in *Pareas iwasakii* (representing the related family Preatidae) the same basic karyotype as occurs in *Lampropeltis triangulum*, and it was also found in 41 out of 43 species of the Viperidae, representing diverse genera from several continents (see literature review by Cole, 1990). The same basic karyotype was also found in *Homalopsis buccata* by Pinthong et al. (2013). Deviations from this ancestral karyotype, as in *Oxybelis aeneus* reported here and in North American natricines (Baker et al., 1972; Eberle, 1972) appear to be recent modifications on a few specific clades.

COMPARISONS TO ALL OTHER SNAKES

In a credible global snake phylogeny, the earliest branches include clades to the Leptotyphlopidae, Typhlopidae, and Booidea (including, among others, Boidae, Xenopeltidae, Pythonidae; and Cyndrophidae; Pyron and Burbrink, 2012). Karyotypes have been determined for representatives of all these groups.

Python molurus (see Singh et al., 1968) and *Xenopeltis unicolor* (fig. 2B; see also Cole and Dowling, 1970) represent the pythonids and xenopeltid karyotyped. Both species have a diploid number of 36 chromosomes, with 16 macrochromosomes and 20 microchromosomes, the sizes and shapes of which are indistinguishable from each other in standard preparations, consistent with the hypothesis that this was the karyotype of their common ancestor. Their karyotypes are also extremely similar to that of *Lampropeltis triangulum*, with the only apparent differences in the centromere position of chromosome numbers 5–8 (compare figs. 1C and 2B). *Simalia amethystina* (formerly *Liasis amethystinus*), *Simalia boeleni* (formerly *Liasis boeleni*), and *Liasis olivaceus* all have similar karyotypes but with one less pair of microchromosomes. Karyotypes are known for several species of the Boidae, representing several clades, and one species of the Cyndrophidae. Of these, *Boa constrictor* (see Bianchi et al., 1969; De Smet, 1978) and *Eunectes murinus* (see De Smet, 1978) have karyotypes extremely similar to those of *Xenopeltis unicolor* (fig. 2B) and *Python molurus*, as have *Charina bottae*, *Lichanura trivirgata* (formerly *L. roseofusca*), *Chilabothrus striatus* (formerly *Epicrates striatus*; see Gorman and Gress, 1970), and *Exiliboa placata* (see Hardy, 1989). Also, *Eryx jaculus* (see Werner, 1959), *Eryx johnii* (see Singh et al., 1968), *Eryx conicus* (see Singh, 1972; De Smet, 1978), and *Acrantophis dumerili* (see Mengden and Stock, 1980) have similar karyotypes but with one less pair of microchromosomes. The only cyndrophid karyotyped is similar to the boids listed above, but with one more pair of macrochromosomes and one less pair of microchromosomes (Toriba, 1992), and the boid *Sanzinia madagascariensis* differs in having an extra pair of macrochromosomes and two less pairs of microchromosomes (Mengden and Stock, 1980). Finally, *Corallus hortulanus* (reported as *Corallus enhydris cookii*; see Gorman and Gress, 1970) has a karyotype that apparently differs from that of *Boa constrictor* by only two centric fissions among the macrochromosomes ($2n = 40$, with 20 macrochromosomes and 20 microchromosomes). The overall pattern of the karyotypes of the pythonids, xenopeltid, boids, and cyndrophid suggests that the basic snake karyotype discussed above for the Colubridae also was the ancestral karyotype for all these snakes, and deviations from the ancestral karyotype were more recently derived on several specific clades (as discussed by Werner, 1959; Bianchi et al., 1969; Baker et al., 1971, 1972; and Gorman, 1973). This appears to extend the age of this ancestral snake karyotype ($2n = 36$ with 16 macrochromosomes and 20 microchromosomes; fig. 2B) back to more than approximately 90 mybp following the phylogeny of Pyron and Burbrink (2012). However, heteromorphic sex chromosomes (as seen, for example, in many colubroids) are not distinguishable in the older lineages.

Very few of the many leptotyphlopids and typhlopids have been karyotyped, but those that have been represent one of the oldest clades in Serpentes (Pyron and Burbrink, 2012). Recently, taxonomy of the leptotyphlopids was reviewed by Adalsteinsson et al. (2009) and that of the typhlopids by Pyron and Wallach (2014).

Chromosomes have been studied for only one species of Leptotyphlopidae, *Myriopholis phillipsi* (formerly *Leptotyphlops phillipsi*). Werner (1959: 197) reported that it has a diploid number of 36 chromosomes, with 16 large macrochromosomes, of which 10 are clearly banded (the largest pair is metacentric, second largest submetacentric, and 6 are submetacentric to telocentric) + 20 microchromosomes and “the set looks very much like the ‘common ophidian karyotype’” (fig. 2B).

For Typhlopidae, Werner (1959: 197) also reported that *Letheobia simoni* (formerly *Typhlops simoni*) has a similar karyotype, but it lacks two pairs of microchromosomes ($2n = 32$), which also was found by De Smet (1978) for *Afrotyphlops punctatus* (formerly *Typhlops punctatus*).

Three species of typhlopids from the Western Hemisphere have been shown to share the same karyotype as each other, with $2n = 34$, having 16 macrochromosomes similar to those of *Xenopeltis unicolor* (fig. 2B) and only one pair of microchromosomes fewer (see Wynn et al., 1987, for *T. jamaicensis* and *T. richardii*; García and Hernando, 2007, for *T. brongersmianus* [= *Amerotyphlops brongersmianus*]). In addition, the pantropical and unisexual *Indotyphlops braminus* (formerly *Ramphotyphlops braminus*) has been karyotyped and appears to be a triploid species with the karyotype comprised of three similar haploid sets of chromosomes (Wynn et al., 1987; Patawang et al., 2016). This haploid karyotype, with $n = 14$, has seven macrochromosomes that are similar in size and centromere position to seven of the eight macrochromosomes in the basic snake karyotype described above (fig. 2B) and seven instead of 10 microchromosomes.

If, based on the similarities of the macrochromosomes and number of microchromosomes among all these leptotyphloid and typhloid snakes, we are correct to hypothesize that these karyotypes were derived from an ancestral karyotype similar to that in figure 2B and consistent with the hypothesis of Werner, 1959, Bianchi et al., 1969, Baker et al., 1971, 1972, and Gorman, 1973, then the age of this ancestral snake karyotype extends back to approximately 140 mybp (Pyron and Burbrink, 2012). This should be tested by determining more karyotypes for the leptotyphloid and typhloid snakes.

The Elapidae is a major group of diverse, pantropical snakes for which chromosome data are available for many species and for which karyotypic diversity is unusually extensive for snakes. The clade is also younger than any of the others we considered above (Pyron and Burbrink, 2012), so it is not necessary to review this variation in the context of our discussion of an ancient snake karyotype. Suffice it to say that considerable variation among Australian species was reported by Mengden (1985), who demonstrated essentially the same basic karyotype described here (fig. 2B) in 16 species representing nine genera and concluded that this was the ancestral karyotype for Australian elapids, which is consistent with our hypothesis.

SIGNIFICANCE OF A 140-MILLION-YEAR-OLD KARYOTYPE

Snakes are an extremely successful group, with thousands of species distributed widely on all the continents that currently support reptilian life (all except Antarctica). As reasonably active amniotes that lost their limbs and eyelids, have internal anatomy adapted to the confines of a long narrow body, but that have extremely well-developed and specialized sensory systems, they have evolved to occupy diverse habitats, such as subterranean, terrestrial, arboreal, freshwater, saltwater, and extremely hot and dry deserts to hot humid tropical evergreen forests, from below sea level to above 4000 m elevation, and they use behavioral modifications to avoid the most extreme conditions in the habitats in which they live (Greene, 1997). Is canalization of the serpent's ancestral karyotype important for preserving coordination of certain necessary genetic elements for continuation of their life styles over a long phylogenetic history? Otherwise, how and why would such an ancient karyotype be maintained? If not canalization, does constant stabilizing selection maintain the ancient karyotype, which appears to be successful or adaptive? It seems very unlikely that this karyotype would be conserved for many millions of years if it were not highly selectively advantageous for snakes. Are recent karyotypic modifications young experiments that may not withstand the test of time? Ancient snakes through the millennia probably experienced numerous karyotypic changes that have not survived to today. Harrington and Reeder (2017) also provided credible estimates of the ages of major branch nodes in snake phylogeny, confirming that the evolution of many diverse morphological characters that are useful for familial and generic diagnoses has occurred while the ancient ancestral karyotype was conserved.

Similar questions have been discussed regarding ancient turtle karyotypes (Bickham and Baker, 1976, 1979; Bickham, 1981). Turtles of one kind or another have survived for a vast period of time as highly distinct amniotes that have the body encased in a shell, have anatomy of the shoulder girdle shifted to occur within the modified rib cage, and have the mouth modified into a toothless beak. The karyotype of Recent batagurines and testudinids was credibly surmised to have occurred in their common ancestor more than approximately 64 mybp (Bickham and Baker, 1976). In addition, general chromosome morphology together with banding methods suggested that in turtles, "some chromosomes have remained unchanged for at least 200 million years" (Bickham, 1981: 1291).

We doubt that it is a matter of chance that ancient karyotypes of snakes and turtles have been conserved for 140–200 million years before the present. We suggest that the basic premise of the hypothesized canalization model for ancient chelonian chromosomes (Bickham and Baker, 1979: 78–79) applies to snakes. "The karyotype, which is a product of [natural] selection for arrangement of genes (supergenes, [coadapted gene complexes], position effect), size and number of linkage groups, centromere position, regulatory function, and others, is an important means of achieving an adaptive phenotype.... Chromosomal stability would characterize lineages that have evolved the optimum karyotype for their adaptive zone." The molecular processes by which ancient karyotypes can become canalized and conserved for such extensive periods of time should be investigated.

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APPENDIX 1

SPECIMENS EXAMINED

The specimens are individually cataloged in the herpetological collections of the American Museum of Natural History (AMNH) or Museum of Life Sciences, Louisiana State University in Shreveport (LSUS), as follows:

Cemophora coccinea: UNITED STATES: *South Carolina*: Horry County; Myrtle Beach, near 68th Avenue N and Somerset Avenue (AMNH R-110651). *Georgia*: Liberty County; St. Catharines Island (AMNH R-107321, R-110750–110751, and AMNH R-110753). *Louisiana*: Bossier Parish; 1.1 mi (by LA Hwy 157) SE intersection of LA Hwy 162 and LA Hwy 157 (LSUS 1048).

Lampropeltis pyromelana: This specimen was hatched in captivity in R.G. Zweifel's captive breeding program. It was one of three offspring from parents collected at different localities, as

follows: AMNH R-112197 from UNITED STATES: Arizona: Cochise County; Greenhouse Canyon, Chiricahua Mountains; and AMNH R-122798 from New Mexico: Hidalgo County; Animas Mountains, SE side Animal Peak at 6800 ft. elev. Two of the three offspring in the clutch were preserved (AMNH R-110546 and R-130940) and one donated to R. Mendez, but individual identity of the one karyotyped was lost in the breeding book records.

Lampropeltis splendida × *L. californiae* (also referred to as *Lampropeltis californiae* × *L. splendida*; formerly *L. getula*): These three specimens (AMNH R-119107, male, R-107372, female, and R-110507, male) were hatched in captivity in R.G. Zweifel's captive breeding program, and they had the same parents. The female parent, AMNH R-104693, was from the United States: New Mexico: Grant County; Hachita. The male parent, AMNH R-102294, was from the United States: "California."

Lampropeltis triangulum: UNITED STATES: *New Jersey*: Middlesex County; Madison Township, about 5 mi S Old Bridge and 2 mi W Route 9 (via Route 520) (AMNH R-108341). *New York*: Orange County; 0.5 mi W Eagle Valley via Eagle Valley Road (AMNH R-109508). *New Jersey*: Somerset County; near Watchung, off Route 22 (AMNH R-109512).

Oxybelis aeneus: Panama: Panama; Isla Taboga, ca. 20 m. elev. (AMNH R-107583 and 107584). Panama: Panama; near Rio Chichebre at Interamerican Hwy. ca. 40 km. NE Panama City (AMNH R-108699). Panama: Bocas del Toro; Isla Bastimentos, near Bastimentos (AMNH R-117793).

Rhinocheilus lecontei: UNITED STATES: *New Mexico*: Chaves County; 27 mi E Roswell on Highway 380 (AMNH R-109444).

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