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Cytogenetics and Molecular Data in Snakes: A Phylogenetic Approach

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Evolution · Mitochondrial genome · Phylogeny · Serpentes · Snake karyotypes · Snake venoms · Toxins

Abstract
Snakes are among the most successful groups of reptiles, numbering about 3,000 extant species. In spite of centuries of comparative anatomical and morphological studies, many aspects of snake systematics remain unsolved. To better understand the evolution and diversity of genomic characteristics in Serpentes, we analyzed online sequence data of mitochondrial and nuclear genes, as well as cytogenetic data and reviewed other genomic characteristics such as toxin genes. After the analysis of the whole-genome and chromosomal organization, we find that: (1) cytogenetic comparisons could provide a useful tool to investigate intergeneric and tribal relationships within the extremely diverse neotropical xenodontine snakes; (2) toxin genes could also help to understand snake evolution if special care is taken to choose the sequences because of the difficulty in avoiding paralogs; (3) snake phylogeny based on mitochondrial genome sequences is largely consistent with the relationship obtained using nuclear genes.

Snakes are important for evolutionary and developmental research, as well as for their ecological role and, from the medical standpoint, due to perspectives for bioprospecting of the biomolecules present in their venoms. Characterization of snake genomes is essential to understanding the overall diversity and evolution of the suborder Serpentes. In addition, recent molecular data has advanced knowledge about snake relationships since classical morphological classification.

Snake monophyly and its lepidosaurian membership have long been recognized. The same morphological features that make snakes such a distinctive group (i.e., overall structural reductions and simplification of the body plan) may also account for many difficulties and uncertainties concerning kinships to which a group of lizards is their closest relative, and also relationships among snake lineages.

Systematic biology provides a single information system that connects all extant and extinct organisms by means of their inherited attributes (morphology and development, physiology and its extensions) or, ultimately, the genes regulating the expression of all this (without forgetting epigenetic process). Evolutionary theory provides an explanation for such recurrence of patterns. The advancement and testing of these ideas about snake evolution depend on an important subject: constraints im-

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Table 1. Higher level classification of snakes used herein

Suborder Serpentes Linnaeus, 1758

Infraorder Scolecophidia Cope, 1864
- Superfamily Anomalepidoidea Taylor, 1939
  - Family Anomalepididae Taylor, 1939
- Superfamily Typhlopoidea Merrem, 1920
  - Family Leptotyphlopidae Stejnejer, 1891
  - Family Typhlopidae Merrem, 1920

Infraorder Alethinophidia Hoffstetter, 1955
- Parvorder Amerophidia Vidal, Delmas & Hedges, 2007
  - Family Aniliidae Stejnejer, 1907
  - Family Tropidophiidae Brongersma, 1951
- Parvorder Henophidia
- Superfamily Uropeltoidea Muller, 1831
  - Family Cylindrophiidae Fitzinger, 1843
  - Family Uropeltidae Müller, 1831
    - Subfamily Anomochilinae Cundall, Wallach & Rossman, 1993
    - Subfamily Uropeltinae Muller, 1831
- Superfamily Pythonoidea Fitzinger, 1826
  - Family Xenopeltidae Bonaparte, 1845
  - Family Loxocemidae Cope, 1861
  - Family Pythonidae Fitzinger, 1826
- Superfamily Booidea Gray, 1825
  - Family Calabaridae
  - Family Boidae Gray, 1825
    - Subfamily Erycinae Bonaparte, 1831
    - Subfamily Boinae Gray, 1825
    - Subfamily Ungaliophiinae McDowell, 1987

Alethinophidia *incertae sedis*:
- Superfamily Bolyerioidea Hoffstetter, 1946
  - Family Xenophidionidae Wallach & Günther, 1998
  - Family Bolyeriidae Hoffstetter, 1946
- Parvorder Caenophidia Hoffstetter, 1939
- Superfamily Acrochordoidea Bonaparte, 1831
  - Family Acrochordidae Bonaparte, 1831
- Superfamily Xenodermatoidea Gray, 1849
  - Family Xenodermatidae Gray, 1849
- Superfamily Colubroidea Oppel, 1811

Colubroidea *incertae sedis*:
- Family Homalopsidae Bonaparte, 1845
- Epifamily Pareatoidae Romer, 1956
  - Family Pareatidae Romer, 1956
- Epifamily Viperoidae Laurenti, 1768
  - Family Viperidae* Laurenti, 1768
    - Subfamily Azemiopinae Liem, Marx & Rabb, 1971
    - Subfamily Crotalinae Oppel, 1811
    - Subfamily Viperinae Laurenti, 1768
- Epifamily Elapoidae Boie, 1827
  - Family Elapidae Boie, 1827
    - Subfamily Elapinae Boie, 1827
    - Subfamily Bungarinae Eichwald, 1831
    - Subfamily Hydrophiinae Fitzinger, 1843
  - Family Lamprophiidae Fitzinger, 1843
    - Subfamily Psammophiinae Dowling, 1967
    - Subfamily Pseudaspidinae Dowling, 1975
    - Subfamily Atractaspidinae Günther, 1858
    - Subfamily Pseudoxyrhophiinae Dowling, 1975
    - Subfamily Lamprophiinae Fitzinger, 1843
- Epifamily Colubroidae Oppel, 1811
  - Family Colubridae* Oppel, 1811
    - Subfamily Calamariinae Bonaparte, 1838
    - Subfamily Grayiinae Meirte, 1992
    - Subfamily Colubrinae Oppel, 1811
  - Family Natricidae* Bonaparte, 1838
    - Subfamily Natricinae Bonaparte, 1838
    - Subfamily Rhabdophiinae Mahendra, 1984
    - Subfamily Hydraethiopsinae Dowling, 1978
  - Family Pseudoxenodontidae McDowell, 1987
  - Family Dipsadidae Bonaparte, 1838
    - Subfamily Carphophiinae Zaher et al., 2009
    - Subfamily Dipsadinae Bonaparte, 1838
    - Subfamily Xenodontinae Bonaparte, 1845

The application of the Principle of Coordination (Article 36 of the International Code of Zoological Nomenclature, 1999), allied to the convention of phyletic sequencing (Nelson, 1973), is sufficient to inform phylogenetic relationships without the paraphernalia of creating new names (as done in some other recent proposals). The epifamily category has been introduced in order to improve subordination and to maintain the stability of some long-standing important clades such as the Colubroidea. Eventual disagreements found in phylogenetic hypotheses (see text for discussion) were properly expressed by *incertae sedis* and **sedis mutabilis* annotations (Wiley, 1979).

posed by our current knowledge on systematic patterns of snake relationships.

Since snake systematics has undergone a major revision recently, with a proliferating number of classification proposals, and given that the present review refers to many outdated taxonomic works, it is important to be explicit about the classification scheme followed by us. Table 1 presents a summary of the present state of snake classification for the family-group categories and higher ones. The higher level taxonomy follows a consensus of current phylogenetic hypothesis [Lawson et al., 2005; Lee et al., 2007; Vidal et al., 2007a, b, 2008, 2009; Wiens et al.,

2008; Wüster et al., 2008; Kelly et al., 2009; Zaher et al., 2009].

Evolution cannot be separated from systematics and evolution is a population genetic process governed by 4 fundamental forces: natural and sexual selection, mutation, recombination, and random genetic drift [Lynch M, 2007]. We reviewed some points of the snake genomes in this manuscript; some are not directly related to the construction of relationships among the snakes but are useful to understand it. The genome size of snakes is small, 2.21 pg/nucleus in average [Olmo, 2008], and GC content profiles are characterized by a great variability among the snakes from 0.4 to 3.6 mg/cm^3 [Hughes et al., 2002]. One important event in differentiation of snakes was the nonsynonymous mutations in the homeobox gene *HOXA-13* that may be responsible for the absence of legs [Kohlsdorf et al., 2008]. One important genetic marker are microsatellites, which are not directly involved in the evolution of snakes, however useful for understanding the genetic processes [Hille et al., 2002; Lukoschek et al., 2008]. We cannot forget to add the Horizontal Gene Transfer to these genetic mechanisms. In this context, many transposable sequences were described in snakes as Bov-B LINE [John et al., 1994; Nakashima et al., 1995; Kordis and Gubensek, 1998; Zupunski et al., 2001], Sauria-SINE [Piskurek et al., 2006], CR1-like [Nobuhisa et al., 1998], and Ty1-like [Flavell et al., 1995].

To better understand the evolution and diversity of genomic characteristics in Serpentes, we elected 3 points to consider in this manuscript. So we have analyzed sequences of available mitochondrial genome (comparing the results with those based solely on nuclear loci), reinterpreted previous cytogenetic data with an up-to-date phylogenetic hypothesis and reviewed some aspects of toxin genes.

Snake Karyotypes

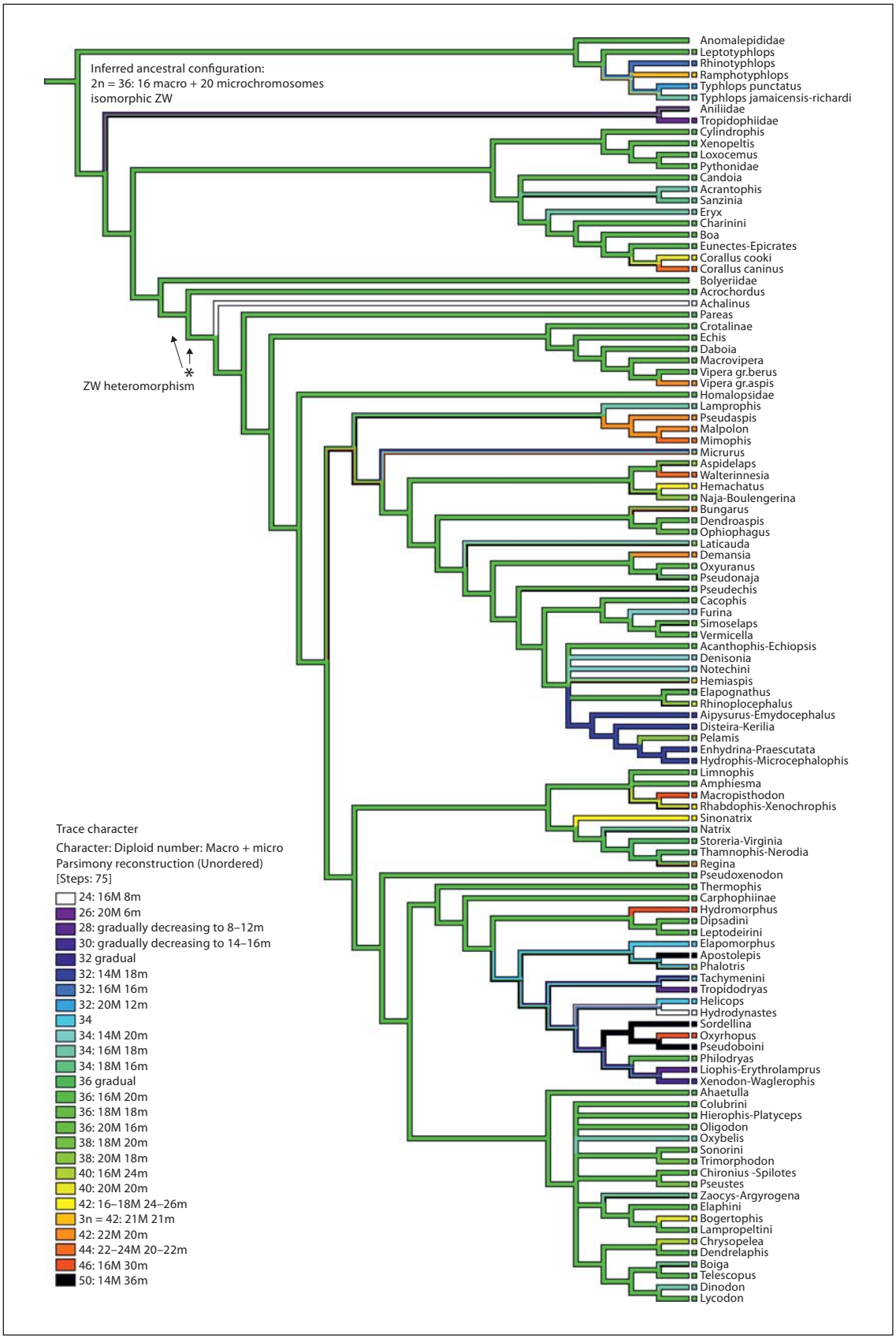
The cytogenetic age, although it provides substantial information for comparative purposes, has not resulted in improvements of snake systematics. Beçak [1965, 1966], Beçak and Beçak [1969], Singh [1972] and Olmo [1986] conducted massive studies in an attempt to better understand chromosome evolution in this group and its relation to systematics, hence providing a general picture of the chromosomal constitution in the group.

The chromosome complement of snakes is relatively conserved, but this does not mean that translocations, inversions, duplications and deletions did not occur. Moreover, fusions and fissions clearly happened in many cases. However, some general features seem to be valid for the whole suborder. Typical karyotypes present: (a) Macro- (M) and microchromosomes (m). (b) 2n = 36 in almost all of the families studied, normally of the 16M + 20m type. (c) Heterochromatin (visualized by C-banding) predominantly centromeric or pericentromeric, and telomeric or subtelomeric. Interstitial C-bands were seldomly reported. (d) AgNORs and rDNA predominantly on microchromosomes. (e) Morphologically differentiated sex chromosomes are of the type ZZ/ZW (or multiple chromosomes in some cases) or not morphologically differentiated in some groups.

Ancestral reconstruction within Serpentes was done by parsimony optimization using the diploid number plus numbers of macro- and microchromosomes available in the literature [main source: Olmo and Signorino, 2005]. These data were complemented with original observation [Batistic and Ferrarezzi, unpublished] for the Tropidophiidae and Elapomorphini. Note that our *Tropidophis paucisquamis* (2n = 26) report consists of the first and single account for the whole family [Batistic et al., 2002], since that currently ascribed as Tropidophiidae in the literature (from *Exiliboa*) actually belongs to the Ungaliophiinae, here included in Charininae, Boidae. Although we are aware this is an oversimplification of a complex character set, it was possible to trace a few preliminary remarks about the pattern that arises. These results are presented in a reduced form in figure 1.

The 2n = 36 (16M + 20m), although not exactly identical in the morphology of the chromosomes for all taxa coded as such, is sufficiently similar to be considered a shared homology among most snake groups. This putative ancestral configuration is notably conservative (remaining apparently unchanged in many extant species) and showing a pervasive distribution throughout the snake tree branches, from which a number of other configurations have been derived. It is suggested to be prim-

Fig. 1. Ancestral reconstruction of karyotype configurations (diploid number plus the number of macro- and microchromosomes) under parsimony optimization on a phylogenetic hypothesis of snakes (adapted from various sources). The 2n = 36 with 16 macro- and 20 microchromosomes (green branches) is indicated as a shared ancestral homology throughout most of the family tree, and from which many other karyotypes have been derived at different taxonomic levels. Sexual chromosome (ZW) heteromorphism is a putative synapomorphy of the superfamily Colubroidea.


Inferred ancestral configuration:
2n = 36: 16 macro + 20 microchromosomes
isomorphic ZW
ZW heteromorphism
Anomalepididae
Leptotyphlops
Rhinotyphlops
Ramphotyphlops
Typhlops punctatus
Typhlops jamaicensis-richardi
Aniliidae
Tropidophiidae
Cylindrophis
Xenopeltis
Loxocemus
Pythonidae
Candoia
Acrantophis
Sanzinia
Eryx
Charinini
Boa
Eunectes-Epicrates
Corallus cooki
Corallus caninus
Bolyeriidae
Acrochordus
Achalinus
Pareas
Crotalinae
Echis
Daboia
Macrovipera
Vipera gr.berus
Vipera gr.aspis
Homalopsidae
Lamprophis
Pseudaspis
Malpolon
Mimophis
Micrurus
Aspidelaps
Walterinnesia
Hemachatus
Naja-Boulengerina
Bungarus
Dendroaspis
Ophiophagus
Laticauda
Demansia
Oxyuranus
Pseudonaja
Pseudechis
Cacophis
Furina
Simoselaps
Vermicella
Acanthophis-Echiopsis
Denisonia
Notechini
Hemiaspis
Elapognathus
Rhinoplocephalus
Aipysurus-Emydocephalus
Disteira-Kerilia
Pelamis
Enhydrina-Praescutata
Hydrophis-Microcephalophis
Limnophis
Amphiesma
Macropisthodon
Rhabdophis-Xenochrophis
Sinonatrix
Natrix
Storeria-Virginia
Thamnophis-Nerodia
Regina
Pseudoxenodon
Thermophis
Carphophiinae
Hydromorphus
Dipsadini
Leptodeirini
Elapomorphus
Apostolepis
Phalotris
Tachymenini
Tropidodryas
Helicops
Hydrodynastes
Sordellina
Oxyrhopus
Pseudoboini
Philodryas
Liophis-Erythrolamprus
Xenodon-Waglerophis
Ahaetulla
Colubrini
Hierophis-Platyceps
Oligodon
Oxybelis
Sonorini
Trimorphodon
Chironius -Spilotes
Pseustes
Zaocys-Argyrogena
Elaphini
Bogertophis
Lampropeltini
Chrysopelea
Dendrelaphis
Boiga
Telescopus
Dinodon
Lycodon
Trace character
Character: Diploid number: Macro + micro
Parsimony reconstruction (Unordered)
[Steps: 75]
24: 16M 8m
26: 20M 6m
28: gradually decreasing to 8–12m
30: gradually decreasing to 14–16m
32 gradual
32: 14M 18m
32: 16M 16m
32: 20M 12m
34
34: 14M 20m
34: 16M 18m
34: 18M 16m
36 gradual
36: 16M 20m
36: 18M 18m
36: 20M 16m
38: 18M 20m
38: 20M 18m
40: 16M 24m
40: 20M 20m
42: 16–18M 24–26m
3n = 42: 21M 21m
42: 22M 20m
44: 22–24M 20–22m
46: 16M 30m
50: 14M 36m

itive to most family-group taxa, with possible exception of the Typhlopidae, Tropidophiidae, and Xenodermatidae (for which just a few or a single species have been karyotyped) and possibly also the Elapidae plus Lamprophiidae clade and the xenodontine dipsadids, both resulting in equivocal optimization due to variation among and within terminal taxa.

Diversification occurred from this ancestral karyotype and from then on possibly all species have suffered at least some modifications in them, altering or not the diploid number. As new tools for chromosomal investigations become available, in addition to the visualization of karyotypes by standard and banding staining, the incorporation of molecular techniques (FISH and others) made it possible to 'see' inside the chromosomes, to localize genes such as the crotamine gene mapped in *Crotalus durissus terrificus* by Rádis-Baptista et al. [2003], the *RABSA*, *DMRT1* and *SOX9* genes to the sexual chromosomes by Matsubara et al. [2006], and others. Chromosomes may undergo many alterations, with or without any perceptible change in their morphologies. Chromosomes may also undergo no changes at all in their morphology or content, as seen in the cryptodiran turtles known for their notable karyotypic stability [Olmo, 1986; Olmo et al., 2002], further corroborated by Mühlmann-Diaz et al. [2001]. These authors used a whole chromosome-1-specific probe from a cryptodiran turtle (*Trachemys*) that resulted in specific hybridization of chromosome 1 on 4 other turtles, revealing a cytogenetic stability of this chromosome during the past 66–144 million years. The authors point out that in one third of that time, various hominid species underwent extensive chromosomal rearrangements.

In Typhlopidae, a poorly sampled family based on chromosomes, 5 out of 6 species present 2n = 32 and 2n = 34 with 16M, as in a 'typical' 36 karyotype. The variation in number is due to a diminishing number of microchromosomes, possibly because of translocations and fusion events. However, in *Typhlops punctatus*, the variation involves macro- and microchromosomes [Olmo and Signorino, 2005; García and Hernando, 2007]. This is also the case for Elapinae, where the karyological alterations involve macro- and microchromosomes. *Micrurus* from Central America varies its chromosome number from 2n = 26 to 34 [Graham, 1977; Gutiérrez and Bolaños, 1979; Gutiérrez et al., 1988; Luykx et al., 1992]. Contrarily, in South America *M. surinamensis surinamensis* has 2n = 38 [Gutiérrez et al., 1988], *M. lemniscatus carvalhoi* presents 2n = 42 [Beçak and Beçak, 1969], *M. corallinus* has 2n = 40 and *M. ibiboboca* has 2n = 42 [Serafim et al., 2007]. Serafim et al. [2007] formulated the hypothesis that the chromosome number in the genus tends to increase in South America and decrease in Central America, changes that especially involve the microchromosomes in Central America and the macrochromosomes in South America, probably due to fusion and fission processes. The high chromosome variability is also true for *Bungarus* and *Naja* and other species in the family. The members of the Hydrophiinae also show considerable variation in the number of chromosomes [see Olmo and Signorino, 2005]. In contrast, the subfamily Crotalinae presents very conservative karyotypes, all with 2n = 36 (16M + 20m) and similar morphology, the 4th pair being the sex chromosomes, with female heterogamety. Minor differences are observed in the morphology of the autosomes from one species to another. Also the W is variable in size and morphology from species to species, but always well differentiated from the Z chromosome. The small morphological differences in the macrochromosomes of different species in the subfamily suggest that translocations and inversions still happen but possibly without disrupting the main linkage groups. Good evidence of these changes was given in the complex of species *Bothrops neuwiedi* by Trajtengertz et al. [1995], showing a translocation of rDNA genes to a macrochromosome, but apparently still in the process of fixation. Contrary to Elapidae and many Xenodontinae, the Crotalinae seem to have achieved an ideal condition in the assemblage of many linkage groups of genes, possibly maintained, at least partially, by the absence of changes in the chromosome number. This seems to fit well with the 'canalization model' and the achievement of the 'optimum karyotype' proposed by Bickham and Baker [1979]. However, it must be stressed that if this is the case, this optimum karyotype was achieved by modifications without changing the chromosome number and that the sex chromosomes must have been already present in the ancestor that gave rise to the family Viperidae. Those snakes present a wide geographic distribution (absent basically in Africa, Europe, Australia and Antarctica), suggesting that the relative stability of the chromosomal set did not interfere with the dispersal of the genus and its adaptation to new environments, accompanied by minor visible rearrangements that might be a consequence of speciation or part of the process.

The opposite situation is found in Elapidae and many Xenodontinae. Their ability to occupy new environments is accompanied by karyotypical alterations. The more conspicuous case is found in the tribe Elapomorphini where the increase in morphological complexity is ac-

companied (or is it its cause?) by karyological alterations especially involving centric fissions of macrochromosomes and the increase in the number of microchromosomes probably also as a result of centric fissions. *Apostolepis*, the genus showing the greatest number of morphological evolutionary novelties in the tribe, has a diploid number of 56 or higher [Batistic and Ferrarezzi, unpublished]. In the Pseudoboini, a common diploid number is 50, with all macrochromosomes being telocentric, except for the sex chromosomes: the Z is meta- or submetacentric and the W is telocentric, normally the biggest one in the karyotype. *Sordellina puctata* has 2n = 52 [Beçak et al., 1990], but there are no evidences of close relationships between it and Pseudoboini; moreover, unpublished molecular data indicate that they belong to different groups [F. Grazziotin, personal communication]. The possible homology detected by parsimony optimization in ancestral reconstruction may be an artifact due to incomplete taxon sampling between these two. If so, we can infer that the fission process occurred independently, repeatedly in the phylogeny of snakes, the above examples are just some of them.

Microchromosomes

A common feature in Reptilia is the presence of macro- and microchromosomes, probably due to karyological ancestral derivation from forms that already presented this attribute. Could microchromosomes be advantageous to the owners? Genes present in microchromosomes are less subject to be bounded in large linkage groups than genes in macrochromosomes, segregating independently. As a result, there is an increase in the variability of the genomes. C-banding showed that not all microchromosomes are heterochromatic. Moreover, even microchromosomes that are apparently totally heterochromatic may have some euchromatin in them. This condition may be or not advantageous, depending on environmental conditions [Olmo and Signorino, 2005; Olmo 2005, 2008; unpublished observations of RF Batistic]. *Hydrodynastes bicinctus bicinctus* has 2n = 24, a very small chromosome number for snakes and no microchromosomes [Beçak and Beçak, 1969], which may be interpreted as a formation of favorable linkage groups. This karyotype is clearly a derived one, when other species of the family are analyzed. In the other extreme, the *Apostolepis* genus presents the highest diploid number so far found in Serpentes and the highest number of microchromosomes, some of them very small [Batistic and Ferrarezzi, unpublished]. This genus belongs to the tribe Elapomorphini, subfamily Xenodontinae, family Dipsadidae [Ferrarezzi, 1994], the diploid number varying from 2n = 34 to 2n = 56 or more. The increase in the number of chromosomes is accompanied by an increase in morphological derivations [Batistic and Ferrarezzi, unpublished]. Olmo [2008] argues that the translocation of microchromosomes to macrochromosomes could result in an increase in R-band numbers. As a consequence an increase in recombination levels and a high rate of chromosome change would have taken place bringing about a reduction in the size of genomes and chromosomes. This could have caused a further increase in the recombination level and an increase in the chromosome change.

Genes thus far mapped for the microchromosomes are the rDNA sequences, detected by silver staining [Trajtengertz et al., 1995] and some other genes recovered from cDNA libraries mapped by FISH [Matsuda et al., 2005; Matsubara et al., 2006]. The rDNA sequences found may be located in the macro- or in the microchromosomes or in both at the same time. The Ag-NOR pattern in the *neuwiedi* group of *Bothrops* (2n = 36) varied in different populations (some considered as subspecies, and nowadays as full species) and within a same population. The active rDNA clusters were always present in 2 chromosomes, as follows: 2 macrochromosomes number 6 or one macrochromosome number 6 and one or 2 microchromosomes [Trajtengertz et al., 1995]. This was the first case of the presence of rDNA genes in macrochromosomes in Viperidae and a very clear example of a translocation of this gene from micro- to macrochromosomes. Despite the translocation, the organization of the rDNA repeats analyzed by Southern blot showed to be highly conserved for the subspecies. In *Liophis poecilogyrus shotti* (2n = 32) as well as in *B. jararacussu* (2n = 36) the AgNORs are located in 2 microchromosomes [Trajtengertz et al., 1995]. In *C. durissus terrificus*, Svartman et al. confirmed by FISH the mapping of rDNA to microchromosomes [personal communication]. The more common situation is the presence of one pair of chromosomes with rDNA, but 2 pairs are not uncommon. As mentioned above, in *B. insularis* (2n = 36) 2 pairs of microchromosomes show Ag-NORs, one pair near the centromere and one pair in the distal region, at the telomere or near it, since 2 dots are clearly visible in 2 microchromosomes [Batistic, unpublished]. Porter et al. [1991], using molecular in situ hybridization with biotinylated probes, determined the location of the 28S ribosomal sequences in 4 species of Caenophidia. In the viperid *Crotalus viridis* and in the colubrid *Mastophis flagellum*, both with 2n = 36 and similar karyotypes, the first presented rDNA sequences on 2 pairs of microchromosomes

and the second in only one pair. In the natricids *Nerodia fasciata* and *Thamnophis marcianus*, both with 2n = 36 (34M + 2m), the hybridization occurred only in one pair of macrochromosomes (long arm of pair 1 or pair 2). Those observations are in agreement with our own observations in many different species [Batistic, unpublished] using silver staining. Microchromosomes were found even in a translocation with sex chromosomes in a population of *Bungarus caeruleus* from West Bengal resulting in a multiple sex chromosome constitution of $Z_1Z_1Z_2Z_2/Z_1Z_2W$. In the same females, the W suffered a dissociation resulting in multiple W chromosomes: W_1 and W_2. A predominance of polymorphic females over the females carrying the original chromosome constitution led the authors to suggest that polymorphic bearers had better adaptive flexibility and higher fecundity [Sing et al., 1979].

Sex Chromosomes

Morphologically differentiated sex chromosomes are spread all over the Metazoa. In vertebrates they occur in all classes but not in all groups within a class. In non-avian reptiles they occur in all Orders but not homogeneously. In Serpentes they are present in some families and not in others. Within families they may be present in some species and absent in others, possibly due to different stages of morphological differentiation. Therefore, snakes are a good model for the study of sex chromosomes.

The great majority of South American Boidae presents no morphological differentiation of sex chromosomes in standard staining [Beçak, 1966]. However, genes for masculinization or feminization may have accumulated in one or more pairs of chromosomes during evolution without morphological differentiation [Beçak, 1965; Singh et al., 1968]. Ray-Chaudhuri et al. [1970] did not find W chromatin in interphasic nuclei in a number of tissues of many Boidae species. *Acrantophis dumerili* was found to have a differentiated W chromosome: the size of the Z and the W were similar but the former is metacentric and the latter is acrocentric. This was the first report of differentiated sex chromosomes in Boidae [Mengden and Stock, 1980], indeed the only known case in non-Caenophidian snakes. In the Colubroidae, and especially in the Natricidae and Dipsadidae, different stages of differentiation of the W chromosome occur: they may differ from the Z by the centromere position or by size or by both [Beçak and Beçak, 1969; Beçak et al., 1990]. Aprea et al. [2006] examined 3 species of the *Vipera aspis* complex and *Cerastes vipera* of the Viperinae using standard and banding staining methods. They found *C. vipera* with a 2n = 36 (16M + 20m) and no sex chromosome differentiation. The 3 species of *Vipera* presented a 2n = 42 karyotype (22M + 20m) and in the examined females of *V. aspis atra* and *V. aspis aspis* C-banding pointed to the existence of a differentiated sex chromosome pair. In this case the W chromosome was almost totally heterochromatic, although morphologically indistinguishable from the Z. *Mimophis*, part of the reptile fauna of Madagascar, is the only psamophiine Lamprophiidae [Vidal et al., 2008] karyotyped to date. Its karyotype (2n = 44: 24M + 20m) revealed the 4th pair as the heteromorphic sex chromosomes: the Z chromosome is biarmed, and the W is uniarmed. The W element was totally heterochromatic (C-banding) except for an interstitial euchromatic region [Aprea et al., 2003].

The Viperidae and Elapidae show a well differentiated W chromosome [Beçak, 1964, 1966; Ray-Chaudhuri et al., 1971]. A multiple sex chromosome system $Z_1Z_1Z_2Z_2/Z_1Z_2W$ was first described in vertebrates in *Bungarus caeruleus* (Elapidae) [Singh et al., 1970]. The males were 2n = 44 and the females 2n = 43 and the W condensed chromatin could be observed in the interphasic nuclei. Later another case of multiple sex chromosomes was found in *Hydrophis fasciatus fasciatus* (Hydrophiidae) of the type ZZ/ZW_1W_2. Also in this case, the interphase nuclei presented in some tissues 2 kinds of heteropycnotic groups, corresponding to the W_1 and the W_2 [Ray-Chaudhuri and Singh, 1972].

What is the first step in the differentiation of the homomorphic autosome that harbors sex-determining genes? Beçak [1964] suggested that the first step was a pericentromeric inversion in one of the homologs, which prevented crossing-over in the region and the accumulation of sex-determining genes in the W. In a second step this region could undergo heterochromatinization and differentiation in morphology [Beçak, 1983]. On the other hand, Ray-Chaudhuri et al. [1971] argued that the first step for this differentiation could be the heterochromatinization of one chromosome that would later undergo morphological modifications. Minor satellite DNA fractions were found in great concentrations in the W chromosome, an evidence that crossing-over may have ceased prior to morphological differentiation between the Z and the W chromosomes [Singh et al., 1976; Jones and Singh, 1985]. Another possibility is that both hypotheses are true, depending on the case under consideration. Matsubara et al. [2006] compared the chromosomes of 3 snakes with different stages of sex chromosome differentiation: *Python molurus bivittatus* (Boidae) with no ap-

parent sex chromosome differentiation, *Elaphe quadrivirgata* (Colubridae) with intermediate differentiation, and *Protobothrops flavoviridis* (Viperidae) with well differentiated ones. *E. quadrivirgata* and *P. flavoviridis*, both presenting the short arm of the W chromosome extensively degenerated, retain the homology between Z and W only in the telomeric regions. Moreover, in *E. quadrivirgata*, a region near the centromere on the long arm of the W chromosome is partially homologous. They advance the hypothesis that the differentiation of sex chromosomes began at the distal region on the short arm of a protosex chromosome in a common ancestor through the occurrence of a sex differentiator on one of the homologs, favoring chromosomal rearrangements with a consequent cessation of meiotic recombination. Supposedly, this favors the accumulation of mutations, of euchromatin deletions and heterochromatinization with accumulation of repetitive DNA sequences. This process progresses from the short to the long arm of the W chromosome, as in the *E. quadrivirgata* and *T. flavoviridis* lineages, where the process would have continued independently after their divergence. Their hypothesis seems to agree with Beçak [1964], although they do not explain how and why this first differentiation occurs.

Have the sex chromosomes of all snakes had the same origin? The study of Matsubara et al. [2006] strongly suggests that all Alethinophidia share the same Z chromosomes, based on gene content, but nothing is so far known in this respect about the Scolecophidia. The other question is: have the sex chromosomes of snakes had the same origin as those of other vertebrates? For instance, were all originated from the same ancestral autosome pair? Many research groups are trying to answer these questions by combining cytogenetics and molecular approaches. Matsuda et al. [2005] constructed comparative cytogenetic maps of a turtle *(Pelodiscus sinensis)*, and of a snake *(Elaphe quadrivirgata)* using cDNA clones of reptile functional genes. They concluded that the homology between turtle and chicken chromosomes are highly conserved but not as high with snake chromosomes. Interestingly, however, turtle chromosome 6q and snake 2p represent conserved synteny with the chicken Z chromosome. Their results point to the conclusion that avian and snake sex chromosomes were derived from different autosomes in a putative common ancestor. Later on, also Matsubara et al. [2006] and Kawai et al. [2007] using molecular techniques associated to cytogenetics came to the same conclusions. However, some observations challenge the view that sex chromosomes have evolved independently in vertebrates, because there is evidence that transitions between ZW and XY systems have occurred many times during the course of evolution in fish, amphibians and reptiles [Ezaz et al., 2006; Janes et al., 2008]. Further investigations are needed to give some definitive answers on the subject.

Toxin Genes

The venom systems in snakes consist of glands that produce toxic substances with or without an injection system. If Viperidae snakes have the most efficient system of venom accumulation and injection, Elapidae snakes show the most toxic venoms [Kochva, 1987]. The venom system presents many levels of complexity: from those undifferentiated serous glands, through many forms artificially taken as intermediaries and assembled as Duvernoy's glands, to the most developed venom gland in Elapidae and Viperidae. The evolutionary homology among venom glands is supported by similarities in embryonic development of the dental, Duvernoy's, and true venom glands [Kochva, 1978; Kardong, 2002; Jackson, 2003]. On the other hand, McDowell [1986] argues that the true venom glands derived from the rictal gland, a small oral gland that has muscles bound to them, while the Duvernoy's gland would have a different origin. According to dentition, the snakes can be aglyphous (with no specialized teeth), opisthoglyphous (rearward grooved fang), proteroglyphous (forward grooved fang) and solenoglyphous (forward pipe grooved fang). After studying the dental ontogeny, Vonk et al. [2008] observed that the front and rear fangs are homologous. Given that the true venom glands (of Viperidae and Elapidae) and the Duvernoy's glands (of the other Colubroidea) originate from different oral glands, the toxins that are part of their arsenal could have different origins, even though they have the same protein structure and/or similar biological activity. Moreover, it is known that the venom toxins are highly polymorphic in all taxonomic levels even within a single species [Chippaux et al., 1991].

The toxin genes have undergone an accelerated evolution that has permitted the endogenous proteins to acquire toxic activities, and after to enlarge their range of activities and targets. This accelerated evolution of sequences was shown by Nakashima et al. [1995] for PLA_2 of *Protobothrops,* where exons showed rates of synonymous and non-synonymous mutations larger than the introns. In the PLA_2 genes from *Vipera palaestinae,* this accelerated evolution is limited to the third exon [Kordis et al., 1998]. The accelerated evolution was also observed for

other snake toxins such as other PLA_2s [Moura-da-Silva et al., 1995; Chuman et al., 2000; Fujimi et al., 2002], disintegrins [Moura-da-Silva et al., 1996, 1997; Juárez et al., 2008], serine proteases [Deshimaru et al., 1996] and 3-finger toxins [Fry et al., 2003; Tamiya and Fujimi, 2006].

Tracing the phylogenetic history among the major groups or between species of snakes using toxins is a complex task. There are abounding doubts about snake taxonomy in the data source [Fry et al., 2003], largely because the systematics of these animals has been neglected in the biomedical literature [Wüster and Harvey, 1996]. The main difficulty is to define whether the genes are orthologous or paralogous, in order to properly examine the phylogenetic relationships. This is particularly important for understanding the evolution of snake venom proteins, for which an exceptionally high amount of copies have been reported, even in one individual [Fujimi et al., 2004; Oguiura et al., 2009]. A third is the lack of information about colubrid toxins due to the difficulty in obtaining the venom. This is currently being solved with studies of transcriptome of Duvernoy's glands of *Philodryas olfersii* [Ching et al., 2006] and cDNAs from other colubrids [Fry et al., 2008].

The family of phospholipase type A_2 (PLA_2) toxins illustrates the complexity of the toxin world and the attempt to use their sequences for phylogeny. The PLA_2 toxins are classified into 2 groups according to their amino acid sequence and pattern of disulfide bridges: group I, PLA_2s from the pancreatic juice of mammals and the venom of the Elapidae, and group II, non-pancreatic PLA_2s of mammals and of the Viperidae. The group I PLA_2s is divided into 2 subgroups: IA that is produced in the venom gland and IB, non-toxic which is produced in the pancreas [Danse et al., 1997].

The gene structures of group II PLA_2 may be divided into 2 types according to the number of exons and introns. PLA_2 genes from *Vipera ammodytes* are organized as non-pancreatic PLA_2 of humans and mice and contain 5 exons and 4 introns. The PLA_2 genes of Crotalinae snakes *(Crotalus* and *Protobothrops)* are organized as pancreatic PLA_2 of humans and dogs and contain 4 exons and 3 introns. Another difference between Viperinae and Crotalinae genes is the presence of Bov-B-LINE sequence in introns [Gubensek and Kordis, 1997].

Davidson and Dennis [1990] analyzed evolutionary trees for all PLA_2s with the amino acid sequences described, but this approach was successful only in the classification of PLA_2s in groups I and II, but not in the taxonomic classification. Indeed, the recruitment of these 2 toxins might have occurred independently in elapid and viperid snakes because the PLA_2 toxins have different origins and structures [Fry and Wüster, 2004]. Lynch VJ [2007] built evolutionary trees of groups I and II separately, and he found the same phylogenetic relationships of the group I PLA_2s in elapid to that found by Slowinski et al. [1997] using different methods of phylogenetic analysis. This success should be consequence of the single origin of elapid PLA_2s and a lack of further events of gene duplication.

Fry and Wüster [2004], with a large number of toxin sequences, amino acids or cDNAs, confirm the conclusion of Strydom [1973] that the toxins were recruited from existing proteins in snakes. Phylogenetic analysis of toxin sequences made by Fry and Wüster [2004] showed that they were not recruited at the same time, despite showing similar structures; from 8 families examined, 5 (Kunitz protease inhibitors, CRISP toxins (cysteine-rich secretory proteins), GBL toxins (galactose-binding lectins), M12B peptidase toxins and NGF toxins (nerve growth factor)) were recruited to the poison before the separation of Viperidae and Elapidae families, and toxins as lectin-like, PLA_2 and natriuretic peptides are clearly the result of 2 independent recruitment events. The 3FTx (3-finger toxin) family is inferred to have been recruited before the split between the elapid and colubrid lineages, and after divergence of the Viperidae, since they are absent in the latter [Fry et al., 2008]. Another example is the disintegrins; the dimeric disintegrins are widely distributed in Viperinae and Crotalinae, whereas short disintegrins appear to be restricted to African and Asian *Echis* and *Eristicophis* genera. This fact indicates that the emergence of dimeric disintegrins represents an early evolutionary event predating the Viperinae-Crotalinae split, whereas short disintegrins have evolved much more recently after the radiation of Viperinae [Juárez et al., 2008]. The sarafotoxins appear to be unique to the genus *Atractaspis* as are the small basic myotoxins, such as crotamine, present only in the venom of the rattlesnakes *Sistrurus* and *Crotalus*. Both toxins were recruited from endogenous proteins such as endothelins [Kochva et al., 1993] and beta-defensins [Torres and Kuchel, 2004; Rádis-Baptista et al., 2004]. However, the extension of the processes from these recruitments and the distinction between the independent and common events remain a vast research field to be explored, from an evolutionary as well as from a systematic point of view. We assumed that the construction of phylogeny using toxin data would not be useful in a discussion of higher level groups of snakes as we did here.

Mitochondrial Genomes

There are 13 protein-coding genes known in snake mitochondrial genomes, 2 ribosomal RNAs genes, and 22 transfer RNAs genes [Kumazawa et al., 1996]. The base compositions are biased in snakes as in other vertebrates with predominance of A-T over G-C base pairs, and a greater A+C content in the gene-rich strand [Asakawa et al., 1991; Yan et al., 2008]. Other features of the complete sequences can be summarized as follows: size ranging from 16,218 bp in *Leptotyphlops humilis* to 18,905 bp in *Boa constrictor*, but can fairly exceed in 3 Dipsadinae, mainly due to extensive control and/or repeated regions, reaching an extreme of 23,038 bp in *Leptodeira septentrionalis*. Alethinophian snakes in general have longer sequences due to the duplicated control region, exhibiting 2 identical copies per genome. These have been maintained stable since their origin and may function as an additional origin of heavy strand replication [Kumazawa et al., 1996; Jiang et al., 2007; Yan et al., 2008].

Since the sequencing of the first snake *(Dinodon semicarinatus)* mitochondrial complete genome by Kumazawa at al. [1996], the comparative mitogenomics of the group have been studied by a number of authors, mainly with phylogenetic purposes [Kumazawa et al., 1996; Kumazawa, 2004; Dong and Kumazawa, 2005; Jiang et al., 2007]. Although the subject has recently been reviewed, the fast increasing sampling of new taxa provides a continuously exciting field of research.

Because the last 2 phylogenetic analyses, published by Yan et al. [2008] and Castoe et al. [2009], included 14 and 15 snake species representing 7 and 10 families, respectively, we carried out here a cladistic analysis of all available snake mitochondrial genomes with a complete coding region (a data set comprising 51 sequences from 41 species, representing 15 families).

Methods of genome sequence alignments and cladistic analysis used here are the following. Protein-coding- genes were extracted from GenBank mitochondrial sequences using the software PEGA 0.99a [Patané, 2009], which parses a GenBank flat file with multiple accessions, outputting only the specified sequence (DNA or protein) of interest from each accession to a multi-FASTA file. Alignment was done with RevTrans [Rasmus and Pedersen, 2003], then adjusted manually, edited and concatenated using BioEdit [Hall, 2007]. Maximum Parsimony Analysis was conducted using TNT [Goloboff et al., 2000], treating the few indels as missing data. The 22 transfer RNAs and the 2 ribosomal RNAs were not included for 2 reasons: multiple alignment ambiguities due to an abundance of indels, and incompleteness of some of these regions in the partial genomic sequences used for a few but important taxa. Gene order information was compiled from the same species, after alignment of the whole mitochondrial sequences using Mauve Genome Alignment Software [Darling et al., 2004]. The Mesquite program [Maddison and Maddison, 2009] was used to edit the character matrix.

The results of mitogenomic analysis are from the raw empirical dataset using parsimony analysis (fig. 2). Our results were then compared, in terms of taxonomic congruence, with the most comprehensive phylogenetic analyses of snake higher taxa, which is based exclusively on nuclear loci [Wiens et al., 2008]. Although the 2 independent data sets are similar in alignment length and variable positions (nuclear: 13,322 bp, 6,783 variable and mitochondrial: 11,539 bp, 7,958 variable), on the one hand, the number of nuclear genes employed is larger (20 vs. 13), but on the other, the number of parsimony informative positions is higher in the mitochondrial set (7,133 vs. 4,766). However, the most important differences regard the sampling of characters and taxa. The nuclear set [Wiens et al., 2008] was previously designed for an analysis of higher level snake relationships (18 of the 20 genes used were newly sequenced for this purpose) with a taxon sampling more homogeneously distributed among the snake family-group categories; whereas the mitogenomic set has been accumulated in a public database during the entire last decade, resulting in the efforts of different authors with different goals, from evolutionary questions to taxonomy and, more recently, phylogeographic purposes [Mulcahy and Macey, 2009].

Thus, the mitogenomic taxon sampling is highly unbalanced, including many duplicated species, with some families overrepresented, while entirely lacking others. On the other side, the character sampling is complete for all terminal taxa with virtually no missing data in the mitogenomic set, whereas in the nuclear set there are many genes differentially lacking for most taxa (so, a higher proportion of missing data). Notwithstanding the considerable differences and a small number of species in common, the nuclear and mitogenomic data sets share a number of suprageneric taxa (e.g. families and subfamilies) whose monophyly has been decisively supported in previous studies, so that both sets can be effectively compared at this level (fig. 3).

The taxonomic congruence obtained between the nuclear and mitogenomic datasets is surprising, showing only 2 incongruent clades, concerning the position of Xenodermatidae as sister to Acrochordoidea or to Colubroidea and the position of Homalopsidae as sister to Elapidae

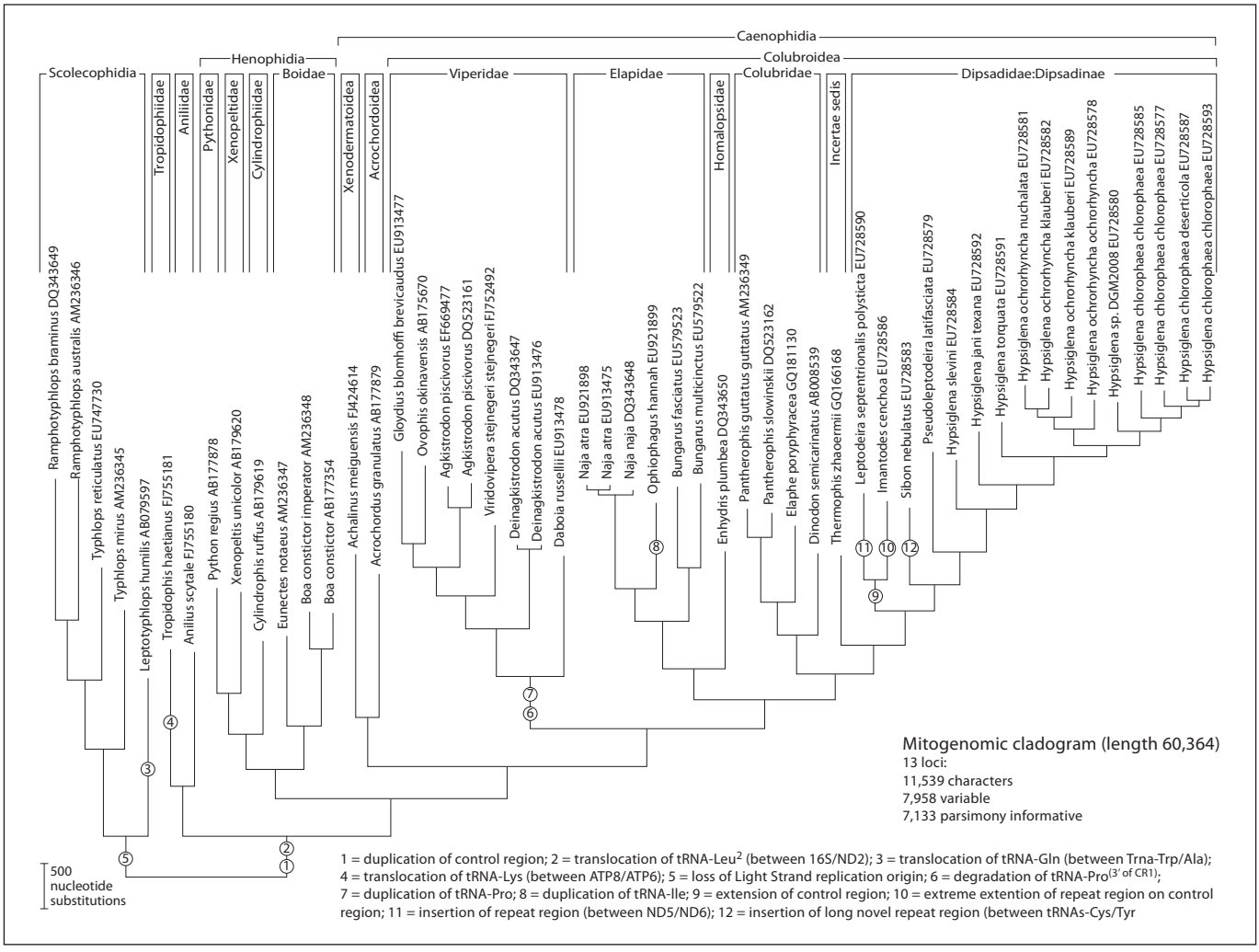


Fig. 2. Most parsimonious cladogram obtained from a cladistic analysis of the nucleotide mitogenomic data set consisting of the concatenate alignments of the 13 protein coding genes (total alignment length: 11,539 bp, of which 7,958 are variables and 7,133 are parsimony informative) for 51 terminal taxa. Inferred synapomorphies and autapomorphies regarding the gene order features are number 1–12. Those 4 from the basal dichotomy were polarized by means of outgroup comparison with genomes of the other Lepidosauria in general, but especially the Anguimorpha. Branch lengths are proportional to the number of nucleotide substitutions (scale bar in the lower left).

or to a group including Elapidae plus other colubroids. Such degree of congruence clashes with some previous assumptions that the mitochondrial DNA evolves too fast and, consequently, the amount of sequence saturation might be too high to give confident phylogenetic reconstructions at the level of deeper ancient branches.

Yan et al. [2008] concluded that: 'The among-lineage and among-gene variation in rate dynamics observed in snakes is the most extreme thus far observed in animal genome'. This point is especially emphasized here, considering that the method of phylogeny reconstruction we have used incorporates no evolutionary models attempting to correct these problems. Even under such extreme condition, the mitogenomic approach performs very well, but the same is not true with regards to the performance of the separate genes. Our results reinforce the utility and importance of continuing sequencing of whole mitochondrial genomes to address important questions about the phylogeny and classification of snake higher taxa (e.g. at and above the family level).

It would be desirable to place some taxon sampling priorities in doing so. Of particular interest at higher level

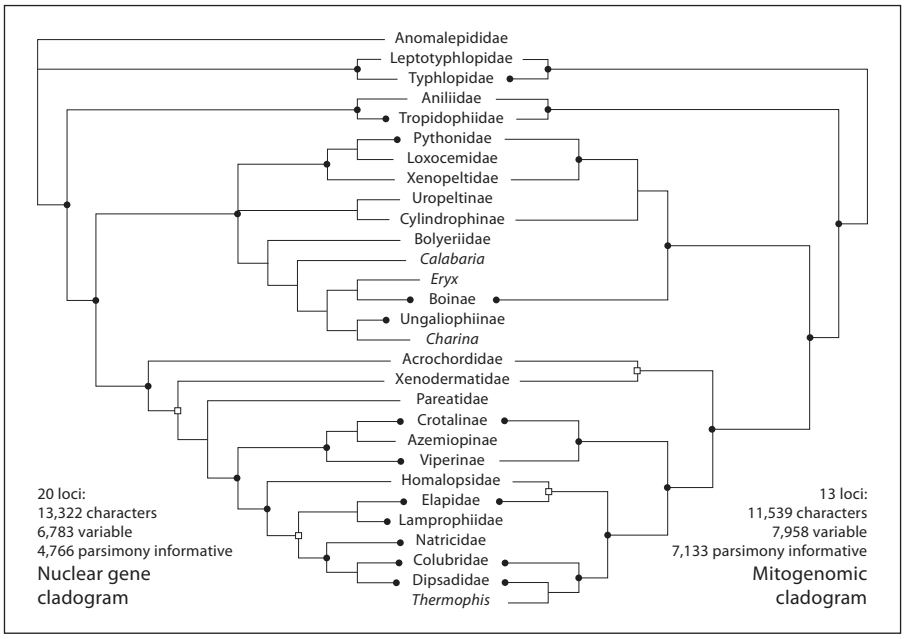


Fig. 3. Taxonomic congruence between nuclear and mitogenomic data sets, at the higher level snake systematics. The left tree was adapted from Wiens et al. [2008], who analyzed 20 nuclear loci, and the right one from our fig. 2. Dots on the nodes denote the congruent clades recovered in common by both data sets; terminal dots also denote monophyletic congruent groups, but a different sampling of subcomponent taxa used in each tree; open squares denote incongruent clades. The character counts presented are from the original complete data sets.

snake systematics would be the completion of genomes for those family group taxa still unrepresented: Anomalepididae, Uropeltidae, *Loxocemus*, *Calabaria*, and *Casarea*. It is also crucial to investigate some additional boid samples, like the erycines/ungaliophines, as well as the indopacific taxa, which have been either poorly sampled or unsatisfactorily resolved using nuclear loci and for which there are exciting historical biogeographical puzzles to be solved. With regard to the advanced snakes, priority should be determined on the same grounds, in order to maximize the covering of phylogenetic diversity: the Pareatidae, Lamprophiidae, Natricidae, and Pseudoxenodontidae.

Conclusions

The most extensive karyotype diversification and divergence from the postulated 2n = 36 generalized configuration are found within the Elapidae, the Natricidae, and the xenodontine dipsadids. Information regarding Anomalepididae, Aniliidae, Uropeltidae, and Bolyeriidae are in prior want to complement the scheme of snake karyotype evolution proposed here. Given the variation and a pattern of taxonomic distribution in apparent consistency with current groupings, cytogenetic comparisons may provide a useful unexploited tool to investigate intergeneric and tribal relationships within the extremely diverse neotropical xenodontine snakes. The incorporation of molecular techniques to the classical cytogenetic methodologies will provide better answers to the great questions about snake evolution, thus far not properly answered.

To overcome the difficulties encountered in the construction of phylogenetic trees from toxin genes, we have to pay attention to some points. First, the toxinologists who produce the majority of toxin sequence data, in addition to specifying the snake species (whose taxonomic status often have a transitory application), inform about its origin, and provide museum collection numbers that were sources of toxin sequences for future validation and identification as well as for other important deductions (for example, different homologous sequences from a same specimen can be easily interpreted as paralogous copies). It is important not only in phylogeny but also in medical care for the treatment of snake bites. Second, understanding organismal phylogeny also provides the means to identify paralogous from orthologous gene copies and to study the events of gene duplications and losses (independent from cladogenesis) that, as a consequence of differential lineage sorting, results in incompatibilities between gene and species trees. Third, the emerging field of phylogenomics enables predicting gene function for sequences obtained in snake transcriptomes of venom glands as well as studying the origin and evolution of these toxic proteins, i.e., their gene-specific history.

In general, nuclear and mitochondrial genes without multiple copies are elected to be used in phylogenetic re-

construction studies. For the Serpentes, the c-*mos* and RAG1 are the most extensively sampled examples, but more than 20 new nuclear loci have been recently sampled and analyzed for a limited, but representative, number of snake taxa [Wiens et al., 2008] providing a real improvement on the available character evidence at the family level and above. Mitochondrial genes, although extensively sampled, such as cyt-*b* and ND4, and rRNAs, are also very useful, but often biased for having hundreds of sequences for a single or a few related species but none or an insufficient representation for many important higher taxa. Of particular interest at higher level snake systematics is the completion of genomes for those family group taxa that are still unrepresented: Anomalepididae, Uropeltidae, *Loxocemus*, *Calabaria*, and *Casarea* as well as Pareatidae, Lamprophiidae, Natricidae, and Pseudoxenodontidae, which will permit an equivalent comparison regarding the taxonomic congruence between nuclear and mitochondrial genomic datasets.

The advantage in using these sequences is the quantity of data available in number of sequences and number of taxa and the possibility of easily concatenating information from different sources in a single original alignment. The sequencing of whole mitochondrial genome is also increasing and the phylogenetic trees obtained with these data are convergent to nuclear data. In addition, we found a different performance in the construction of phylogenetic trees using mitogenomic or separate gene approaches; therefore, we reinforce the usefulness and importance of continuing to sequence the whole mitochondrial genome.

The accelerated evolution detected in toxin genes and mitochondrial genome architecture, combined with the genetic variety that microchromosomes can provide, could explain the extraordinary physiology and metabolic flexibility of snakes that enable the snake's adaptation in such diverse habitats and conditions.

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