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Molecular systematics of racers, whipsnakes and relatives (Reptilia: Colubridae) using mitochondrial and nuclear markers

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Abstract

Four protein-encoding mitochondrial genes (cytochrome *b*, NADH-dehydrogenase subunits 1, 2 and 4) and one nuclear (c-mos) gene were sequenced to infer phylogenetic relationships among Old and New World representatives of racers and whipsnakes, *Coluber* (sensu lato). New World *Coluber* (*Coluber* sensu stricto, including *Masticophis*) and *Salvadora* proved to have affinities with the Old World non-racer colubrine genus *Ptyas* (and possibly *Elaphe* s.l. and *Coronella*), whereas Old World '*Coluber*' form several basally related clades; these are (1) *Hemorrhois*-(*Spalerosophis-Platyceps*); (2) *Hierophis*, with *Eirenis* nested within this paraphyletic genus and (3) '*Coluber*' *dorri* as the sister taxon to *Macroprotodon cucullatus*. The position of '*Coluber*' *zebrinus* along with *Hemerophis socotrae* located at the base of the Old World racer radiation forming the possible sister group to all remaining Palearctic racers and whipsnakes remains less well supported. Nevertheless, inter- and subgeneric relationships among many of the Old World racer groups have been resolved.

Key words: Phylogeny – molecular systematics – Reptilia: Colubridae: *Coluber* s.l. – mitochondrial DNA – c-mos – molecular clock

Introduction

Some 40 species of snakes are considered to belong to that group of the Colubrinae known as the whipsnakes and racers (Reptilia: Colubridae: *Coluber* s.l.; Terentjev 1961, Schätti and Wilson 1986). These represent a very widespread group, with their distributional area covering four continents: Africa, Asia, Europe and North America. Formerly, racers were collectively considered to form a natural group. More recently, however, the generic and subgeneric taxonomy of racers has come into question, with concerns over their monophyly, the identity of their closest relatives and indeed over the phylogeny of the entire group. The systematics of the group has until recently been almost exclusively based on morphological investigations (Ortenburger 1928, Inger and Clark 1943, Schätti 1986a,b, 1987, 1988a,b). In spite of this considerable body of work and one more recent study utilizing molecular data (Schätti and Utiger 2001), which answered some questions, many controversial problems remain.

Regarding the nomenclature of the racers, Schätti (1986a) writes that 'there is hardly any generic name which has been more in dispute than *Coluber* L. 1758' (sensu lato). The type species of the genus, the Nearctic *Coluber constrictor*, the common black racer, was so designated by Fitzinger (1843).

Inger and Clark (1943) established five genera of racers based exclusively on differences in scale row reductions: (1) *Coluber* Linnaeus; (2) *Masticophis* Baird and Girard; (3) *Zamenis* Wagler; (4) *Platyceps* Blyth and (5) *Hemorrhois* Boie. However, this arrangement has never been fully accepted, and a number of racer species were not considered by Inger and Clark.

Nevertheless, in the last few decades many external and internal characters including scalation, morphology of skull bones, vertebrae and hemipenis, as well as visceral topography have been investigated in attempts to clarify racer relationships. Generally, because the observed sets of taxa were restricted, these studies, though often giving acceptable results with closely related species, brought only modest resolution to questions of genus definition or intra- and intergeneric relationships.

The most recent attempt at a fairly comprehensive investigation of Old World racer relationships is that of Schätti and Utiger (2001) who in part used molecular methods. Their key findings were the confirmation of the genera *Hemorrhois* and *Platyceps*, the naming of a new genus *Hemerophis* for *Coluber socotrae*, the possible paraphyly of *Hierophis* with regard to *Eirenis* and the close relationship between *Spalerosophis* and Old World racers. However, their molecular trees suffered from low bootstrap support and a limited number of species tested.

The purpose of the present study is to confirm or refute the results of Schätti and Utiger (2001) by a different and much expanded set of mitochondrial and nuclear genes and by increasing the taxon sample. Because recent reports suggest that genera that have not been traditionally associated with *Coluber* s.l. (e.g. *Coronella*, *Spalerosophis*, *Eirenis*, *Elaphe*) might be related to racers (Nagy et al. 2000, Helfenberger 2001, Schätti and Utiger 2001), we include a number of representatives of these genera in our study. Additionally, we greatly increase the number of molecular characters. A total of 3809 nucleotide positions from four mitochondrial protein coding genes were analysed thereby increasing bootstrap values to support our phylogenetic conclusions. In addition, we partially sequenced the nuclear proto oncogene c-mos, a gene which is known to evolve at a slower rate than mitochondrial DNA (Saint et al. 1998).

Materials and methods

Taxon sampling

A list of the 42 taxa sampled for this study along with their voucher numbers when known and including locality of collection is given in Table 1.

The rarity of some species belonging to the genus *Coluber* s.l. prevented the inclusion of every known species in this strictly molecular study. Nevertheless, apart from these rarities our sampling of taxa is quite comprehensive. For our ingroup we included six species currently included in the genus *Hierophis*, five species of *Eirenis*, seven species of *Platyceps*, four of *Hemorrhois*, the monotypic Socotran endemic *Hemerophis socotrae* and a single species of the genus

Table 1. Taxa used for analysis, collection numbers and localities of voucher specimens

Species			
Coluber s.l.	Genus according to Inger and Clark (1943)[1]/Schätti and Utiger (2001)[2]	Geographic origin	Voucher specimen
algirus	*Hemorrhois*[2]	Morocco, 16 km SW of Tan-Tan Plaque	HLMD RA-1187
atayevi		Turkmenistan, Ashkabad, 2 km SE of Saivan	CAS 185188
caspius	*Zamenis*[1], *Hierophis*[2]	Greece, Serifos	NHMW KCC1
constrictor priapus	*Coluber*[1]	USA, Florida Monroe Co.	CAS 201502
(constrictor) mormon	*Coluber*[1]	USA, California Lake Co., Mendocino National Forest	CAS 212760
dorri		Senegal, Niokolo-Koba NP	HLMD RA-2906
florulentus	*Platyceps*[1,2]	Egypt, Sinai	HLMD RA-3040
gemonensis	*Zamenis*[1], *Hierophis*[2]	Croatia, Narina	HLMD J3
hippocrepis	*Hemorrhois*[1,2]	Spain, Cadiz Province, 11.4 km NE Benalup de Sidonia	MNCN 11988
jugularis	*Zamenis*[1], *Hierophis*[2]	Turkey, Antalya Province, 2 km NE Finike	MVZ 230242
karelini		Turkmenistan, Krasnovodsk, 14.2 km SW Madau	CAS 184636
najadum	*Platyceps*[1,2]	Armenia, Kotajsk Region	ZISP 27780
nummifer	*Hemorrhois*[2]	Armenia, Kotajsk Region	ZISP 27709
ravergieri	*Platyceps*[1], *Hemorrhois*[2]	Armenia, Kotajsk Region	ZISP 27733
rhodorachis	*Platyceps*[1,2]	Turkmenistan, Ashkabad, 12 km NW Fyrusa	CAS 185035
rogersi	*Platyceps*[1,2]	Egypt, Sinai	NHMW KCR2
rubriceps		Jordan	HLMD J14
schmidti		Turkmenistan, Mary Region, Mt. Dushak, 20 km of Dushak	CAS 182953
socotrae	*Hemerophis*[2]	Yemen, Socotra	HLMD RA-2973
spinalis	*Coluber*[1]	China, Ningxia Hui Aut. Region, Yinnan	MVZ 211019
viridiflavus	*Zamenis*[1], *Hierophis*[2]	Spain, Aragon, Huesca, Banos de Benasque	MVZ 178418
zebrinus		Namibia, N of Warmquelle	CAS 214764
Other genera			
Salvadora mexicana		Mexico, Michoacan, 17 km S of Quatro Caminos	No voucher
Spalerosophis diadema		Jordan, Wadi Ram	HLMD J62
Masticophis f. flagellum		USA, Florida, Citrus Co., Inverness,The Highlands	CAS 218708
Masticophis flagellum piceus		USA, California, Kern Co.	CAS 219734
Ptyas korros		Myanmar, Mandalay Div., Nwahtogyi, Pyin Si	CAS 205259
Ptyas mucosus		Myanmar, Mandalay Div., Kyaukse Pyaw Bwe, Po Ywa	CAS 208434
Macroprotodon cucullatus		Spain, Andalusia, Cadiz Prov., 22.1 km N of Facinas	MVZ 186073
Coronella austriaca		Turkey, Ilgaz	HLMD RA-2608
Coronella girondica		Morocco, Marrakesch Prov., 8 km N of Oukaimeden	MVZ 178073
Elaphe quatuorlineata		Turkey, European Turkey	LSUMZ 40626
Elaphe (Rhinechis) scalaris		Spain	LSUMZ 37393
Eirenis eiselti		Turkey, 24 km N of Göksun (Pr. Kahramanmaras)	CS 4653
Eirenis aurolineatus		Turkey, N Karaisali (Pr. Adana)	CS 4655
Eirenis levantinus		Turkey, S Osmaniye	CS 4651
Eirenis modestus		W Turkey	Not collected/HLMD J159
Eirenis punctatolineatus		No data	Not collected/HLMD J175
Malpolon monspessulanus		Spain, Andalusia, Cadiz Prov., 5.8 km E of Puerto Real	MVZ 186256
Afronatrix anoscopus		Liberia	ROM 19842
Thamnophis godmani		Mexico, Oaxaca	MZFC ART 145
Xenochrophis punctulatus		Myanmar, Ayeyarwade Div., vic Mwe Hauk Village	CAS 206594

Museum acronyms used: CAS, California Academy of Sciences, San Francisco; CS, private collection of J.F. Schmidtler; HLMD, Hessisches Landesmuseum Darmstadt (RA codes refer to specimen collection nos., other HLMD codes refer to the reptile tissue collection); LSUMZ, Louisiana State University, Museum of Zoology; MNCN, Museo Nacional de Ciencias Naturales; MVZ, Museum of Vertebrate Zoology, Berkeley; MZFC, Museo de Zoología de la Facultad de Ciencias la Universidad Nacional Autónoma de México; NHMW, Naturhistorisches Museum Wien (W. Mayer tissue collection no.); ROM, Royal Ontario Museum, Toronto; ZISP, Zoological Institute of St Petersburg.

Spalerosophis. Old World representatives of species currently remaining in the genus *Coluber* included in our study are *Coluber zebrinus* and *C. dorri*.

As representatives of New World *Coluber* s.s. and its close relatives we included two geographically widely separated examples of the common racer, *C. constrictor* and two geographically widely separated examples of the New World whipsnake, *Masticophis flagellum*, and the Mexican patch-nosed snake *Salvadora mexicana*. Among colubrines outside – but possibly related to *Coluber* s.l. – we included representatives of the genera *Coronella*, *Elaphe* s.l., *Ptyas* and the monotypic *Macroprotodon cucullatus*. For the purposes of tree rooting we chose three natricine genera and one psammophiine genus. These are

represented by *Afronatrix anoscopus*, *Xenochrophis punctulatus*, *Thamnophis godmani* and *Malpolon monspessulanus*, respectively. These four genera while clearly outside the Colubrinae are within the family Colubridae.

Laboratory procedures

As a source of DNA we used alcohol-preserved tissue samples, some from museum specimens, or we used whole blood drawn from live animals and stored in 70–90% ethanol or in an ethylenediaminetetraacid buffer (Arctander 1988) until needed.

We used a standard method for obtaining total genomic DNA (Sambrook et al. 1989). Template DNA for the polymerase chain reaction (PCR) was prepared by diluting the stock DNA with TE buffer to give a spectrophotometric absorbance reading of between 0.2 and 0.7 at A260. Amplification of target DNA was carried out in 100 µl reactions. Primers for amplification and sequencing, as well as conditions for the PCR were as detailed in Burbrink et al. (2000) and de Queiroz et al. (2002).

Following clean-up of PCR products using the Promega Wizard PCR Preps Purification System (Promega, Madison, WI, USA) according to manufacturer's instructions, cycle sequencing was performed as in Burbrink et al. (2000) and sequences were determined using either the ABI Prism Genetic Analyzer model 3100 (Applied Biosystems, Foster City, CA, USA) or the ALFExpress II (Amersham Pharmacia Biotech, Uppsala, Sweden) automatic sequencers.

Phylogenetic analyses

Because there were few indels in the nucleotide sequences of the mitochondrial protein coding genes, alignment by eye was simple and was accomplished by using either of the computer programs ESEE (Cabot and Beckenbrack 1989) or SEQUENCHER 4.0 (Gene Codes Corporation, Inc., Ann Arbor, MI, USA). The nuclear c-mos sequences were aligned in a similar manner and as indels were confined to a single two-codon region, alignment was again readily accomplished by eye.

To test for differences in phylogenetic information content among the four mitochondrial genes we used the incongruence length difference test (ILD) (Farris et al. 1994) provided in PAUP* 4.0b10 (Swofford 2002) as the partition homogeneity test. For this test we first removed invariable characters (Cunningham 1997a,b, Yoder et al. 2001, Caranza et al. 2002). Tests were performed using heuristic searches with 1000 replicates.

In addition to these tests we examined congruence between maximum parsimony (MP) trees derived from each of the genes separately. Testing of the combined mtDNA data for the presence of phylogenetic signal was done by using g statistics to examine parsimony trees for skewness and kurtosis (Snedecor and Cochran 1967) as suggested by Hillis and Huelsenbeck (1992).

Measuring of substitution saturation has been done using DAMBE 4.1.15 (Xia 2000, Xia and Xie 2001) for mitochondrial and c-mos sequences, respectively. Additionally, we present plots for both data matrices showing transversions/transitions versus divergence (F84 genetic distances calculated by DAMBE), which also enable the assessment of saturation in a simple way (Fig. 1).

To infer phylogenies we used the methods of MP and maximum likelihood (ML), these analyses were carried out using PAUP* 4.0b10. We also used the newer but increasingly utilized method of Bayesian inference (BI) (Yang and Ranala 1997). For this procedure we used the computer program MRBAYES 2.01 (Huelsenbeck 2001) analysing the total mtDNA data in four chains for 500 000 generations with a consensus being drawn for the last 450 000 trees. For ML analyses, an appropriate model of sequence evolution was inferred using MODELTEST 3.06 (Posada and Crandall 1998) using hierarchical likelihood ratio tests (hLRTs) and the Akaike information criterion (AIC). MP analyses were conducted with heuristic searches, tree-bisecting-reconnection and simple stepwise addition. In performing MP analyses we used equal weighting for all characters as well as with codon third positions removed. In choosing to emphasize equal weighting for all nucleotide sites we are making the assumption that at the taxonomic level of our ingroup, the value of increased resolution at subterminal nodes provided by codon third positions will outweigh possible perturbations at deeper nodes.

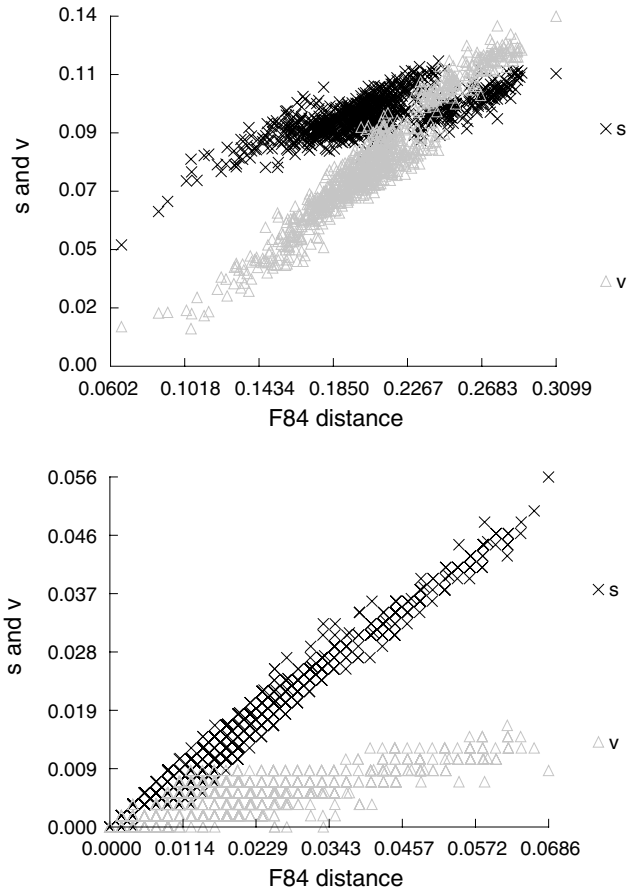

Fig. 1. Transversions (v)/transitions (s) versus divergence by mitochondrial (at the top) and c-mos sequences (at the bottom), respectively

Support for clades was assessed by bootstrapping (Felsenstein 1985). In this study, bootstrap values refer to 1000 replicates. In our interpretation of bootstrap values we follow Felsenstein and Kishino (1993). Accordingly, we define weak, moderate and strong support as 50–69, 70–89 and 90–100%, respectively, for all bootstrap values.

We used a molecular clock to estimate divergence times at nodes within our ML tree. We first checked our data set for constancy of mutation rate (relative rate test) and for this purpose the computer program PHYLTEST 2.0 (Kumar 1996) was used. Calibration of the clock for our tree was done by using data points obtained from the literature and from our own unpublished data. These calibration points were: divergence of *Hemorrhois* subgroups from a common ancestor, max. 16 millions of years ago (mya); the divergence of *Hierophis* subgroups including *Eirenis*, 18 mya (based on palaeoherpetological data of Ivanov 2002); the origin of the Old World whipsnakes and racers excluding *Coluber* s.l. *zebrinus*, max. 30 mya; the origin of the Colubrinae, 35 mya (Rage 1988, Szyndlar 1991, Rage et al. 1992, Ivanov et al. 2000).

All new sequences produced for this study have been deposited in GenBank; accession numbers: AY486911-AY487075.

Results

Pseudogenes and phylogenetic signal

Although it is not possible to completely rule out the presence of paralogous genes, for the following reasons we believe that our sequence data are free from pseudogenes: (1) In a total of over 53 000 codons examined, no unexpected stop codons

occur. Conversely, stop signals are always present where expected. (2) Among all taxa there are no unexpectedly great divergences in amino acid sequence within genes. (3) The mutation ratio between second and third codon positions falls within the expected range.

Statistical tests comparing our MP tree (based on 1785 parsimony informative sites) with one million random trees gave a g_1 value of −0.5159 and a g_2 of 0.4003, indicating that our data differ significantly from random; that is, a phylogenetic signal is present.

Saturation analyses demonstrated that both data sets (mitochondrial and c-mos sequences) bear little substitution saturation (the observed Iss values are significantly lower than Iss.c); thus, our data could be well used for phylogenetic analyses. The slope of the curves shown in Fig. 1 also point out the strength of our sequence data.

Gene characteristics

There is some variation in length of the ND1 sequence within our ingroup (Table 2). In almost all of our ingroup taxa the signal for termination of translation is a terminal T which is post-transcriptionally adenylated to form a functional stop codon TAA. Three of the ingroup taxa (*Coronella austriaca*, *Eirenis levantinus* and *Eirenis modestus*) have the stop codon AGG. Disregarding the taxa with codon deletions and the terminal T, the ND1 gene of our ingroup taxa is 963 nucleotides or 321 amino acid codons in length.

There is also length variation in the ND2 gene sequence. In approximately half of the taxa the nominal length of the gene is 1032 nucleotides (=344 codons, see Table 2) including the stop codon TAG present in all taxa. The remaining taxa have a single codon deletion at nucleotide positions 277–279 or at 280–282 corresponding to codon positions 93 and 94 (Table 2).

Table 2. Length variation observed in marker genes. In the sequenced fragment of ND4, no indel site was found

Gene (most common length in our colubrid samples)	Species	Length (bp)
ND1 (964 bp)	*Elaphe quatuorlineata*	955
	Elaphe scalaris	955
	Platyceps florulentus	955
	Malpolon monspessulanus	961
ND2 (1032 bp)	*Afronatrix anoscopus*	1026
	Coluber (s.l.) *dorri*	1029
	Coronella girondica	1029
	Eirenis spp.	1029
	Hemerophis socotrae	1029
	Hierophis spp.	1029
	Malpolon monspessulanus	1029
	Platyceps spp.	1029
	Spalerosophis diadema	1029
	Thamnophis godmani	1029
	Xenochrophis punctulatus	1029
cyt *b* (1117 bp)	*Afronatrix anoscopus*	1113
	Coluber (s.l.) *zebrinus*	1114
	Hierophis spinalis	1113
	Malpolon monspessulanus	1114
	Spalerosophis diadema	1111
c-mos (567 bp[1])	*Hemorrhois nummifer*	561
	Hemorrhois ravergieri	561
	Malpolon monspessulanus	570

[1]Sequenced part of gene.

We sequenced only approximately the 3' two-thirds of the ND4 gene, this gave 696 nucleotides, 232 codons including the stop codon which was usually TAA but was TAG in a few taxa (*Hemorrhois nummifer*, *H. ravergieri*, *Spalerosophis diadema*, *Masticophis flagellum piceus*, *Ptyas korros*, *Rhinechis scalaris* and *T. godmani*), as well as AGA in *Malpolon monspessulanus*. This segment of the ND4 gene contained no indels.

The cytochrome *b* gene of snakes has been studied in a number of snake groups, boids and pythonids (Campbell 1997), elapids (Slowinski and Keogh 2000, Slowinski et al. 2001), viperids (Lenk et al. 1999, 2001) and colubrids. From these studies, we know that as a whole there is variation in the number of nucleotides at the 3' end of the cytochrome *b* gene. However, within colubrids the nucleotide number appears to be constant at 1117 including a terminal T (Burbrink et al. 2000). Within our ingroup most taxa fit this pattern of 1117 nucleotides including the 3' post-transcriptionally adenylated T, exceptions are *Hierophis spinalis* (1113 bp) and *A. anoscopus* (1113 bp), which have normal mitochondrial stop codons.

The highly conserved nuclear c-mos fragment with a sequence length of 561–570 bp showed only 52 parsimony informative characters. However, because of this conservation in nucleotide sequence, this gene was useful in identifying

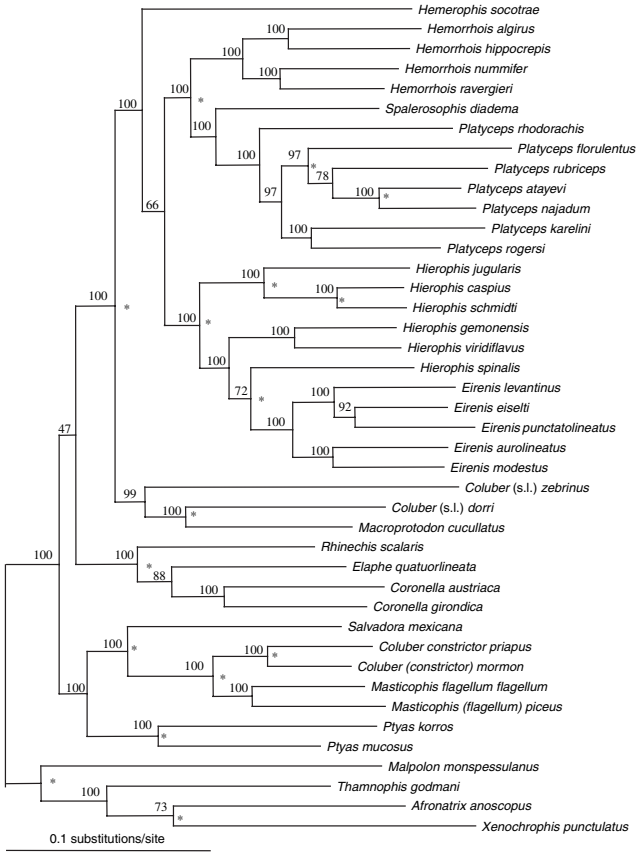

Fig. 2. Tree of Bayesian inference (BI) analysis (GTR + I + Γ model, the topology is identical by Maximum likelihood method without estimating molecular clock) based on combined analysis of 3809 base pairs of the mitochondrial genes ND1, ND2, ND4 and cyt *b*. *Malpolon monspessulanus* and three natricine species were used as outgroups. Numbers are clade credibility values from the BI analysis. Asterisks mark nodes supported by ML analysis of c-mos sequences of 570 base pairs

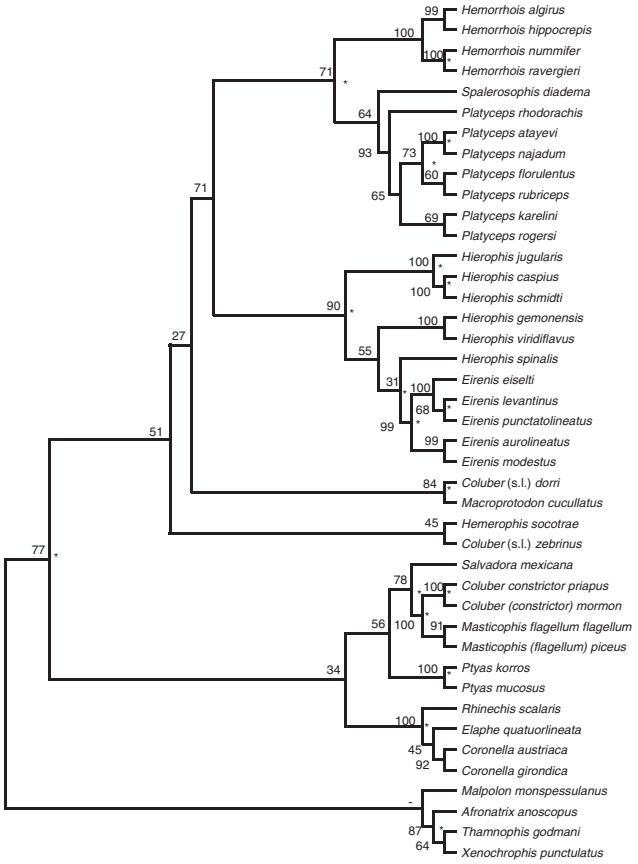

Fig. 3. Bootstrap consensus tree obtained by maximum parsimony (MP) analysis, based on mitochondrial sequences of 3809 base pairs. Numbers are bootstrap values in percent from 1000 replicates. Asterisks mark nodes supported by MP analysis of c-mos sequences

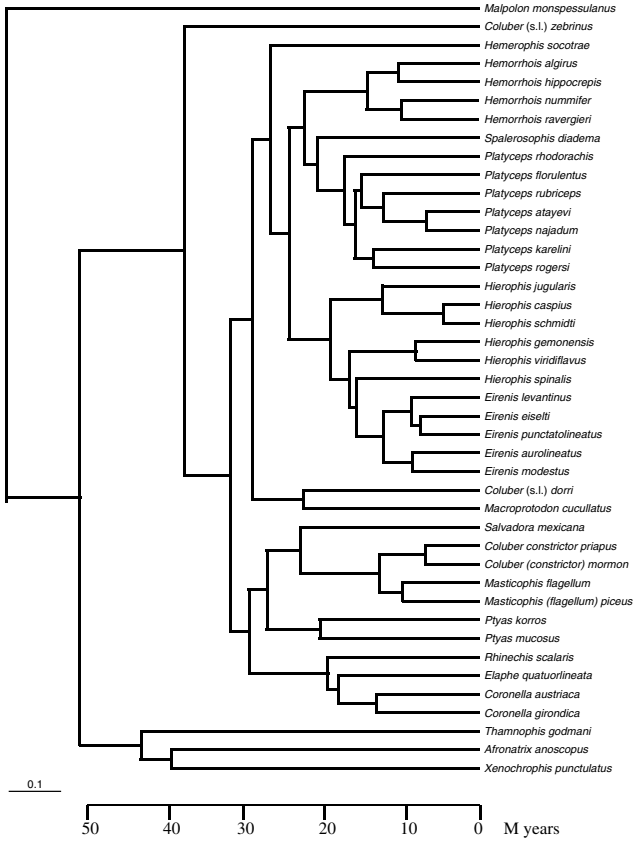

Fig. 4. Maximum likelihood chronogram of the evolution of the colubrid taxa studied. Time data are maximum estimates. See Materials and methods for details

support for the deeper nodes within our phylogenetic trees. The c-mos sequence support for branching events is indicated on the trees by asterisks (Figs 2 and 3).

Phylogeny

The ILD test of the four mitochondrial genes (ND1, ND2, ND4 and cyt *b*) tested simultaneously was non-significant, $p = 0.397$, suggesting that the level of congruence in phylogenetic signal among these genes is similar and that they should be combined for phylogenetic analysis. The ILD test, performed pairwise on the four genes, gave the following probabilities. Cyt *b*/ND1, $p = 0.864$; cyt *b*/ND2, $p = 0.598$; cyt *b*/ND4, $p = 0.495$; ND1/ND2, $p = 0.418$; ND1/ND4, $p = 0.484$; ND2/ND4, $p = 0.024$.

An initial unrooted MP analysis clustered the four outgroup taxa well outside of an apparently monophyletic colubrine clade composed of all of the ingroup taxa. Among the possible evolutionary models in ML analysis of the combined mitochondrial sequence data, a general time reversible model (GTR + Γ + I) was chosen by MODELTEST by both methods (hLRTs and AIC). The value of the single best ML tree is 58648.6670. The c-mos sequence data were analysed under ML criteria with the HKY + Γ model determined by MODELTEST, the best tree's value is 1757.2724.

BI and MP trees with credibility values of Bayesian analysis and bootstrap values are shown in Figs 2 and 3, respectively. MP trees of the four mitochondrial genes analysed separately (not shown) all show slightly different topologies but do show a number of clades in common (Table 3). In general, clades on these individual gene trees are supported by only low to very low bootstrap values.

A MP tree (not shown, but see Table 3) of the combined mitochondrial genes but with codon third positions excluded (2540 characters remain of which 639 are parsimony informative) is similar but not identical in topology to the MP tree showed in Fig. 3. However, bootstrap values supporting clades are lower than on the MP tree (Fig. 3). The BI/ML analyses (both methods gave the same tree topology) of the combined mitochondrial sequence data (Fig. 2) supported by evidence from the c-mos sequence can be summed up as follows.

The entire ingroup is divided between three major clades. One is composed entirely of Old World taxa and contains all Old World representatives of the genera *Coluber* s.l., *Hemerophis*, *Hemorrhois*, *Spalerosophis*, *Platyceps*, *Hierophis*, *Eirenis* and *Macroprotodon*. The second clade contains Old World ratsnakes (*Elaphe* s.l.) and the smooth snakes, *Coronella*. The third clade contains both New and Old World taxa; *Coluber* s.s., *Masticophis*, *Salvadora* and *Ptyas*, respectively.

The Old World racers are divided between two clades, the most basal of which consists of *Coluber* s.l. *dorri*, *C.* s.l. *zebrinus* and *Macroprotodon*. While most Old World racers, including the genera *Hierophis*, *Platyceps* and *Hemorrhois*, encompassing also *Eirenis* and *Spalerosophis*, form a large monophyletic unit, the endemic Socotran racer, *Hemerophis*

Table 3. Results of maximum parsimony analyses of partitioned mitochondrial sequences, with respect to the most relevant results of the combined analysis (Fig. 3). +/− indicate whether a clade was recovered

Clade	ND1, 2 & 4 + cyt *b*; third position excluded	ND1	ND2	ND4	cyt *b*
Monophyly of *Hemorrhois*–(*Spalerosophis*–*Platyceps*)	+[1]	+	−[2]	+[1]	+[1]
Monophyletic *Eirenis* embedded in *Hierophis*	+	+	+	+	+
Hierophis spinalis as sister species of *Eirenis*	+	+	−	+	−
Monophyly of Palearctic whipsnakes/racers (*Eirenis*, *Hierophis*, *Hemorrhois*, *Platyceps*, *Spalerosophis*)	+	+	+	−	+
Hemerophis as sister group of Palearctic racers	+	−	+	−	+
Coluber s.l. *dorri*–*Macroprotodon*	+	+	+	−	+
Monophyly of Old World racers	−[3]	−[3]	−[3]	−[4]	+
Ptyas-(*Salvadora*–(*Coluber* s.s.–*Masticophis*))	+	+	−	−	+
(*Elaphe* s.l.–*Coronella*) linked to New World racers	+	−	+	−	+

[1](*Hemorrhois*–*Spalerosophis*)–*Platyceps* supported weakly.
[2]The position of *Spalerosophis* is not supported.
[3]Excluding *C.* s.l. *zebrinus*.
[4]*Ptyas* groups with Old World racers.

socotrae, is linked in the BI tree as sister taxon to this large clade of what may be loosely termed Palearctic racers. This relationship is not supported by parsimony criteria which place *Hemerophis* as sister taxon to the southwest African species *Coluber* s.l. *zebrinus* but without credible bootstrap support. MP places this sister taxon pair basal to all other Old World racers (Fig. 3).

The Palearctic racer taxa are divided between two clades, one composed of a monophyletic *Hemorrhois*, a monophyletic *Platyceps* with *Spalerosophis* linked basally to it. The second clade composed of a monophyletic *Eirenis* directly linked to *Hierophis spinalis* and then to certain Western Palearctic species of the genus *Hierophis*, before linking to a second clade of *Hierophis* species. Thus, *Hierophis* as so composed is paraphyletic.

Except in the following aspects, the MP tree (Fig. 3) is identical with the ML tree in topology: (1) *Coluber* (s.l.) *zebrinus*, instead of connecting to *Macroprotodon* and *Coluber* (s.l.) *dorri* links directly to *Hemerophis socotrae*, however, with low bootstrap support. These two taxa together are placed outside of the remaining ingroup taxa. (2) There is a difference in the pattern of clustering among the species of *Eirenis* when the ML and MP trees are compared (Figs 2 and 3). (3) The relation of the three major ingroup clades remains unresolved by almost all analyses.

The relative rate test performed with PHYLTEST (Kumar 1996) gave a Z statistic of $Z = 6.957 \times 10^{-4}$, indicating that constancy of mutation rate at the 5% level is not rejected for our data. Consequently, Fig. 4 represents an ML tree with a time bar calibrated (see section Materials and methods) to show the approximate time of past branching events in millions of years before the present.

Discussion

Quantity and quality of sequence data needed

In recent years, a number of studies (Cao et al. 1994, 1998, Cummings et al. 1995, Russo et al. 1996, Zardoya and Meyer 1996, Corneli and Ward 2000, Hauf et al. 2000) have focused on attempts to assess the phylogenetic utility of different mitochondrial protein coding genes or combinations of these genes in recovering a phylogenetic tree of higher level taxa. The results of these studies, though not in complete agreement, had a number of findings in common. Certain genes were found to be better than others at recovering tree topologies thought to be correct; among these are ND4 and cyt *b*. Corneli and Ward (2000) found that no single gene produced the correct topology, though ND1 and ND2 came fairly close to doing so, with other genes failing in greater degrees. A second finding was that certain combinations of genes totalling 2–3.5 kb in length performed fairly well with several combinations finding the 'correct' topology. It appeared that an ideal combination would contain the genes ND1, ND2 and cyt *b* and be within the region of three kb in length.

In a recent paper (de Queiroz et al. 2002), the amount of mitochondrial gene nucleotide sequence necessary to produce a robust phylogeny for a single genus of snakes was thoroughly investigated. In the case of the North American garter snakes, genus *Thamnophis*, it was shown that a plateau of resolution was reached with about 2000–2500 nucleotides of protein coding sequence. However, in the present study we are dealing with several genera and cannot assume that the same number of nucleotides will be sufficient to give optimal results. Indeed, several studies have shown that at higher taxonomic levels even twice this number of nucleotides might not be enough for best results (Cummings et al. 1995, Otto et al. 1996, Springer et al. 2001). Nevertheless, it is clear from the study of de Queiroz et al. (2002) that increasing the number of nucleotides does improve resolution as judged by the increase in number of fully resolved clades and does improve clade support as judged by increase in bootstrap values in MP analyses.

Congruence between phylogenies estimated from independently derived data is a powerful re-enforcer of the total phylogenetic signal. Additionally, one gains in the robustness of clades independently supported (Miyamoto and Fitch 1995). Thus, when genes are unlinked, each with its own separate history, each provides an estimate of a species phylogeny (Slowinski and Page 1999). In this manner, the conserved nuclear c-mos gene identifies branching events independently from the mitochondrial sequence data and provides corroborative support for some of the earlier branching events within our phylogenies.

When applied to our data which consist of a concatenation of four mitochondrial protein coding genes of nearly 4 kb in

length, congruence support from a conserved nuclear gene, along with a dense taxon sampling, we believe the combination of factors described above provides the information to recover a robust phylogeny for the whipsnakes and racers.

The origin of modern racers and whipsnakes

Invoking the molecular clock hypothesis it appears that the Palearctic racers had unambiguously a common if ancient origin, but are paraphyletic with the dwarf snakes, *Eirenis*, nested within them. At about the same time, max. 25–30 mya (Fig. 4) that the Old World racers were starting to diverge from an ancestor which was to produce the genera *Hierophis*, *Hemorrhois*, *Platyceps* and *Spalerosophis*, New World racers were arising from a common ancestor with the progenitor of the extant Old World genus *Ptyas*. Seemingly, the colubrine colonization of North America occurred rather early, about 25 mya. Whether the New World racers, with *Ptyas*, are the sister group to the Old World genera *Elaphe*, *Rhinechis* and *Coronella*, in agreement with the observations of Lopez and Maxson (1996), cannot be resolved with our data. This is demonstrated by the differences between our likelihood and parsimony trees (Figs 2 and 3).

Old World racers

According to the topology of our likelihood and parsimony trees and their well-supported clades (Figs 2 and 3), early divergences are evident in the phylogeny of the Old World racers. Afrotropical groups here represented by *Coluber* s.l. *zebrinus*, *C*. s.l. *dorri* and *Hemerophis* appear to have separated very early from the Palearctic stem. It is likely that these snakes will have unexpected and yet to be discovered relationships to other African colubrine genera. An example of just such a relationship is that between *C*. s.l. *dorri* and the monotypic genus *Macroprotodon* revealed by this study and described below. It is also supported by the c-mos data, whereas the relative position of *C*. s.l. *zebrinus* and of *Hemerophis socotrae* differ in c-mos and mitochondrial phylogenies (in c-mos trees *Hemerophis socotrae* clustered in the *Hierophis viridiflavus* group!). We regard the latter as being still unresolved.

Among the true Palearctic racers including the dwarf snakes, *Eirenis*, there are two main branches which diverged from a common ancestor early in Old World racer evolution (ca. 25 mya). Parallel evolution of these two lineages has resulted in similar numbers of genera and species in each clade, *Hemorrhois*, *Spalerosophis* and *Platyceps* in the one and *Hierophis* and *Eirenis* in the other. Support for the early divergence of these two Old World racer groups is supplied by the highly conserved c-mos sequences (Figs 2 and 3). Moreover, parallel evolution through time in these two major Old World racer groups has resulted, except for the dwarf snakes, in a number of instances of convergence in body form and ecological adaptations between members of each group. Additionally, both groups have come to occupy a similar geographic range.

Hierophis and Eirenis. Relationships between the Palearctic racers of the genus *Hierophis* and the dwarf snakes, genus *Eirenis* are close. This unexpected relationship was demonstrated by Schätti and Utiger (2001) who showed on the basis of the analysis of their DNA sequence data that the species *E. modestus* was nested, though with low bootstrap support, within *Hierophis*, making this genus paraphyletic. Our findings, while agreeing with the general observation of Schätti and Utiger (2001) that *Eirenis* and *Hierophis* are indeed phylogenetically close, differ from theirs in a number of important details. Because we included in our data set a number of *Eirenis* species comprised of members of the *E. modestus* complex (sensu Schmidtler 1993) as well as more distantly related species we were able to demonstrate that the dwarf snakes form a monophyletic group which is strongly supported as being the sister group to the Middle Asian species *Hierophis spinalis* (Figs 2 and 3; Table 3). The clade composed of *Eirenis* and *H. spinalis* is a subclade of one which includes also the sister taxon pair of *H. gemonensis* with the type species of the genus; *H. viridiflavus*. Linked to this clade is a separate one composed of the sister taxon pair *H. caspius* with *H. schmidti* as well as *H. jugularis* (Figs 2 and 3).

The remarkable differences in external morphology between members of the genus *Hierophis* and the dwarf snakes, *Eirenis*, which are typified by a great reduction in body size and in characters of pholidosis may at first make a close relationship between the two genera seem unlikely. However, hemipenis morphology and juvenile colour are very similar in *Eirenis* and *Hierophis* (Schätti 1988b, Schmidtler 1993). A potential homology uniting *Eirenis* and *Hierophis* is the deletion in the ND2 gene (Table 2), but as it also occurs in *Spalerosophis* and *Platyceps* as well as in outgroups such as *Malpolon* and *Afronatrix* also, it cannot be ruled out that this homology is a plesiomorphy. It appears that, based on our molecular evidence, the dwarf snakes represent a derived group. Their peculiar morphological characters probably evolved in concert with an adaptation to a cryptic lifestyle (see also Inger and Marx 1965, Schmidtler 1993, 1997). This ecological niche once invaded has allowed rapid speciation to occur within the genus. Some of their special morphological character states, such as 17 dorsal scale rows, are shared by *Hierophis spinalis* which in our trees is sister to *Eirenis*. This allows us to hypothesize that these character states represent synapomorphies rather than homoplasies.

Although the monophyly of *Eirenis* has not been in doubt the genus has been split between two subgenera (Dotsenko 1989). The type species of *Eirenis* is *E. modestus*. Our data show that *E. aurolineatus*, a taxon only recently raised to species level (Schmidtler 1993) is closely related to *E. modestus*. Schmidtler (1993) also described another new species, *E. levantinus*, and included it in the *E. modestus* complex. In contrast to this view, we find *E. levantinus* more closely related to *E. eiselti*, a member of the second subgenus (*Collaria* sensu Dotsenko). Clearly, interspecific relationships within *Eirenis* would benefit from further molecular studies and these are presently in progress (Nagy et al. 2003).

The taxonomic implications arising from the paraphyly of *Hierophis* with respect to *Eirenis* are discussed below.

Hemorrhois. The genus *Hemorrhois* includes only four species which on the basis of geographic distribution form two distinct groups. A western group is composed of *H. hippocrepis* of North Africa, the Iberian Peninsula and a number of Mediterranean islands and *H. algirus* also inhabiting North Africa and some Mediterranean islands. Geographically well separated from this sister species pair (Figs 2 and 3) is the eastern group consisting of the closely related *H. ravergieri* and *H. nummifer* both inhabiting Central Asia largely in sympatry. Within the Palearctic region such geographic separation is found in a number of vertebrates

and among snakes is seen, for example, in the genus *Natrix* (unpublished) and in a clade of viperids composed of *Macrovipera mauritanica, M. deserti, 'Vipera' palaestinae* and *Daboia russelii* (Lenk et al. 2001). The geographical gap separating these two species groups of *Hemorrhois* corresponds to that separating *Macrovipera deserti* and *V. palaestinae* in Libya and Egypt or that between *Natrix maura* and *N. tessellata*. These gaps are maintained by the arid conditions found in this part of North Africa where the pre-Saharan semidesert virtually reaches the Mediterranean coast. We note that all three pairs of taxa are separated by similar sequence divergence, ca. 12–14% (uncorrected distance) in cytochrome *b*. We may assume that the ancestors of each geographic group were formerly distributed continuously along the southern Mediterranean coast until eastern and western groups were separated by an advancing zone of aridity. It has been thought that speciation in Mediterranean racers occurred in the Upper Miocene and Pliocene (Pozuelo 1974).

All analyses of our data (Figs 2 and 3) indicate a strongly supported close sister group relationship between the genera *Hemorrhois* and *Platyceps* with *Spalerosophis*. This differs from the phylogenetic hypothesis of Schätti and Utiger (2001) where *Spalerosophis* is sister taxon to *Hemorrhois*. However, these authors could not provide sufficient statistical support.

Platyceps and Spalerosophis. Encompassed within the genus *Platyceps* are species from North Africa and Western and Middle Asia, thus in its entirety the genus occupies the large Saharo-Sindian biogeographic region. In addition to those species, which can be unambiguously referred to this genus, there exist a number of taxa of East African and Arabian distribution which have uncertainly been assigned to the genus and these include the wide ranging and polytypic *P. florulentus* (Schätti 1988a) as well as probably *Coluber* s.l. *insulanus*, *C.* s.l. *messanai*, *C.* s.l. *sinai, C.* s.l. *somalicus* and *C.* s.l. *smithi* (Schätti and Utiger 2001).

Platyceps appears monophyletic. Our results (Figs 2 and 3) go some way in resolving intrageneric relationships within this genus. Both the BI/ML and MP trees are largely in agreement. In the BI/ML tree *Platyceps atayevi* and *P. najadum* are sister taxa which together with *P. rubriceps* and *P. florulentus* form a four-taxon clade to which *P. karelini* and *P. rogersi* are linked as another sister taxa. Basally linked to this six-taxon clade is *P. rhodorachis*. The MP tree differs only in having *P. rubriceps* and *P. florulentus* also as sister taxa.

In writing about a *P. karelini–P. rhodorachis–P. ventromaculatus* complex, Khan (1997) was of the opinion that *P. karelini* and *P. rhodorachis* were sister species. However, as noted above, our findings are of a strongly supported sister taxon relationship between *P. karelini* and the Arabian *P. rogersi*, thus excluding *P. rhodorachis*.

In their original description of *P. atayevi* as a new species, Tuniyev and Shammakov (1993) note the distinction between this species and *P. najadum* and suggest a closer relationship between *P. najadum* and *P. rubriceps*. These relationships differ in detail from our findings. The sequence divergence between *P. atayevi* and *P. najadum* is in fact quite low indicating a relatively recent speciation event.

All analyses of our data (Figs 2 and 3) show the genus *Spalerosophis* as represented in our study by a single specimen of *S. diadema*, linked directly with strong statistical support as sister taxon to the genus *Platyceps*. So placed, *Spalerosophis* becomes the basal member of a *Platyceps/Spalerosophis* clade.

Afrotropical racers. Because of their rarity, the Afrotropical racers are the least well represented group in our study. It appears, based on DNA sequence differences, that these are a diverse assemblage which probably does not represent a natural group. Because of differences in tree topology between our different analyses, the phylogeny of the group is at present ambiguous. This is the only subset of taxa within our ingroup where the BI/ML and MP analyses differ significantly in the placement of some of these terminal taxa. However, all analyses of our data agree on the well-supported sister taxon pairing of *C.* s.l. *dorri* and *Macroprotodon cucullatus* (Figs 2 and 3, see also Table 3). It is clear that whatever the phylogenetic history of the Afrotropical racers may eventually prove to be, judging by our representatives, *C.* s.l. *zebrinus*, *C.* s.l. *dorri*, *Hemerophis socotrae* and *Macroprotodon cucullatus*, there is no close relationship between this rather non-cohesive group and any of the Palearctic racers. The supposed relationship between *C.* s.l. *zebrinus* and *Platyceps florulentus* (Broadley and Schätti 1997) is by analysis of our data untenable.

The sister taxon relationship between *Coluber* s.l. *dorri*, a typical representative of the Sahelian fauna and the southern Mediterranean *Macroprotodon cucullatus* was an unexpected finding. The separation of these two taxa from their common ancestor was likely brought about by the emerging Sahara desert. Both mitochondrial and c-mos sequence distances indicate a considerable independent phylogenetic history for each of these taxa. Following the split came the subsequent evolution of the opisthoglyph condition in *Macroprotodon*. The appearance of rear fanged dentition has been an often occurring phenomenon in colubrid evolution and apparently within our ingroup has little bearing on phylogeny. However, in an even more drastic way than in *Eirenis*, it defines a different adaptive level for *Macroprotodon* and precludes the inclusion of *dorri* in that genus.

The Nearctic racers and their allies

Investigation of the phylogeny and taxonomy of the New World racers and other related nearctic snakes has commonly been undertaken as an issue independent from the Palearctic racer fauna. However, it was not until pioneering serological and molecular investigations began (see Dessauer et al. 1987 for a review) that the wide evolutionary gap between these two faunas was realized, this in spite of the fact that fundamental differences in hemipenial morphology between the two had long been known (Schätti 1986a). The type species of the genus *Coluber* is *C. constrictor*, the common North American racer. This polytypic species ranges, apart from its absence in the high elevations of the West, from the Atlantic to the Pacific coasts. Closely related to *C. constrictor* is the North American whipsnake, *Masticophis flagellum* which is also polytypic with a similar transcontinental distribution. Some of the earliest non-morphological attempts directed at the systematics of snakes demonstrated the phylogenetic closeness of the genera *Coluber*, *Masticophis* and *Opheodrys* and their distance from Old World racer fauna (Pearson 1966, 1968).

It is perhaps not surprising that the New World racers should show a closer phylogenetic affinity with Old World colubrid taxa that belong to an evolutionary lineage distinct

from the Old World racers. Analysis of our data produces a clade composed of the New World racer fauna and species of the Asian colubrid genus *Ptyas*. Though not reinforced by the c-mos data, our findings provide support for this relationship earlier concluded by Dowling et al. (1983).

In a study of relationships by comparing electrophoretic mobility of alleles of slowly evolving proteins, Dowling et al. (1996) found that *Ptyas korros* and *Salvadora grahami* were in the same clade; whereas *Ptyas mucosus, Masticophis lateralis* and *C. constrictor* were closer to *Elaphe* s.l. Our data (Figs 2 and 3) clearly indicate that *Ptyas* is not paraphyletic and that *Salvadora* is sister taxon to a *C. constrictor*/*Masticophis* clade.

Taxonomic recommendations

We recommend restricting the usage of the name *Coluber* to the New World taxa currently contained within that genus. Whether the closely related *Masticophis* should also be included in *Coluber*, thus reducing the name *Masticophis* to a synonym of *Coluber*, cannot be decided on the basis of our current data.

Among the Old World racers, the names *Platyceps* and *Hemorrhois* should now be used consistently for their respective groups with *Spalerosophis* and *Hierophis* being already widely used. The paraphyly of *Hierophis* with *Eirenis* nested within it presents a taxonomic problem. We do not recommend the inclusion of *Eirenis* in *Hierophis* as it is a long-established, morphologically and ecologically distinct species-rich genus. A subdivision of *Hierophis* into three monophyletic genera seems more appropriate. The name *Hierophis* would in this case be restricted to the European group as *H. viridiflavus* is the type species. Thus constituted, *Hierophis* would contain the taxa *H. viridiflavus* and *H. gemonensis*. The name *Eirenis* should be retained in its current usage to encompass the dwarf snakes with the probable inclusion of *H. spinalis*. For the third group, the Eastern Mediterranean and Asian group composed of *H. jugularis*, *H. caspius* and *H. schmidti* as well as probably *H. gyarosensis* and *H. cypriensis* (Schätti 1988b) the name *Dolichophis* Gistel, 1868 is available.

An alternative scheme would be to reduce *Eirenis* to a subgenus which would restore the monophyly of *Hierophis* but would violate the principle that members of a genus should share an ecologically and morphologically homogenous adaptive level (Illies 1970, Dubois 1988). *Eirenis* is clearly at a different adaptive level from the other two groups within the *Hierophis* clade. For this reason we do not recommend this course.

With the name *Coluber* confined to New World taxa, the Afrotropical racers should no longer be accommodated under this umbrella. However, they do not have close affinities to any of the other racer genera included in this study. Until possible phylogenetic links with other African colubrine genera (e.g. *Meizodon*) can be established or ruled out, new designations for these snakes are not possible and for the time being they must remain as *Coluber* in the broad sense.

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Zusammenfassung

Phylogenetische Beziehungen zwischen altweltlichen und neuweltlichen Vertretern der Natterngattung Coluber (sensu lato) wurden mit Hilfe von Sequenzen von vier mitochondrialen Genen (Cytochrom b, NADH-Dehydrogenase-Untereinheiten 1, 2 und 4), sowie des Kerngens c-mos ermittelt. Die neuweltlichen Zornnattern (*Coluber* sensu stricto, einschließlich *Masticophis*) und *Salvadora* erwiesen sich als Verwandte der altweltlichen Rattenschlangen der Gattung *Ptyas* und möglicherweise der Gattungen *Elaphe* (s.l.) und *Coronella*, während sich altweltliche Zornnattern in mehrere basal miteinander verbundene Gruppen gliedern: A, *Hemorrhois* mit *Spalerosophis* und *Platyceps*; B, *Hierophis* mit den Zwergnattern (*Eirenis*), die innerhalb dieser paraphyletischen Gattung entspringen; C, „*Coluber*" *dorri* mit der Kapuzennatter *Macroprotodon cucullatus* als Schwesterart. Die Stellung zweier weiterer Taxa, „*Coluber*" *zebrinus* und *Hemerophis socotrae* an der Basis der altweltlichen Radiation der Zornnattern, als möglicher Schwestergruppe der paläarktischen Zornnattern und ihrer Verwandten ist nicht eindeutig belegt. Inter- und subgenerische Beziehungen der meisten altweltlichen Taxa wurden aber eindeutig geklärt.

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