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The phylogeny of squamate reptiles (lizards, snakes, and amphisbaenians) inferred from nine nuclear protein-coding genes

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Abstract

Squamate reptiles number approximately 8000 living species and are a major component of the world’s terrestrial vertebrate diversity. However, the established relationships of the higher-level groups have been questioned in recent molecular analyses. Here we expand the molecular data to include DNA sequences, totaling 6192 base pairs (bp), from nine nuclear protein-coding genes (C-mos, RAG1, RAG2, R35, HOXA13, JUN, α-enolase, amelogenin and MAFB) for 19 taxa representing all major lineages. Our phylogenetic analyses yield a largely resolved phylogeny that challenges previous morphological analyses and requires a new classification. The limbless dibamids are the most basal squamates. Of the remaining taxa (Bifurcata), the gekkonids form a basal lineage. The Unidentata, squamates that are neither dibamids nor gekkonids, are divided into the Scinciformata (scincids, xantusiids, and cordylids) and the Episquamata (remaining taxa). Episquamata includes Laterata (Teiformata, Lacertiformata, and Amphisbaenia, with the latter two joined in Lacertibaenia) and Toxicofera (iguanaids, anguimorphs and snakes). Our results reject several previous hypotheses that identified either the varanids, or a burrowing lineage such as amphisbaenians or dibamids, as the closest relative of snakes. Our study also rejects the monophyly of both Scleroglossa and Autarchoglossa, because Iguania, a species-rich lineage (ca. 1440 sp.), is in a highly nested position rather than being basal among Squamata. Thus iguanians should not be viewed as representing a primitive state of squamate evolution but rather a specialized and successful clade combining lingual prehension, dependence on visual cues, and ambush foraging mode, and which feeds mainly on prey avoided by other squamates. Molecular time estimates show that the Triassic and Jurassic (from 250 to 150 Myr) were important times for squamate evolution and diversification. To cite this article: N. Vidal, S.B. Hedges, C. R. Biologies 328 (2005).

Résumé

La phylogénie des squamates (lézards, serpents, et amphibiennes) inférée à partir de neuf gènes nucléaires codants. Les squamates comprennent environ 8000 espèces actuelles et forment une composante majeure de la faune de vertébrés terrestres. Les relations phylogénétiques entre les familles actuelles de squamates sont inférées par analyses de séquences de neuf gènes nucléaires codant pour des protéines (C-mos, RAG1, RAG2, R35, HOXA13, JUN, α-énolase, amélogénine, MAFB). Notre jeu...
1. Introduction

The order Squamata includes lizards (ca. 4750 sp.), snakes (ca. 3000 sp.), and amphisbaenians (ca. 160 sp.). Together with the two extant species of tuataras from New Zealand, they form the Lepidosauria [1–5]. Squamates are divided into two major clades based on morphology: the Iguania (Iguanidae, Agamidae, Chamaeleonidae) and the Scleroglossa (Dibamidae, Amphisbaenia, Serpentes, Gekkonidae, Scincidae, Cordylidae, Teiidae, Gymnophthalmidae, Scincoida (Scincidae, Cordylidae), and Anguimorpha (Anguidae, Xenosauridae, Shinisauridae, Helodermatidae, Varanidae, Serpentes) (see reviews by Evans [2] and Lee et al. [20]).

Most previous molecular studies of squamates have not sampled a diversity of taxa and thus conclusions have been limited. In early 2004, the first molecular study to sample all major squamate lineages [22], including all lizard and amphisbaenian families and most snake families, discovered some unconventional relationships among squamates. With sequences of two nuclear genes (C-mos and RAG1), snakes were found not to be nested within Anguimorpha. In addition, a close relationship was found between lacertid lizards and amphisbaenians, and between those taxa and the teiid lizards. The classical association of snakes with either the varanid lizards or amphisbaenians was therefore rejected. Moreover, the classical squamate dichotomy between Iguania and Scleroglossa was not supported. Instead, Iguania (one fifth of all living species of squamates) was found to be a derived lineage, clustering high in the tree, together with snakes and anguimorphs. Later in 2004, a second molecular study [23] reached similar conclusions with additional sequence data and support. Nonetheless, several major nodes in both studies were weakly supported or unresolved.

In this work, we expand upon those earlier studies with new sequences from additional genes in an attempt to obtain a more robust phylogeny of squamate reptiles. Here we present analyses of sequence data from nine nuclear protein coding genes (C-mos, RAG1, RAG2,
R35, HOXA13, JUN, MAFB, α-enolase, and amelogenin) obtained from all major squamate lineages. It is the first study to use the JUN, MAFB, and amelogenin genes to elucidate squamate phylogenetic relationships, and expands the taxonomic coverage of other genes (RAG2, R35, and HOXA13).

2. Materials and methods

2.1. Taxonomic sampling and DNA sequencing

Tissue samples were obtained from the tissue collections of Nicolas Vidal and S. Blair Hedges (see Appendix A). We avoided taxa characterized by an increased rate of evolution in order to decrease the influence of long-branch attraction artifacts. Therefore, within snakes, we used henophidian representatives, which evolve more slowly than scolecophidians and caenophidians [24]. For the same reason, within Iguania, we used iguanid representatives, which evolve more slowly than Acrodonta (Chamaeleonidae and Agamidae) [22,23]. DNA extraction was performed using the DNeasy Tissue Kit (Qiagen). The sets of primers used for amplification and sequencing are listed in Appendix B.

Both strands of the PCR products were sequenced using the BigDye sequencing kit (Applied Biosystems) in the ABI Prism 3100-Avant Genetic Analyser. The two strands obtained for each sequence were aligned using the BioEdit Sequence Alignment Editor program [25]. Accession numbers of sequence data obtained from GenBank are listed in Appendix C. The new sequences have been deposited in GenBank.

2.2. Sequence analysis

Sequence entry and alignment (19 taxa) were performed manually with the MUST2000 software [26]. Amino acid properties were used, and ambiguous gaps deleted. This resulted in 360 C-mos sites, 723 RAG2 sites, 732 R35 sites, 444 HOXA13 sites, 330 JUN sites, 81 α-enolase sites, 336 amelogenin sites, and 324 MAFB sites. The RAG1 dataset (2862 sites) was obtained from Townsend et al. [23]. Accession numbers of sequence data obtained from GenBank are listed in Appendix C. The new sequences have been deposited in GenBank.

3. Results and discussion

3.1. Higher-level squamate relationships

Our nuclear dataset allows us to resolve with strong support all major squamate splits but one (Fig. 1).
The limbless dibamids are the most basal lineage, followed by the gekkonids. All squamates but Dibamidae have a variously bifurcated tongue (from slightly notched in gekkonids to deeply forked in varanids and snakes) [7,16,40]. The only other exception lies within Chamaeleonidae, which have lost the bifurcated condition and have secondarily acquired a highly specialized tongue that is projected to capture prey [7,16]. As the tongue of Sphenodon is not notched, the presence of a bifurcated tongue is therefore a shared derived trait uniting the squamates composing the closest relative of dibamids (Fig. 2).

Dibamids and gekkonids are the only two squamate lineages possessing paired egg teeth [40,41]. The paired egg teeth condition is therefore primitive for squamates, and the presence of a single median egg tooth is a shared derived trait uniting all squamates composing the closest relative of gekkotans (Fig. 2).

The next higher-level group comprises xantusiids, cordylids, and scincids, with scincids in a basal position,
Fig. 2. Evolution of prey discrimination and prehension in squa-
mates. Squamates relied primitively on visual prey discrimination, jaw
prehension and a low metabolic rate. Chemical prey discrim-
ination (whether olfactory or vomeronasal) evolved with Bifurcata
(bifurcated tongue). Gekkota rely mostly on olfactory and visual prey
discrimination, and retain a relatively low metabolic rate (mostly am-
bush foragers). Unidentata are more active foragers (higher metabolic
rate) and rely primarily on vomeronasal and visual prey discrimina-
tion. The vomeronasal prey discrimination culminates with Episqua-
matata, which includes the squamates with the most deeply bifurcated
tongues, best-developed Jacobson’s organs, and the highest metabolic
rates. Iguanians display several reversals in these traits.

which is consistent with previous molecular studies [22,
23, 42]. This group is the closest relative of a large
cianeans and snakes, and which is divided into two major
groups. The first group comprises amphibiaenians, lac-
ertids, and teiioids; within this clade, the teiioids are the
most basal lineage, and amphibiaenians and lacer
tids form a monophyletic group, all results previously found
by Vidal and Hedges [22], Townsend et al. [23], and Fry et al. [43].

Within amphibiaenians, rineurids are the most basal lineage, followed by bipedids, which are the
closest relatives to a clade including amphibiaenids and
trogonophids, a result in accordance with previ-
ous molecular studies [22, 23, 43–45]. The second major
group comprises iguanians, anguiomorphs, and snakes,
with snakes as the most basal lineage, although this last
result is not strongly supported. The presence of toxin
secreting oral glands is a shared derived trait of this
clace, demonstrating a single early origin of the venom
system in squamates, instead of two independent ori-
gins (one among caenophidian snakes and one among
helodermatid anguiomorphs) [43] (Fig. 2). Within an-
guiomorphs, varanids are in a basal position, as found by
Vidal and Hedges [22], Townsend et al. [23], and Fry et al. [43].

The position of snakes among squamates has been
a controversial topic. The closest relatives of snakes
are neither the varanids, nor burrowing lineages such
as amphibiaenians and dibamids (which are limbless
or have reduced limbs), but are the anguiomorphs and/or
the iguanians. The novelty of this phylogenetic arrange-
ment mainly lies in the clustering of iguanians with
anguiomorphs and snakes [43]. Our study therefore re-
jects the monophyly of both Scleroglossa and Autar-
choglossa, because iguanians are in a highly nested
position among Squamata. The two lepidosaurian lin-
eages using tongue prehension of food, the tuataras
and the iguanians, have therefore acquired their feed-
ing modes independently. As the iguanians are the only
squamate lineage using tongue prehension of food, and
are highly nested within squamates, we can robustly in-
fer that they have lost the jaw prehension trait used by all
other squamate lineages, and have secondarily acquired
their tongue prehension trait, an inference also made
by Townsend et al. [23] (Fig. 2). In parallel, iguanians
have switched from visual and vomeronasal prey dis-
triment to visual (only) prey discrimination (Fig. 2).

Iguanians thus do not represent a primitive state of evo-
lution in squamates but are a species-rich (ca. 1440 sp.)
and specialized lineage combining lingual prehension,
dependence on visual cues, and ambush foraging mode,
and which feeds mainly on low-energy and noxious
prey avoided by other squamates such as ants, other hy-
menopterans, and beetles [21]. Given this type of food,
the functional significance (e.g., in defense) of the toxin
secerting mandibular and maxillary glands present in
iguanians [43] remains to be investigated.

3.2. Taxonomic implications

Our proposed taxonomic changes are indicated in
Fig. 1. Names are not required for every node of a phy-
ology, but squamates are widely used in ecological and
evolutionary studies, and higher-level taxonomic names
such as these facilitate research and communication in
the field. For taxonomic stability, we prefer to use avail-
able names. However, when the definition (characters)
and content of some taxa are altered substantially, it is
less confusing and more practical to abandon those old
names and use new names, as we have done in several
cases here. In each case, the taxon named refers to the
most recent common ancestor of the included groups
and all descendants.

Lacertiformata (new name) includes Lacertidae. Lac-
certibaenia (new name) includes Lacertiformata and Am-
phisbaenia, and is a name that includes elements of both words. Teiformata (new name) includes Teiidae and Gymnophthalmidae. Although one solution to the problem of the paraphyly of Lacertiformes would be to redefine this taxon to include Amphisbaenia, this would conflict with the meaning of the name (‘lizard-shaped’) and lead to confusion over content. Therefore, we propose a new name, defined by a morphological character that is consistent with the molecular phylogeny. Laterata (new name) includes Lacertibaenia and Teiformata, referring to the presence of tile-like (squarish or quadrangular, and sometimes raised) scales that form the rings in Amphisbaenia, and are also present ventrally in Lacertiformata and Teiformata (while recognizing that squarish scales occur in other taxa, such as xantusiids and some anguimorphs). Toxicofera (new name) includes Iguania, Anguimorpha, and Serpentes, referring to the presence of venom. Episquamata (new name) includes Toxicofera and Laterata, referring to its derived position in the tree of squamates (‘top squamates’). Scinciformata (new name) includes Scincidae, Xantusiidae, and Cordylidae. Although one solution to the problem of the paraphyly of Autarchoglossa would be to redefine this taxon to include Iguania, this would conflict with the meaning of the name (‘free-tongued’) and lead to confusion over content. Unidentata (new name) includes Episquamata and Scinciformata and refers to the presence of one egg tooth. Bifurcata (new name) includes Unidentata and Gekkota, referring to the presence of a bifurcated tongue. With the repositioning of Iguania to a derived position in the tree, the name ‘Scleroglossa’ now becomes obsolete in terms of its meaning and content and is discarded.

Our proposed classification of Squamata is summarized here:

Squamata
   Dibamia
   Bifurcata
      Gekkota
      Unidentata
   Scinciformata
      Episquamata
      Toxicofera
         Serpentes
         Unnamed clade
         Anguimorpha
         Iguania
   Laterata
      Teiformata
      Lacertibaenia
         Lacertiformata
         Amphisbaenia

3.3. Biogeography

The new relationships and divergence times provide a better understanding of the historical biogeography of squamates. Molecular time estimates show that the Triassic and Jurassic (from 250 to 150 Myr) were important times for squamate evolution and diversification (Fig. 1). For example, the early divergences among the major groups (Dibamia/Bifurcata, Gekkota/Unidentata, Scinciformata/Episquamata) apparently took place largely during the Triassic at a time when all of the continents were joined in a single supercontinent Pangaea. Therefore, we should not expect a strong geographic influence in the ancestral distributions of these groups. However, the major clades of episquamats contain lineages that diverged during the Jurassic and later, after the initial breakup of Pangaea, and therefore we should expect to see more geographic patterns in their historical (e.g., Mesozoic) distributions (Cenozoic distributions show considerable dispersal among continents). For some groups, such as anguimorphs (Laurasia), snakes (possibly Gondwana) and iguanians (possibly Gondwana), this has already been noted [2]. The association of those three groups in Toxicofera now raises the possibility that vicariance has played a role in their origin. Another striking pattern in the tree (Fig. 1) is the relatively recent (Cenozoic) origin of three lineages (families) of amphisbaenians: Bipedidae, Trogonophidae, and Amphisbaenidae. Currently, the major limitation of drawing biogeographic inferences is the relatively poor fossil record of squamates from the southern hemisphere [2]. Nonetheless, this new molecular phylogeny and timescale of squamate evolution should help encourage development of new hypotheses and searches for new fossils.

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Appendix A. Tissue samples used

The following list identifies the collection number of the sample used and the locality. Collection numbers have the following prefixes: LACM (Los Angeles County Museum), MVZ (University of California Museum of Vertebrate Zoology, Berkeley), NV (Nicolas Vidal, ethanol preserved tissue collection), SBH (S. Blair Hedges, frozen tissue collection), and USNM (United States National Museum, Smithsonian, Washington, D.C.).

**Sphenodontida**: *Sphenodon punctatus* (SBH 266085; New Zealand; RAG2, R35, HOXA13, JUN, amelogenin), *Squamata*: *Xantusidae*: *Xantusia vigilaris* (LACM 136813; California, San Bernadino County, Hesperia; RAG2), *Xantusia henshawi* (LACM 136789; California, Riverside County, 4.7 miles SE Banning; R35), *Xantusia riversiana* (LACM 125513; California, Ventura County, San Nicolas Island; HOXA13), *Cricosaura typica* (SBH 190532; Cuba, Santiago de Cuba Province, 2.8 km N. Uvero; JUN, MAFB, amelogenin), *Amphisbaenidae*: *Amphisbaena cubana* (SBH 161956; Cuba, Guantánamo Bay Naval Station; RAG2, R35, HOXA13, JUN, MAFB, amelogenin, α-enolase), *Rhineuridae*: *Rhineura floridana* (SBH 172913; Florida, Plant City; RAG2, R35, HOXA13, JUN, MAFB, amelogenin, α-enolase), *Bipedidae*: *Bipes biporus* (MVZ 137543; Baja California Sur; RAG2, HOXA13), *Bipes canaliculatus* (SBH 267134; Guerrero, Mexico; R35, JUN, MAFB, amelogenin, α-enolase), *Trogonophidae*: *Trogonophis wiegmanni* (MVZ 11124; North Africa; RAG2, R35, HOXA13, JUN, MAFB, amelogenin, α-enolase), *Lacertidae*: *Podarcis hispanica* (SBH 266666; Morocco, Asilah; RAG2, JUN), *Takydromus sexlineatus* (NV; locality unknown; R35, HOXA13, MAFB, amelogenin), *Teioidae*: *Teiidae*: *Ameiva auberi* (USNM 306540; Cuba, Guantánamo, 8.9 km SW Hatibonicó; RAG2, HOXA13, JUN, MAFB), *Gymnophthalmidae*: *Gymnophthalmus underwoodii* (SBH 102274; Guadeloupe, Grande Terre, Playa Anse Laborde; amelogenin), *Iguanidae*: *Cyclura nubila* (SBH 104677; Puerto Rico; C-mos, RAG2, R35, HOXA13, JUN, MAFB, amelogenin, α-enolase), *Leiocephalus barahonensis* (SBH 101427; Dominican Republic, Barahona Province, Barahona; C-mos, RAG2, R35, HOXA13, JUN, MAFB, amelogenin), *Anolis sagrei* (SBH 160990; Jamaica, St James Province, 3.2 km W. Mocho; α-enolase), *Gekkonidae*: *Gekko vittatus* (NV; Indonesia; RAG2, R35, HOXA13, JUN, MAFB, amelogenin), *Pygopodinae*: *Lialis burtonis* (SBH 266057; Papua New Guinea, Wipim; RAG2, R35, MAFB, amelogenin), *Lialis jicari* (NV, Irian Jaya, JUN), *Cordylidae*: *Cordylus giganteus* (SBH 266055; South Africa; RAG2, R35, HOXA13, JUN, MAFB, amelogenin), *Scincidae*: *Eumeces inexpectatus* (SBH 191579; Florida; RAG2, R35, JUN, MAFB, amelogenin), *Helodermatidae*: *Heloderma suspectum* (SBH 194118; locality unknown; RAG2, R35, HOXA13, JUN, MAFB, amelogenin, α-enolase), *Dibamidae*: *Dibamus novaeguineae* (SBH 266054; Philippines; RAG2, R35, HOXA13, JUN, MAFB, amelogenin), *Anguidae*: *Aniella pulchra* (SBH 194106; California, Santa Barbara County, Santa Maria; RAG2, R35, HOXA13, JUN, MAFB, amelogenin, α-enolase), *Varanidae*: *Varanus dumerilii* (SBH 266058; locality unknown; RAG2, R35, JUN, MAFB, amelogenin, α-enolase), *Serpentes*: *Pythonidae*: *Liasis savuensis* (NV; Savu Island, Indonesia; RAG2, R35, HOXA13, JUN, MAFB, amelogenin, α-enolase).

Appendix B. Primers used

Amplification was performed using the following sets of primers: G73, 5'-GCG-GTA-AAG-CAG-GTG-AAG-AAA-3' [46], G74, 5'-TGA-GCA-TCC-AAA-GTC-TCC-AAT-C-3' [46] for the C-mos gene; L460, 5'-AAC-AAT-GAN-CTT-TCT-GAT-AA-3' (original), L562, 5'-CCT-RAD-GCC-AGA-TAT-GGY-CAT-AC-3' (original) and H1306, 5'-GHG-AAY-TCC-TCT-GAT- TCT-TC-3' (original) for the RAG2 gene; L29, 5'-CTG AAA-ATK-CAG-ACC-AAA-A-3' (original), L29B, 5'-CTG-AAA-ATG-CAG-ACC-AAA-AGT-AC-3' (original), L42, 5'-GAA-CAA-AGG-TAC-WTG-ATT- AAC-3' (original), L75, 5'-TCT-AAG-TGT-GGA-TGA-TYT-GAT-3' (original), H786, 5'-TTG-GRA-GCC-ARA-GAA-TGA-CTT-3' (original), H792, 5'-CAT-CAT-TGG-RAG-CCA-AAG-AAA-3' (original), H792B, 5'-CAT-CAT-TGG-GAG-CCA-RAG-AAT-GA-3' (original) for the R35 gene; F2, 5'-ATC-GAG-CCC-ACC-GTC-ATG-TTT-CTC-TAC-GAC-3' [47], F35, 5'-GTC-ATG-TTY-CTY-TAC-GAC-AG-AG-3' (original), F54, 5'-ACA-ACA-GCY-TGG-ARG-AGA-TYA-ACA-A-3' (original), R2, 5'-TGG-TAG-AAA-GCA-AAC-TCC-

Appendix C. Sequence data obtained from GenBank

**Sphenodontida**: Sphenodon punctatus (RAG1: AY662576; C-mos: AF039483), Squamata: Xantusiidae: Xantusia vigilis (RAG1: AY662642; C-mos: AF148703), Lepidophyma sylvatica (α-enolase: AY218079), Amphisbaenidae: Amphisbaena cubana (C-mos: AF487346), Amphisbaena xera (RAG1: AY662619), Rhineuridae: Rhineura floridana (RAG1: AY662618; C-mos: AY662617; C-mos: AY662676; C-mos: AY444025), Lacertidae: Eremias sp. (RAG1: AYY662615), Lacerta kulzeri (C-mos: AF148712), Mesalina guttulata (α-enolase: AY218056), Teiioidea: Aspidoscelis tigris (RAG1: AY662620), Kentropyx calcarata (C-mos: AF420864), Tupinambis quadrilineatus (α-enolase: AY218076), Gekkonidae: Gekko gecko (RAG1: AYY662625; C-mos: AY444028), Gehyra mutilata (α-enolase: AY218045); Pygodinidae: Lialis javanicus (C-mos: AF090850), Lialis jacari (RAG1: AY662628), Cordylidae: Cordylus polyzonus (RAG1: AY662643), Cordylus cordylus (C-mos: AF148711), Scincidae: Eumeces inexpectatus (RAG1: AY662632; HOXA13: AF083100; α-enolase: AY218075), Eumeces skiltonianus (C-mos: AF315396), Helodermatidae: Heloderma suspectum (RAG1: AY662606; C-mos: AY487348), Dibamidae: Dibamus novaeguinea (C-mos: AY487349), Dibamus sp. (RAG1: AYY662645), Anguidae: Anniella pulchra (RAG1: AY662605; C-mos: AY487350), Varanidae: Varanus dumerilii (HOXA13: AF083102), Varanus rosenbergi (C-mos: AY099976), Varanus griseus (RAG1: AYY662608), Serpentes: Pythonidae: Liais savuenensis (C-mos: AF544726), Cylindrophiidae: Cylindrophis ruffus (RAG1: AYY662613), Iguanidae: Leiocephalus carinatus (RAG1: AYY662598), Sauropterygia (RAG1: AYY662591).

References


