Evolution of developmental sequences in lepidosaurs

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Background. Lepidosaurs, a group including rhynchocephalians and squamates, are one of the major clades of extant vertebrates. Although there has been extensive phylogenetic work on this clade, its interrelationships are a matter of debate. Morphological and molecular data suggest very different relationships within squamates. Despite this, relatively few studies have assessed the utility of other types of data for inferring squamate phylogeny.

Methods. We used developmental sequences of 20 events in 29 species of lepidosaurs. These sequences were analysed using event-pairing and continuous analysis. They were transformed into cladistic characters and analysed in TNT. Ancestral state reconstructions were performed on two main phylogenetic hypotheses of squamates (morphological and molecular).

Results. Cladistic analyses conducted using characters generated by these methods do not resemble any previously published phylogeny. Ancestral state reconstructions are equally consistent with both morphological and molecular hypotheses of squamate phylogeny. Only several inferred heterochronic events are common to all methods and phylogenies.

Discussion. Results of the cladistic analyses, and the fact that reconstructions of heterochronic events show more similarities between certain methods rather than phylogenetic hypotheses, suggest that phylogenetic signal is at best weak in the studied developmental events. Possibly the developmental sequences analysed here evolve too quickly to recover deep divergences within Squamata.

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12 major clades of extant vertebrates. Although there has been extensive phylogenetic work on this
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14 very different relationships within squamates. Despite this, relatively few studies have assessed
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19 two main phylogenetic hypotheses of squamates (morphological and molecular).

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21 resemble any previously published phylogeny. Ancestral state reconstructions are equally
22 consistent with both morphological and molecular hypotheses of squamate phylogeny. Only
23 several inferred heterochronic events are common to all methods and phylogenies.

24 **Discussion.** Results of the cladistic analyses, and the fact that reconstructions of heterochronic
25 events show more similarities between certain methods rather than phylogenetic hypotheses,
26 suggest that phylogenetic signal is at best weak in the studied developmental events. Possibly the
27 developmental sequences analysed here evolve too quickly to recover deep divergences within
28 Squamata.

29

30 **Introduction**
31 With over 10,000 species, Squamata (lizards, snakes and amphisbaenians) are one of the most
32 species-rich extant tetrapod lineages (Uetz, Freed & Hošek, 2016). However, our understanding
33 of its evolutionary history is confounded by the conflict between phylogenetic hypotheses based
34 on morphology and molecular data (e.g. Losos, Hillis & Greene, 2012). Morphological analyses
35 suggest that the first divergence within Squamata was between Iguania (iguanas, agamas,
36 chameleons and kin) and Scleroglossa (all other lizards and snakes) (e.g. Estes, de Queiroz &
37 Gauthier, 1988; Conrad, 2008; Gauthier et al., 2012), while molecular studies indicate that
38 iguanians are highly derived lizards, closely related to anguimorphs (e.g. monitor lizards) and
39 snakes, and that limbless dibamids or gekkotans (geckos and kin, sometimes also including
40 dibamids) are the first-diverging branch of squamates (e.g. Townsend et al. 2004, Vidal &
41 Hedges, 2005; Wiens et al., 2010, 2012; Pyron, Burbrink & Wiens, 2013). Increasing the number
42 of taxa and characters in these analyses has not led to an improvement of our understanding of
43 squamate phylogeny, but rather has only increased the discordance between the hypotheses
44 based on those two lines of evidence. Combined morphological and molecular analyses (e.g.
45 Wiens et al., 2010; Reeder et al., 2015) generally favour the molecular topology (but see Lee,
46 2005). However, some authors argue that molecular data may not be ideal for resolving the
47 higher-level phylogeny of squamates because of the large genetic distance between squamates
48 and their closest living relative – the tuatara (*Sphenodon punctatus*), the only extant
49 rhynchocephalian – and thus the only reasonable proximal outgroup to Squamata in phylogenetic
50 analyses (McMahan et al., 2015). Despite numerous publications on this subject (Gauthier et al.,
51 2012; Losos, Hillis & Greene, 2012; Reeder et al., 2015), the debate continues and still new

52   approaches to the problem are being taken (McMahan et al., 2015; Harrington, Leavitt & Reeder,
53   2016; Pyron, 2017).

54   Developmental data may be useful for phylogenetic inference (e.g. Laurin & Germain, 2011) but
55   they rarely have been used in squamate phylogenetics. Notable exceptions are the studies of
56   Maisano (2002) and Werneburg & Sánchez-Villagra (2015), using ossification sequences. The
57   former found that these sequences are useful for determininig relatively shallow divergences but
58   failed to recover deeper nodes, possibly because of their high rate of evolution (Maisano, 2002).
59   Werneburg & Sánchez-Villagra (2015) found that developmental data were most congruent with
60   the close relationship between snakes and varanids, as postulated by some morphological studies
61   (e.g. Lee, 1997) but also some combined morphological and molecular analyses (e.g. Lee, 2005).
62   Sequences of other developmental traits were studied by Andrews, Brandley & Greene (2013)
63   but the authors regarded relationships of squamates as "well defined" and reconstructed the
64   ancestral states only on the molecular topologies. Moreover, their study did not consider the
65   tuatara, a taxon critical in studying lepidosaur evolution. We attempt to supplement their data
66   with the developmental sequence of the tuatara and reconstruct ancestral states using both
67   molecular and morphological topologies. We also assess phylogenetic utility of timing of
68   organogenesis using several different methods.

69

70   **Materials & Methods**
71   **Character construction and cladistic analyses.** Developmental sequences of 20 characters in
72   21 species representing most major squamate lineages (Tables 1-2) were obtained from
73   Andrews, Brandley & Greene (2013). Developmental sequences of seven other squamate species
74   were taken from the literature (Table 1). The developmental sequence of the tuatara was
75   compiled from Dendy (1899) and Sanger, Gredler & Cohn (2015) (see also Moffat, 1985). These
76   sequences were transformed into continuous characters, where the first event has a value of 0,
77   and the last one – a value of 1 (Germain & Laurin, 2009; Laurin & Germain, 2011). These values
78   constituted the basis for cladistic characters, which were created following Werneburg &
79   Sánchez-Villagra (2015) – values between 0 and 0.09 were coded as 0, between 0.1 and 0.19
80   were coded as 1, and so on. The missing data were coded as unknown (?), while limb characters
81   in snakes were coded as inapplicable (-). Cladistic analyses employing these characters were
82   conducted in TNT v. 1.1 (Goloboff, Farris & Nixon, 2003, 2008), using the traditional search
83   option, with 10 replications of Wagner trees. These trees were held in RAM and subjected to tree
84   bisection reconnection, holding 10 trees per replicate. In the first analysis, all characters were
85   unordered (non-additive), and in the second one, all were ordered (additive) (see Werneburg &
86   Sánchez-Villagra, 2009; Laurin & Germain, 2011). In both analyses, *Sphenodon* was used as the
87   outgroup.

88   Another set of cladistic characters was created using the event-pairing method (Smith, 1997;
89   Velhagen, 1997; Jeffery et al., 2002a, 2005). Comparing 20 developmental events in 29 species
90   resulted in 190 event pairs. These characters were analysed in the same way as continuous
91   characters.

92   With these cladistic characters and files with both molecular and morphological topology in
93   memory, Templeton test (Templeton, 1983) was performed in TNT (using a script written by

94   Alexander Schmidt-Lebuhn, https://www.anbg.gov.au/cpbr/tools/templetontest.tnt). Four
95   replications were conducted: using either ordered or unordered characters; and employing
96   continuous or event-paired characters.

97   **Ancestral state reconstruction and heterochronic events.** Reconstruction of ancestral states
98   was performed in Mesquite v. 3.2 (Maddison & Maddison, 2017). Developmental sequences
99   were mapped on two competing phylogenetic hypotheses of lepidosaurs – first one, from Pyron,
100   Burbrink & Wiens (2013), using seven nuclear and five mitochondrial genes, and the second
101   one, from Gauthier et al. (2012), the largest morphological analysis to date. Ancestral states were
102   reconstructed using both maximum parsimony and maximum likelihood for event-paired data
103   and square-changed parsimony for continuous data. The branch length may have a significant
104   effect on reconstruction of ancestral states (e.g. Andrews, Brandley & Greene, 2013; Boyd,
105   2015), so analyses using maximum likelihood and square-changed parsimony were performed on
106   both molecular and morphological topologies. In the first analysis, all branches were given an
107   equal length (= 1), while in the second, the branch lengths were calibrated to reflect the fossil
108   record of a given group. The oldest-known fossil of a total group was used to calibrate the tree
109   rather than that of a crown group (Table 3). Only fossils unquestionably placed within a given
110   group were included. When the fossil record of a group was unknown (mostly in relatively
111   recently diverged species), the branch length was set, arbitrarily, as 3. Square-changed
112   parsimony reconstruction using continuous data was performed using root node reconstruction in
113   PDAP:PDTREE module of Mesquite (Midford, Garland & Maddison, 2011). This module
114   calculates 95% confidence intervals (Garland & Ives, 2000) for each character of a hypothetical
115   ancestor of all taxa included in a tree (in this case, ancestral lepidosaur). A statistically
116   significant heterochronic event occurs when a value of character state of a given taxon is beyond
117   the confidence interval. In the second analysis, *Sphenodon* was pruned from the tree, and
118   reconstruction was made for the ancestral squamate and compared to the values of terminal taxa.

119   Event-pair synapomorphies were mapped on both topologies using synapomorphy mapping in
120   TNT. These synapomorphies were subjected to event-pair cracking, following the procedure
121   described in detail by Jeffery et al. (2002a). Only deviations from their methods are described
122   below. Clades supported by only one event-pair synapomorphy, two synapomorphies involving
123   four different events and so on were excluded because the number of developmental changes was
124   insufficient for determining the background pattern and heterochronies. In the ordered dataset,
125   when degree of change was ambiguous (e.g. from 0 to 1 or 2), a mean was taken (in this
126   example, 1.5). Characters in which the direction of change could not be unambiguously
127   reconstructed (i.e. from 1 to 0 or 2) were excluded from further analysis. This should not have
128   significant effect on the analysis, as there was only a few such characters (Tables S1-S8). Only
129   events with total relative change (TRC) beyond the 95% confidence interval calculated for the
130   mean TRCs at a given node were regarded as heterochronic. This is more conservative approach
131   than the one taken by Jeffery et al. (2002a) but will make the analysis more comparable to the
132   continuous analysis described above.

133

134   **Results**

135   **Cladistic analyses.** Cladistic analyses conducted using the transformed continuous data
136   generated trees that are not similar to trees obtained in either morphological or molecular
137   analyses. Analysis using unordered characters yielded 214 most parsimonious trees (MPT; tree
138   length = 109, consistency index = 0.560, retention index = 0.628), the strict consensus tree of
139   which is almost completely unresolved. This analysis failed to recover clades of very closely
140   related species such as *Liolaemus* (Fig. 1A). When all characters were ordered, it resulted in 174
141   most parsimonious trees (TL = 133, CI = 0.459, RI = 0.625). The strict consensus tree is mostly
142   unresolved – the only groups that were monophyletic in all MPTs are *Liolaemus*, *Tropidurus* +
143   *Strophurus*, *Calyptommatus* + *Anolis* and a clade including *Uta*, *Agama*, *Furcifer*, *Mabuya*,
144   *Gehyra*, *Chamaeleo* and *Zootoca*. A 50% majority rule tree does not resemble published
145   morphological or molecular phylogenies (Fig. 1B).

146   Similar to the continuous dataset, the event-paired data did not result in a topology matching any
147   previously published phylogeny. Analysis of unordered characters generated 10 MPTs (TL =
148   185, CI = 0.530, RI = 0.552). In the strict consensus tree *Furcifer* and *Varanus indicus* are in
149   trichotomy with the clade including all other squamates. This clade is divided into a group
150   containing seven species of iguanians, gekkotan *Strophurus*, snake *Thamnophis*, scincoid
151   *Mabuya* and lacertiform *Zootoca*, and the second group to which all other squamates belong
152   (Fig. 2A). Analysis using ordered characters yielded 16 MPTs (TL = 220, CI = 0.464, RI =
153   0.599). The strict consensus tree is poorly resolved but excluding *Varanus indicus* from it
154   significantly improves resolution. After this, squamates are divided into two clades – the first one
155   includes eight species of iguanians, *Thamnophis* and *Mabuya*, while the second group includes
156   all other squamates (Fig. 2B).

157   Mapping of continuous characters indicates slight differences in tree length between
158   morphological and molecular topologies. With all branches being assigned equal length (= 1),
159   the former is 1.49630768 steps long and the latter – 1.51610078. With the fossil-calibrated tree,
160   the morphological topology is 0.19257679 steps long and molecular – 0.17638729. Mapping of
161   unordered event-paired characters gives the molecular topology a length of 250 steps and the
162   morphological – 252 steps. With ordered characters, the molecular topology is 322 steps long,
163   while the morphological is 327 steps long.

164   Neither replication of the Templeton test detected any statistically significant differences
165   between morphological and molecular phylogenies under both present continuous and event-
166   paired character datasets (p > 0.05 in all cases).

167   **Developmental diagnoses.** There are several event-pair synapomorphies diagnosing some
168   higher-level taxa (i.e. family-level clades or higher). However, at least some of these groups are
169   represented by only a few members (e.g. Anguimorpha, Scincoidea), so these apomorphies may
170   in fact diagnose less inclusive clades (Table 4).

171   **Heterochronic events.** Inferred heterochronic events show more consistency between given
172   methods than between phylogenies (e.g. event-paired data for morphological phylogeny are more
173   similar to event-paired data for molecular topology than to continuous data for morphological
174   tree). Only a few of these events are common to all methods and phylogenies (Figs. 3-15).

175

176 **Discussion**
177 Developmental cladistic characters failed to recover topology similar to those based on other
178 data (i.e. molecular or morphological). This was also found in similar studies (Maisano, 2002;
179 Werneburg & Sánchez-Villagra, 2009, 2015). This may be a consequence of uneven sampling of
180 different squamate clades in the present analysis – out of 28 included species, 11 are iguanians
181 and six are gekkotans, while there are only three anguimorphs (and all of them belong to a single
182 clade, *Varanus*) and one scincoid. Members of other important clades, like Amphisbaenia and
183 Dibamidae, were not included. Some of these groups only recently were studied in terms of
184 development (e.g. Gregorovicova et al., 2012). Moreover, development of lepidosaurs included
185 in this analysis is incompletely known. Thorough study of developmental sequences of these and
186 other members of these diverse clades will be beneficial to future analyses. However, it may be
187 that homoplasies are very common in developmental sequences of squamates. Moreover, the
188 phylogenetic signal in organogenetic events (at least those used in this study) may be weak or
189 detectable only in deeper nodes of the phylogenetic tree (cf. Jeffery et al., 2002b; Maisano,
190 2002). This may be indicated by higher congruence between methods in reconstructing
191 heterochronic events than between given phylogenies.

192 The only cladistic analyses that slightly resembled published phylogenies employed event-paired
193 characters, especially ordered ones (Fig. 1B). In this analysis, eight of eleven included iguanian
194 species formed a monophyletic group with *Thamnophis* and *Mabuya* that was sister to all other
195 squamates. This resembles the morphological topology, where iguanians are sister group to all
196 other squamates (e.g. Estes, de Queiroz & Gauthier, 1988; Conrad, 2008; Gauthier et al., 2012).
197 This may suggest that developmental sequences of most iguanians and the tuatara are relatively
198 similar. Under morphological topology, these similarities would represent symplesiomorphies
199 but under molecular one, would be considered homoplasies. Reeder et al. (2015) suggested that
200 support for basal placement of Iguania comes from the cranial characters. This is not the case in
201 the present analysis. Character mapping and ancestral states reconstructions of event-paired data
202 suggest that potential symplesiomorphies between the tuatara and iguanians (as a whole or one of
203 their major subgroups – Acrodonta and Pleurodonta) are connected with the relatively later
204 torsion completion, rather than of some events concerning head development. Other groups
205 recognized by morphological analyses also receive some support. For example, Scleroglossa are
206 supported by earlier occurrence of torsion completion (simultaneous with occurrence of
207 hyomandibular slit and allantois bud), unlike in tuatara and Iguania. Scincomorpha are supported
208 by simultaneous development of otic placode, allantois bud and secondary optic vesicle.

209 Gekkotans differ from other squamates in later development of the allantois (Andrews, Brandley
210 & Green, 2013) but in that trait they resemble the tuatara. Under molecular topology, earlier
211 development of the allantois bud supports the Unidentata (Table 4). This may represent a
212 genuine signal of monophyly of that group, however, caution is warranted. Gekkotans display
213 many paedomorphic features, including their morphology (e.g. Daza, Bauer & Snively, 2014)
214 and development (Jonasson, Russel & Vickaryous, 2012). Thus, the condition in gekkotans may
215 represent reversal to the primitive condition (presumably, as displayed by the tuatara) rather than
216 plesiomorphy. This situation is similar to the development of a single egg tooth, which
217 purportedly supports the monophyly of Unidentata (see discussion in Assis & Rieppel, 2011). To

218   gain more insight into that matter, it would be crucial to sample development of dibamids, the
219   only other non-unidentate squamates.

220   In the fossil time-calibrated continuous analysis, only one event in two species is inferred to
221   show heterochrony in relation to the ancestral lepidosaur. This may seem surprising, as some
222   squamates show heterochrony to the ancestral squamate (much closer phylogenetically).
223   However, if all studied taxa are extant (as is the case in the present analysis), the long branches
224   would result in wider confidence intervals and thus ancestral state reconstructions for deep nodes
225   of the phylogenetic tree would be less certain (Germain & Laurin, 2009). Integration of data
226   from fossils would be useful in that regard but it seems highly unlikely that information on
227   organogenesis can be preserved in the fossil record, despite recent significant advances in
228   developmental palaeobiology (e.g. Skawiński & Tałanda, 2015).

229   In the continuous analyses (both calibrated and uncalibrated and using either molecular or
230   morphological topology), values of all developmental events of the tuatara are located within the
231   confidence interval of the ancestral squamate. This suggests that present data are equally
232   consistent with either hypothesis of squamate phylogeny (cf. Germain & Laurin, 2009).

233   In this study only two major phylogenetic hypotheses of squamates were used. It is not beyond
234   imagination that neither of these phylogenies is fully correct. For example, in the analysis
235   combining morphological and molecular data conducted by Lee (2005) the "fossorial group" is
236   polyphyletic, as suggested by molecular analyses (e.g. Wiens et al., 2012; Pyron, Burbrink &
237   Wiens, 2013), but division of squamates into Iguania and Scleroglossa is retained, as in
238   morphological analyses (e.g. Conrad, 2008; Gauthier et al., 2012). This could, to some extent,
239   explain the discrepancies in reconstructions of heterochronic events, as none of these would be
240   done on the basis of the correct tree.

241

242   **Conclusions**
243   Cladistic analyses conducted using characters generated by event-pairing and continuous
244   analysis do not resemble any previously published phylogeny. Ancestral state reconstructions are
245   equally consistent with both morphological and molecular hypotheses of squamate phylogeny.
246   Results of the cladistic analyses, and the fact that reconstructions of heterochronic events show
247   more similarities between certain methods than phylogenetic hypotheses, suggest that
248   phylogenetic signal is at best weak in the studied developmental events.

249

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254

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Figure 1

Results of the cladistic analysis using characters from the continuous analysis.

Strict consensus tree. (A) Unordered characters; TL = 109, CI = 0.560, RI = 0.628. (B) Ordered characters; TL = 133, CI = 0.459, RI = 0.625. Colour represents clade to which given species belongs.
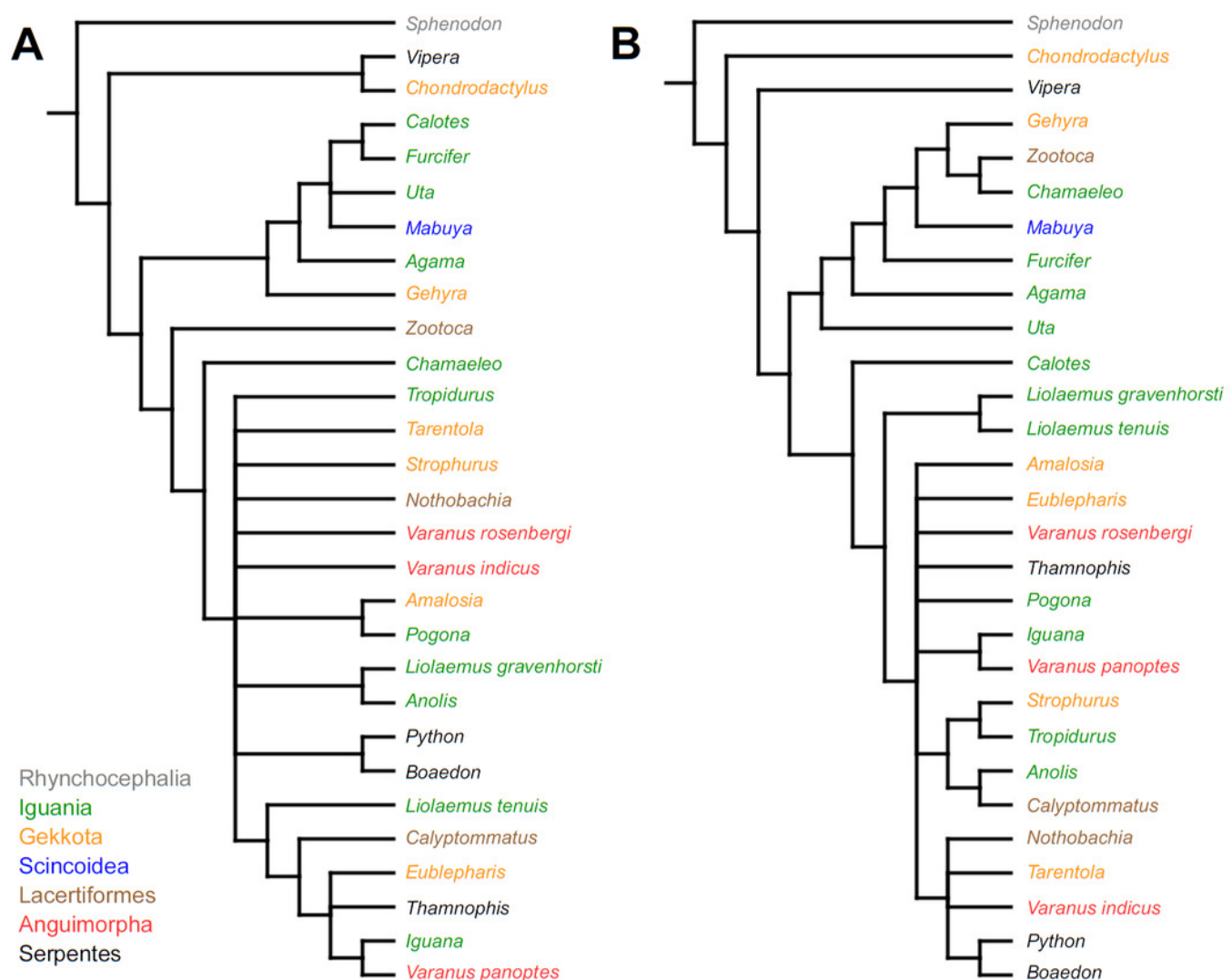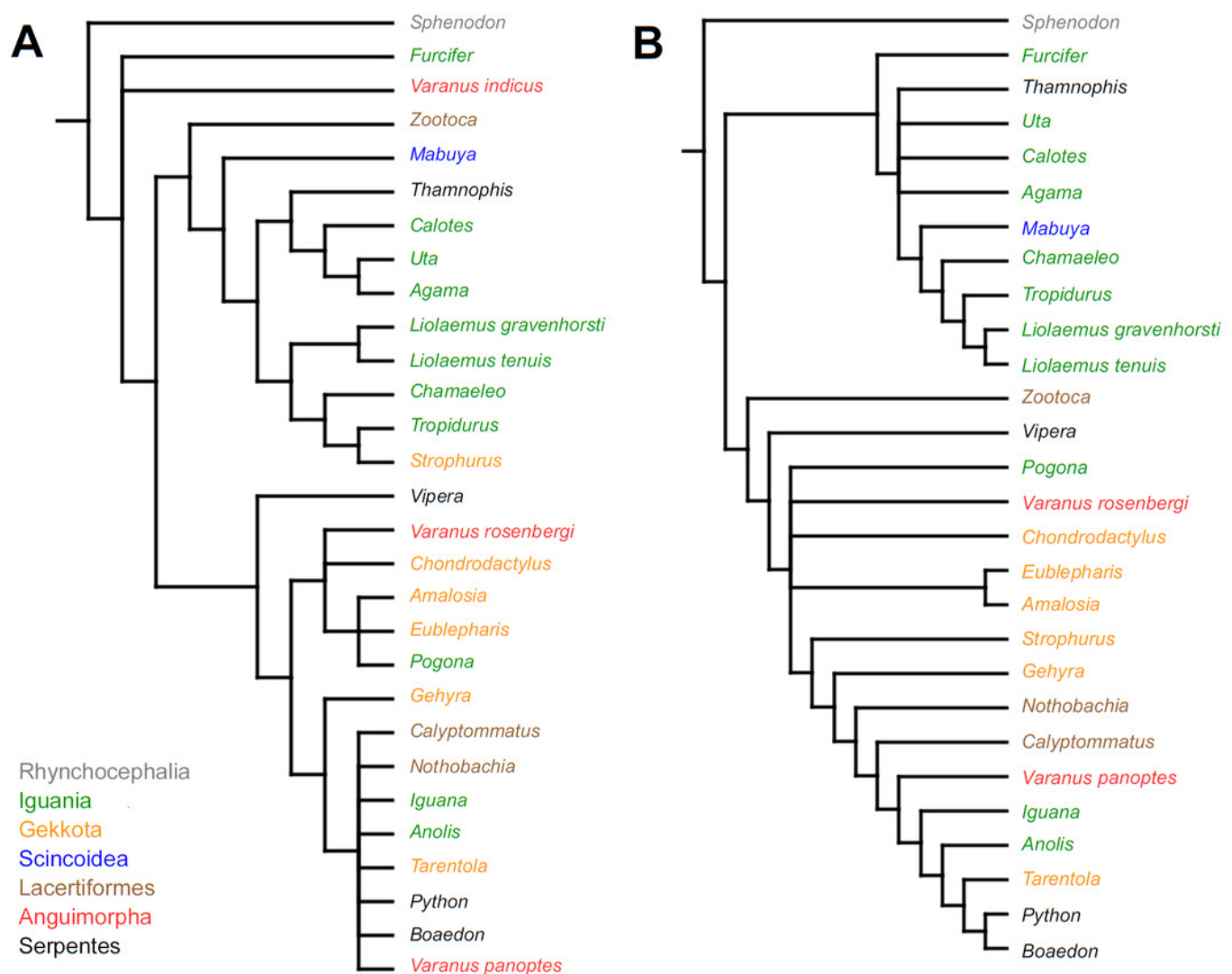
Figure 2

Results of the cladistic analysis using characters from event-pairing.

Strict consensus tree (in B after excluding *Varanus indicus*). (A) Unordered characters; TL = 185, CI = 0.530, RI = 0.552. (B) Ordered characters; TL = 220, CI = 0.464, RI = 0.559. Colour represents clade to which given species belongs.
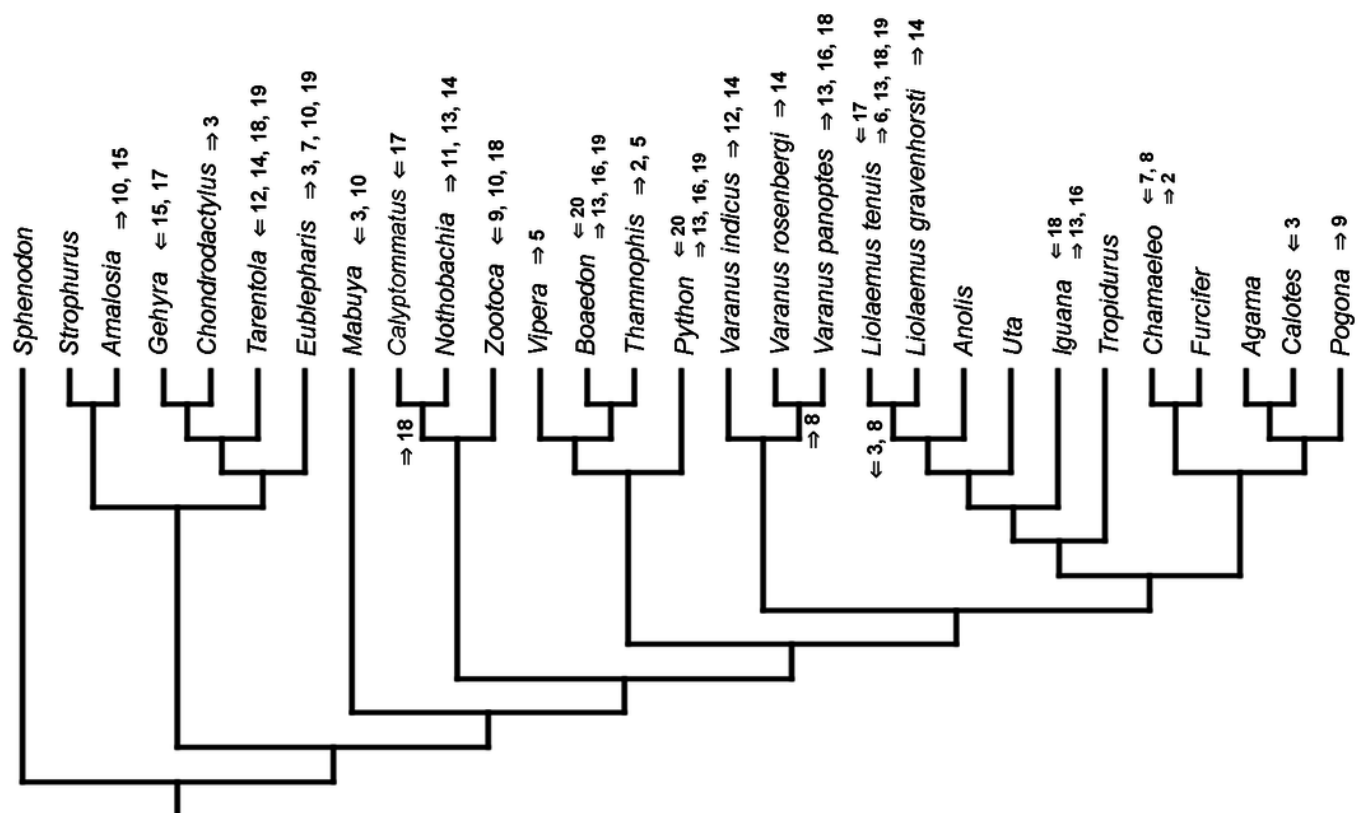
Figure 3

Heterochronic events in lepidosaur evolution.

Mapped onto molecular phylogeny, using continuous data, in relation to the ancestral lepidosaur. Length of all branches equals 1. Numbers within boxes refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
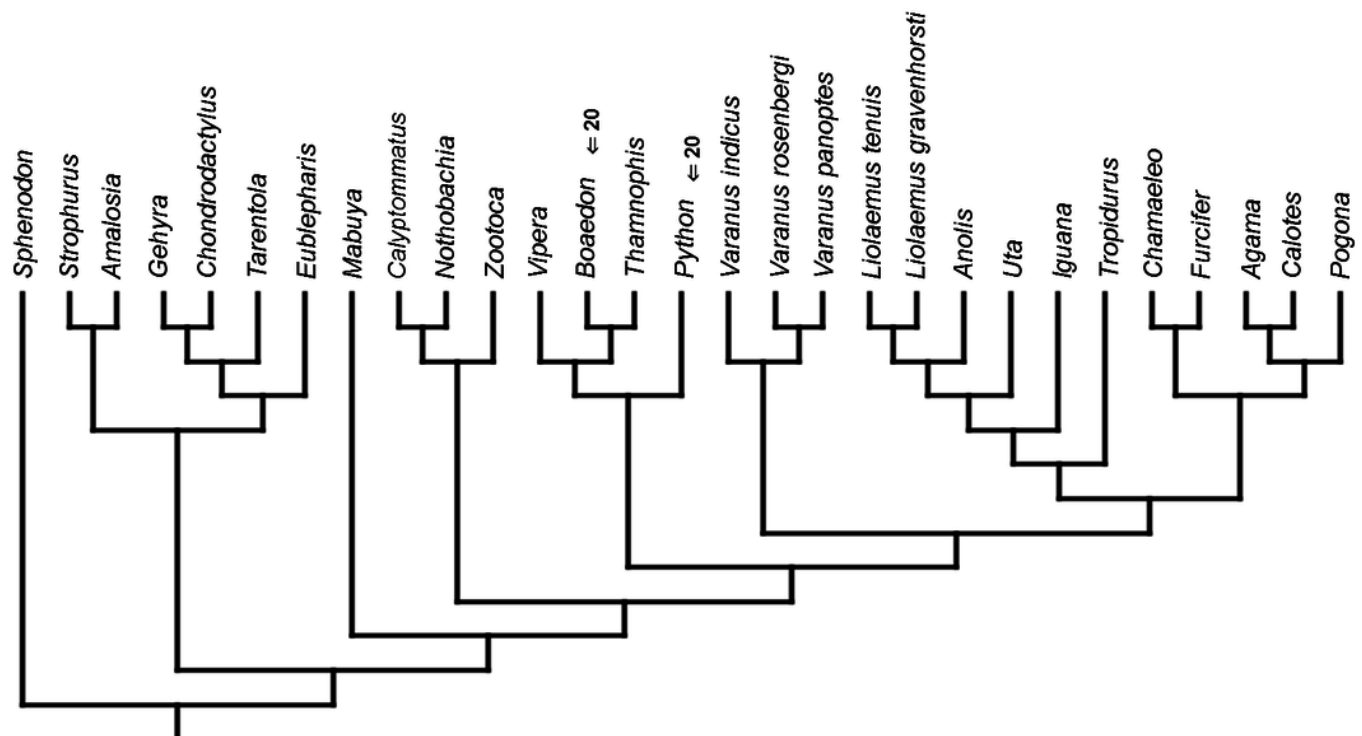
Figure 4

Heterochronic events in lepidosaur evolution.

Mapped onto molecular phylogeny, using continuous data, in relation to the ancestral squamate. Length of all branches equals 1. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
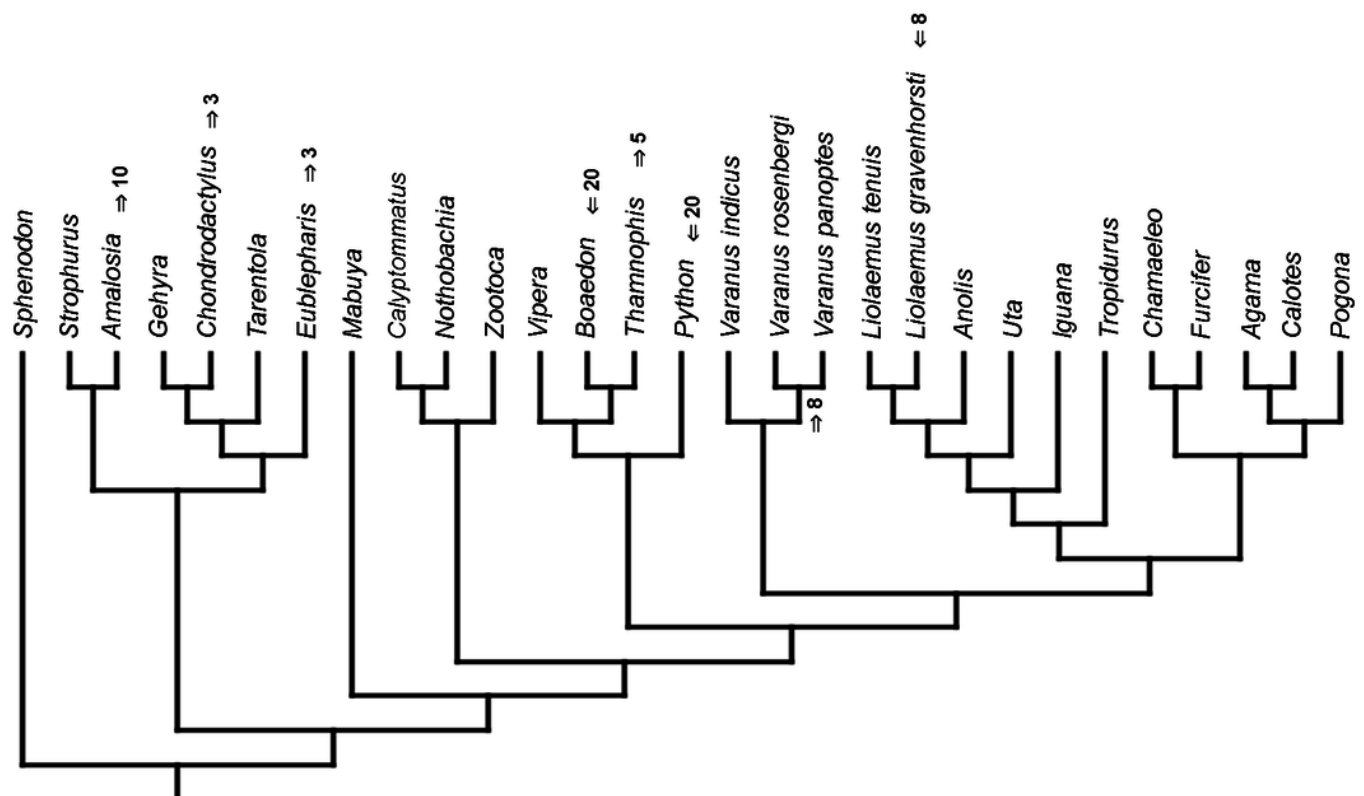
Figure 5

Heterochronic events in lepidosaur evolution.

Mapped onto molecular, stratigraphically calibrated phylogeny, using continuous data, in relation to the ancestral lepidosaur. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.

Figure 6

Heterochronic events in lepidosaur evolution.

Mapped onto molecular, stratigraphically calibrated phylogeny, using continuous data, in relation to the ancestral squamate. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
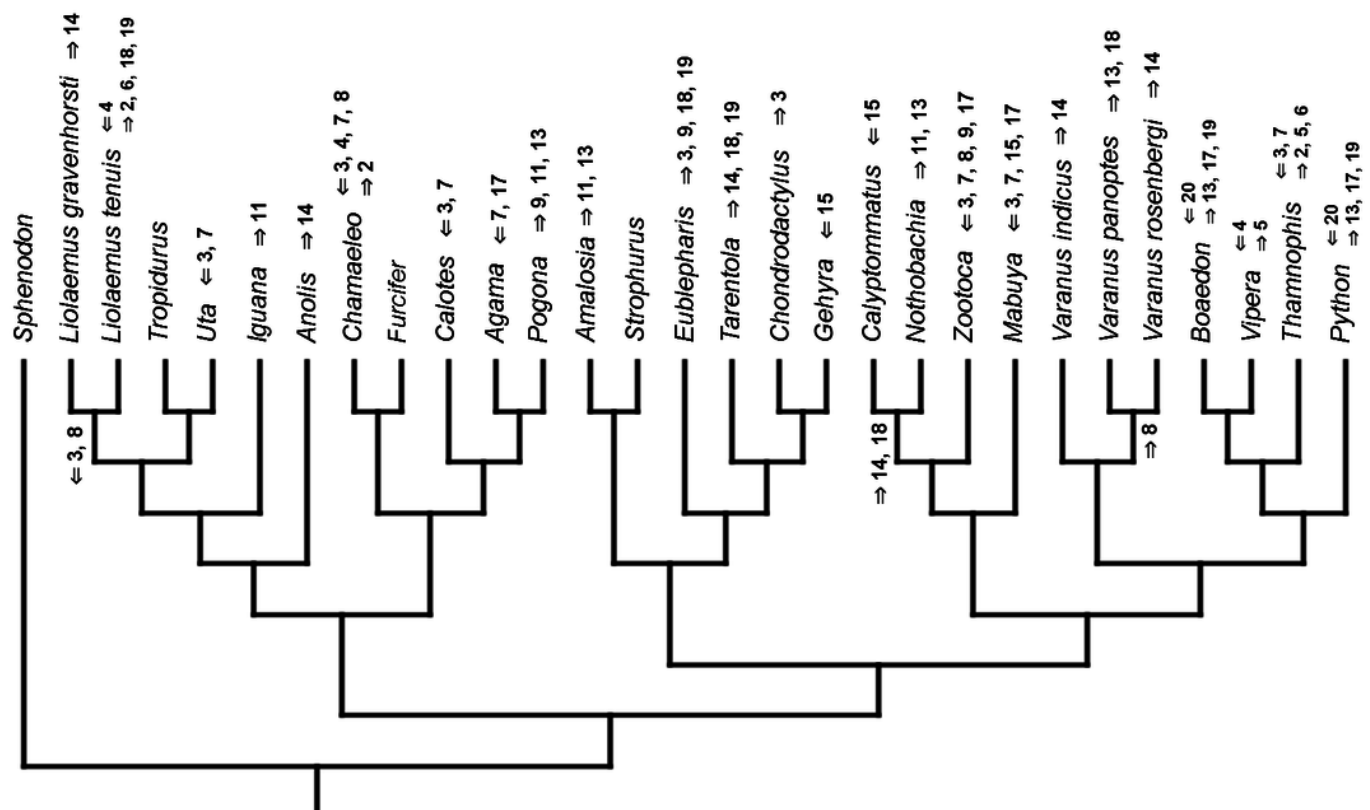
Figure 7

Heterochronic events in lepidosaur evolution.

Mapped onto morphological phylogeny, using continuous data, in relation to the ancestral lepidosaur. Length of all branches equals 1. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
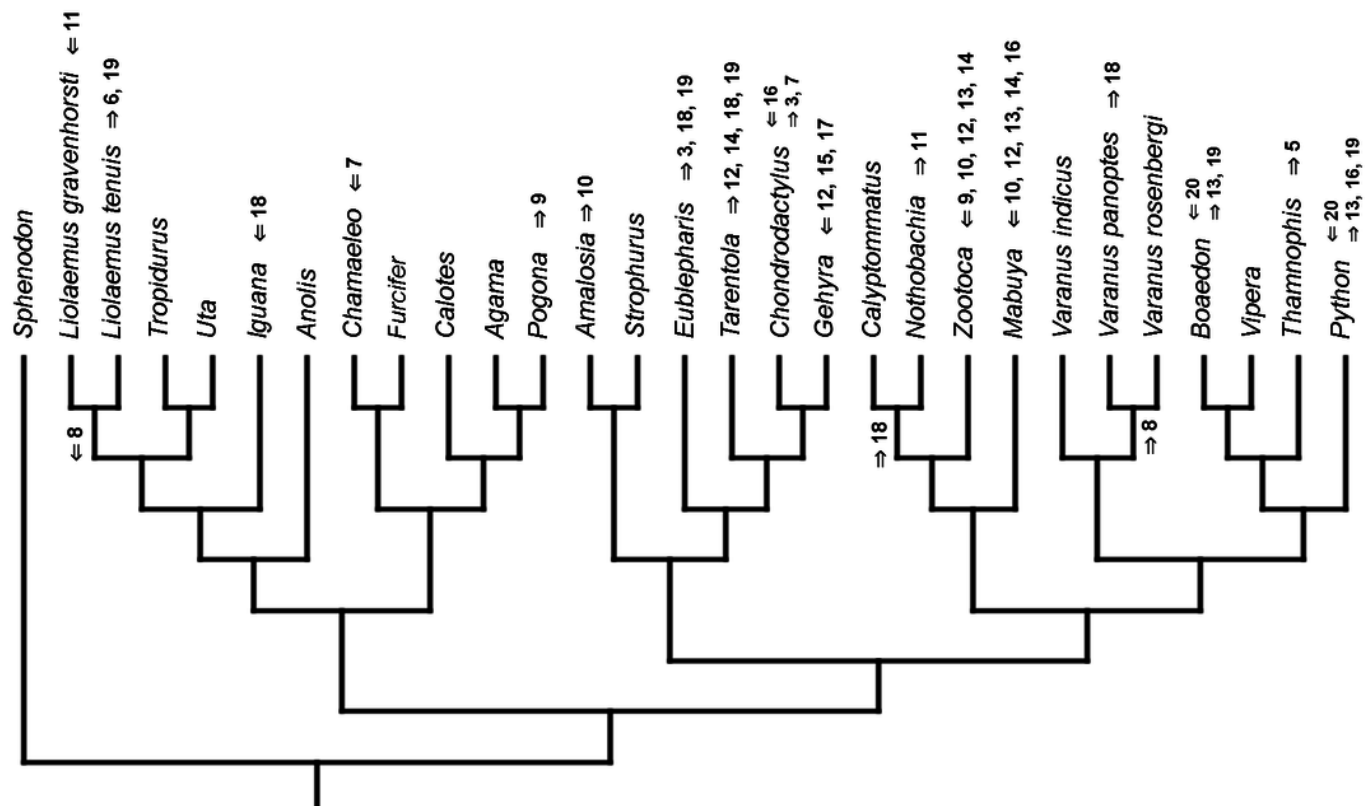
Figure 8

Heterochronic events in lepidosaur evolution.

Mapped onto morphological phylogeny, using continuous data, in relation to the ancestral squamate. Length of all branches equals 1. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.

Figure 9

Heterochronic events in lepidosaur evolution.

Mapped onto morphological, stratigraphically calibrated phylogeny, using continuous data, in relation to the ancestral lepidosaur. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
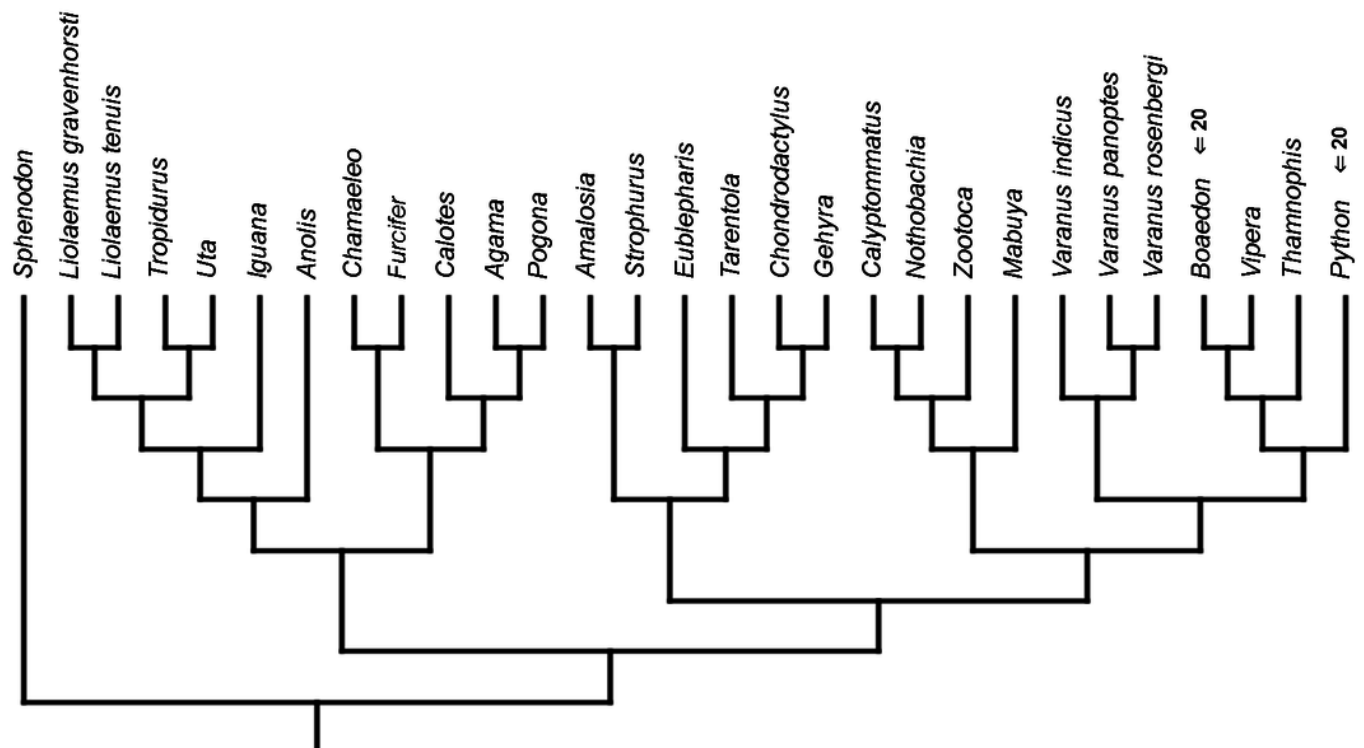
Figure 10

Heterochronic events in lepidosaur evolution.

Mapped onto morphological, stratigraphically calibrated phylogeny, using continuous data, in relation to the ancestral squamate. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
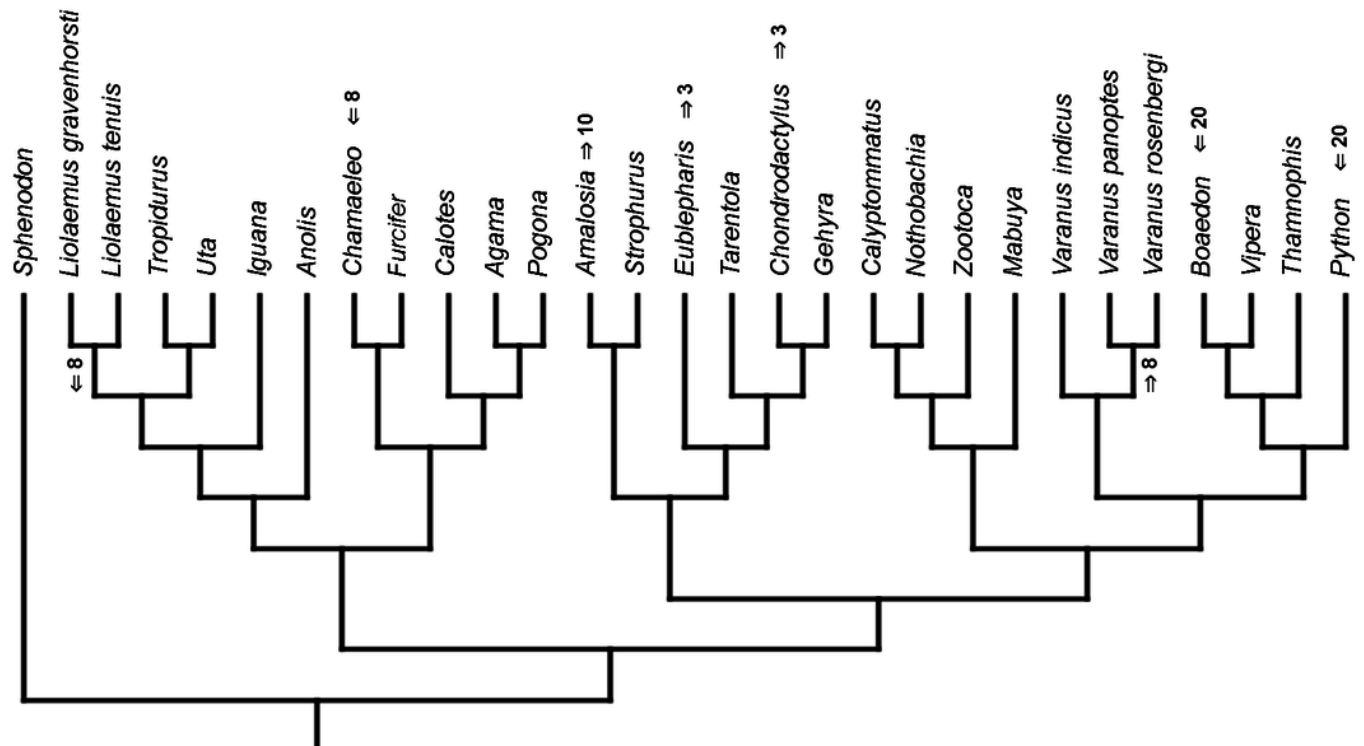
Figure 11

Heterochronic events in lepidosaur evolution.

Mapped onto molecular phylogeny, using unordered event-paired characters. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
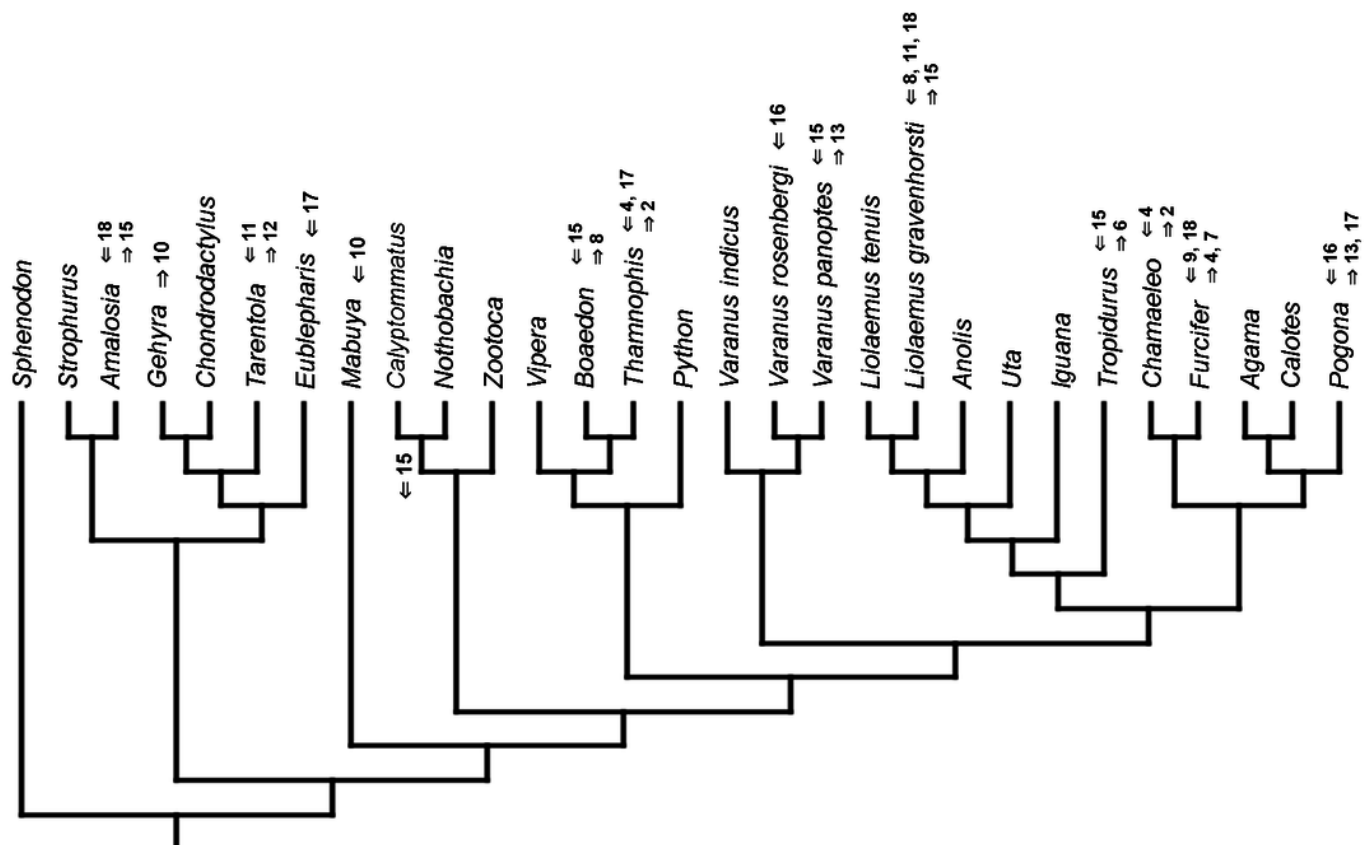
Figure 12

Heterochronic events in lepidosaur evolution.

Mapped onto molecular phylogeny, using ordered event-paired characters. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.

Figure 13

Heterochronic events in lepidosaur evolution.

Mapped onto morphological phylogeny, using unordered event-paired characters. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.

Figure 14

Heterochronic events in lepidosaur evolution.

Mapped onto morphological phylogeny, using ordered event-paired characters. Numbers refer to developmental events (Table 2). Down arrow denotes earlier development of a given structure, while up arrow represents later development.
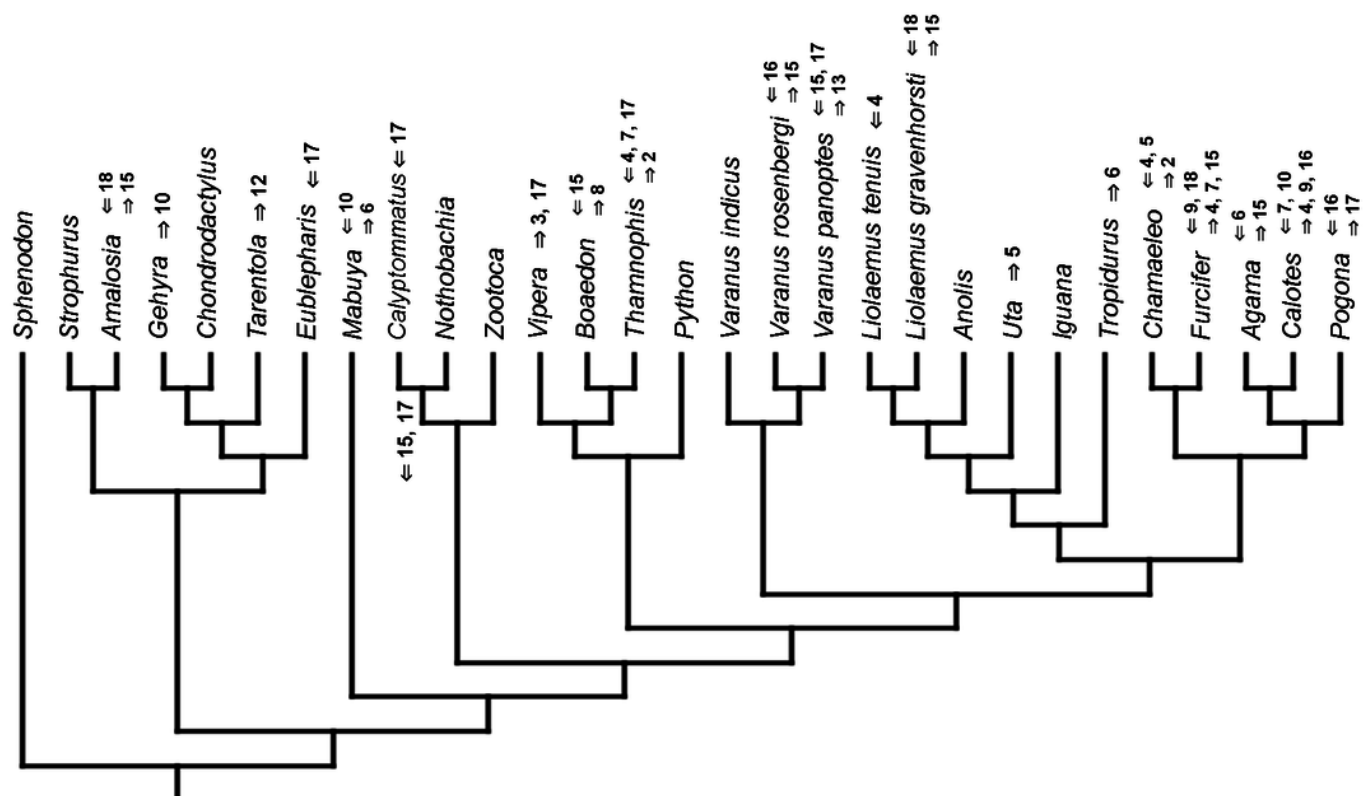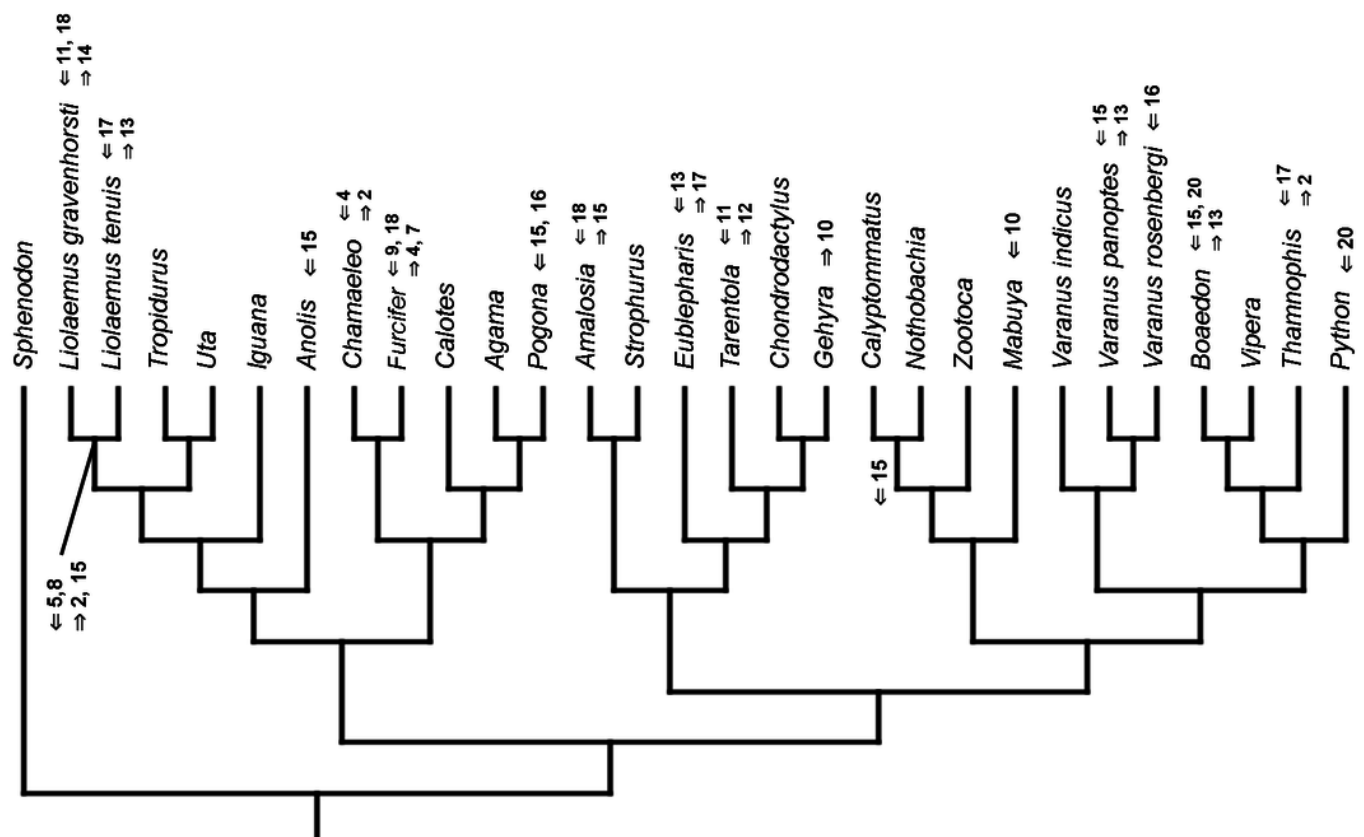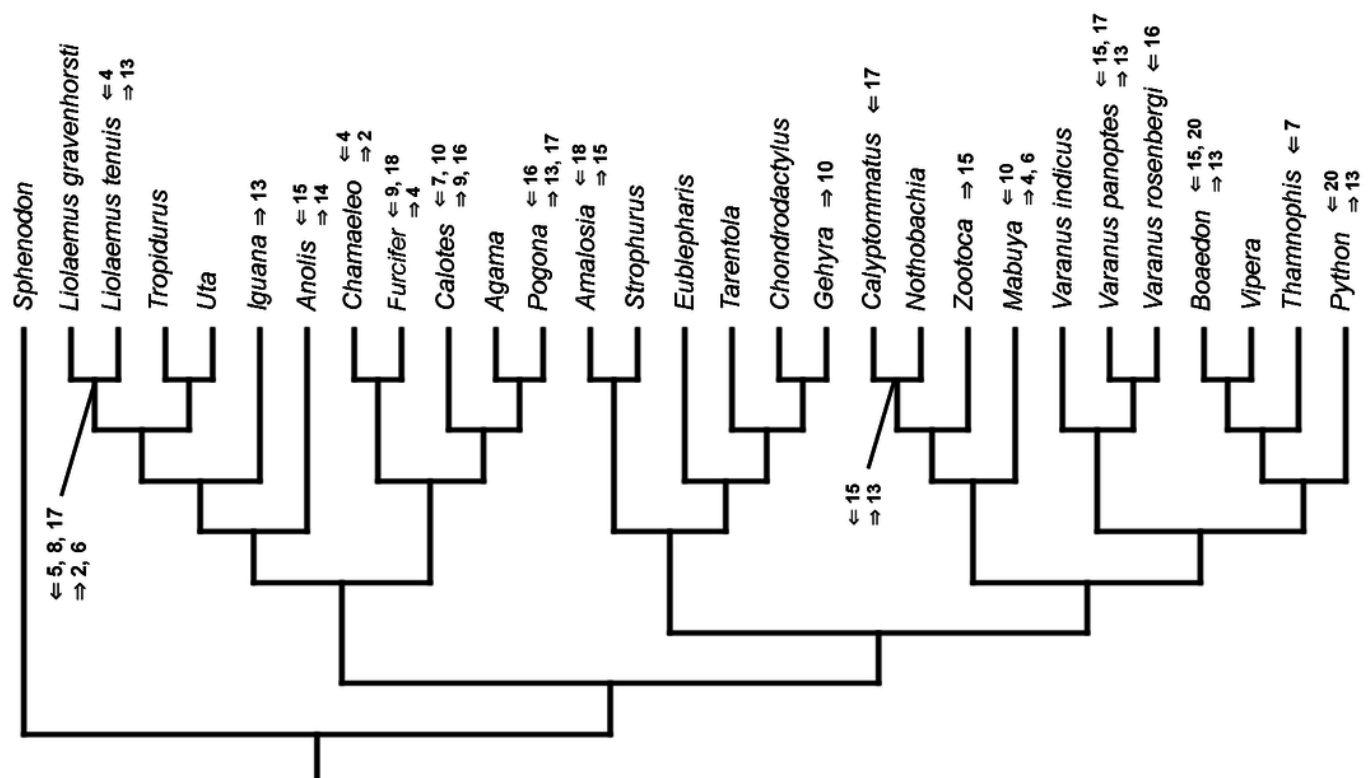
Table 1(on next page)

Species included in this study, their taxonomic position and sources of information on their development.

Species	Higher taxon	Source
Sphenodon punctatus (Gray, 1842)	Rhynchocephalia: Sphenodontidae	Dendy, 1899; Moffat, 1985; Sanger, Gredler & Cohn, 2015
Amalosia lesueurii (Duméril & Bibron, 1836)	Gekkota: Diplodactylidae	Andrews, Brandley & Greene, 2013
Strophurus williamsi (Kluge, 1963)	Gekkota: Diplodactylidae	Andrews, Brandley & Greene, 2013
Eublepharis macularius (Blyth, 1854)	Gekkota: Eublepharidae	Andrews, Brandley & Greene, 2013[1]
Tarentola annularis (Geoffroy Saint-Hilaire, 1827)	Gekkota: Phyllodactylidae	Khannoon, 2015
Chondrodactylus turneri (Gray, 1864)	Gekkota: Gekkonidae	Andrews, Brandley & Greene, 2013
Gehyra variegata (Duméril & Bibron, 1836)	Gekkota: Gekkonidae	Andrews, Brandley & Greene, 2013
Mabuya sp.	Scincoidea: Scincidae	Andrews, Brandley & Greene, 2013
Calyptommatus sinebrachiatus Rodrigues, 1991	Lacertiformes: Gymnophthalmidae	Andrews, Brandley & Greene, 2013
Nothobachia ablephara Rodrigues, 1984	Lacertiformes: Gymnophthalmidae	Andrews, Brandley & Greene, 2013
Zootoca vivipara (Lichtenstein, 1823)	Lacertiformes: Lacertidae	Andrews, Brandley & Greene, 2013
Python sebae (Gmelin, 1789)	Serpentes: Pythonidae	Boughner et al., 2007
Natrix natrix (Linnaeus, 1758)	Serpentes: Colubridae	Rupik, 2002
Thamnophis sirtalis (Linnaeus, 1758)	Serpentes: Colubridae	Andrews et al., 2013

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[1] Wise, Vickaryous & Russell (2009) presented slightly different developmental table for *Eublepharis macularius* but we used data from Andrews, Brandley & Greene (2013), as they span the whole development.

Boaedon fuliginosus (Boie, 1827)	Serpentes: Lamprophiidae	Boback, Dichter & Mistry, 2012
Vipera aspis (Linnaeus, 1758)	Serpentes: Viperidae	Andrews, Brandley & Greene, 2013
Varanus rosenbergi Mertens, 1957	Anguimorpha: Varanidae	Andrews, Brandley & Greene, 2013
Varanus indicus (Daudin, 1802)	Anguimorpha: Varanidae	Gregorovicova et al., 2012
Varanus panoptes Storr, 1980	Anguimorpha: Varanidae	Werneburg, Polachowski & Hutchinson, 2015
Iguana iguana (Linnaeus, 1758)	Iguania: Pleurodonta: Iguanidae	Lima, 2015
Uta stansburiana Baird & Girard, 1852	Iguania: Pleurodonta: Phrynosomatidae	Andrews, Brandley & Greene, 2013
Anolis sagrei Duméril & Bibron, 1837	Iguania: Pleurodonta: Dactyloidae	Andrews, Brandley & Greene, 2013
Liolaemus gravenhorsti (Gray, 1845)	Iguania: Pleurodonta: Liolaemidae	Andrews, Brandley & Greene, 2013
Liolaemus tenuis (Duméril & Bibron, 1837)	Iguania: Pleurodonta: Liolaemidae	Andrews, Brandley & Greene, 2013
Tropidurus torquatus (Wied-Neuwied, 1820)	Iguania: Pleurodonta: Tropiduridae	Py-Daniel et al., 2017
Chamaeleo calyptratus Duméril & Duméril, 1851	Iguania: Acrodonta: Chamaeleonidae	Andrews, Brandley & Greene, 2013
Furcifer lateralis (Gray, 1831)	Iguania: Acrodonta: Chamaeleonidae	Andrews, Brandley & Greene, 2013
Pogona vitticeps (Ahl, 1926)	Iguania: Acrodonta: Agamidae	Andrews, Brandley & Greene, 2013
Calotes versicolor (Daudin, 1802)	Iguania: Acrodonta: Agamidae	Andrews, Brandley & Greene, 2013
Agama impalearis Boettger, 1874	Iguania: Acrodonta: Agamidae	Andrews, Brandley & Greene, 2013

1

Table 2(on next page)

Developmental events used in this study.

From Andrews, Brandley & Greene (2013).

Number	Event
1	Primary optic vesicle
2	Otic placode
3	Allantois bud (small thick-walled out-pouching)
4	Torsion complete
5	Secondary optic vesicle
6	Hyomandibular slit
7	Allantois vesicle (thin-walled bag)
8	Choroid fissure open (horseshoe-shaped)
9	Limb ridge
10	Allantois contacts chorion (allantois flattened above embryo like umbrella)
11	Maximum pharyngeal slits
12	Limb Apical Ectodermal Ridge (AER)
13	Hemipenal buds form on cloacal lip
14	Three-segmented limb (stylo-, zeugo-, autopodium)
15	Jaw initiated
16	Eyelid forms as a thin ribbon-like sheet of tissue overlapping the eyeball
17	Pharyngeal slits closed
18	Digits differentiated in limb paddle
19	Jaw complete; mandible meets tip of maxilla
20	Scale anlagen visible

1

Table 3 (on next page)

Calibration points for the fossil time-calibrated analyses.

See "Material & Methods" for details.

Taxon	Age	References	Notes
Sauria	256 Ma	Ezcurra, Scheyer & Butler, 2014; Ezcurra, 2016	
Rhynchocephalia	238 Ma	Jones et al., 2013	
Iguania	105 Ma (99 + 3 + 3)	Daza et al., 2016	Much older, Jurassic, fossils may represent iguanians (e.g. Evans, Prasad & Manhas, 2002) but their systematic position is ambiguous (e.g. Jones et al., 2013).
Acrodonta	102 Ma (99 + 3)	Daza et al., 2016	
Chamaeleonidae	99 Ma	Daza et al., 2016	
Agamidae	99 Ma	Daza et al., 2016	
Chamaeleo	13 Ma	Bolet & Evans, 2014	
Tropiduridae	ca. 15 Ma	Conrad, Rieppel & Grande, 2007	
Iguanidae	56 Ma	Nydam, 2013	
Anolis	20 Ma	Sheratt et al., 2015	
Gekkota	150 Ma	Gauthier et al., 2012; Caldwell et al., 2015	See also Daza, Bauer & Snively, 2014
Gekkonidae	15 Ma	Daza, Bauer & Snively, 2014	
Diplodactylidae	20 Ma	Daza, Bauer & Snively, 2014	
Serpentes	167 Ma	Caldwell et al., 2015	
Pythonidae	35 Ma	Head, 2015	
Colubridae	31 Ma	Head, Mahlow &	

		Müller, 2016	
Lamprophiidae	17 Ma	Head, Mahlow & Müller, 2016	Based on the elapid *Naja romani* (Head, Mahlow & Müller, 2016).
Viperidae	20 Ma	Head, Mahlow & Müller, 2016	
Anguimorpha	145 Ma	Head, 2015; Caldwell et al., 2015	
Lacertiformes	99 Ma	Daza et al., 2016	
Gymnophthalmidae	66 Ma	Venczel & Codrea, 2016	Gymnophthalmid fossils are currently unknown (Nydam & Caldwell, 2015) but teiids are universally accepted as gymnophthalmid sister group, so the oldest known teiid is used to provide a calibration point for gymnophthalmids in the analyses.
Scincoidea	150 Ma	Evans & Chure, 1998; Gauthier et al., 2012	See also Conrad, 2008 and Tałanda, 2016 – regardless of that, the oldest known scincoids seem to be Late Jurassic in age.

1

Table 4(on next page)

Event-paired developmental synapomorphies of higher-level squamate clades.

Asterisk denotes synapomorphies present only in analysis using ordered characters, while plus denotes synapomorphies present only in analysis employing unordered characters.

a) Molecular phylogeny	
Clade	**Synapomorphies**
Gekkota except Diplodactylidae	(1) pharyngeal slits closed simultaneous with three-segmented limb*
Unidentata	(1) secondary optic vesicle simultaneous with allantois bud, (2) hyomandibular slit not earlier than allantois bud
Scincoidea (*Mabuya*)	(1) hyomandibular slit later than secondary optic vesicle, (2) allantois vesicle earlier than torsion completion, (3) allantois contacts chorion simultaneous with torsion completion, (4) allantois contacts chorion simultaneous with hyomandibular slit, (5) allantois contacts chorion earlier than choroid fissure open, (6) allantois contacts chorion earlier than limb ridge*, (7) pharyngeal slits closed later than eyelid forms as a thin ribbon-like sheet of tissue overlapping the eyeball*
Gymnophthalmidae	(1) jaw initiated simultaneous with maximum pharyngeal slits, (2) jaw initiated earlier than hemipenal buds form on cloacal lips, (3) pharyngeal slits closed simultaneous with hemipenal buds form on cloacal lips*, (4) pharyngeal slits closed simultaneous with three-segmented limb*, (5) jaw completion simultaneous with digits differentiated in the limb paddle
Toxicofera	(1) secondary optic vesicle later than allantois bud, (2) allantois vesicle simultaneous with secondary optic vesicle*
Serpentes	(1) pharyngeal slits closed no later than hemipenal buds form on cloacal lips*, (2) pharyngeal slits closed earlier than eyelid form as thin ribbon-like sheet of tissue*
Thamnophis + Vipera	(1) jaw initiated later than hemipenal buds form on cloacal lips*, (2) eyelid form as thin ribbon-like sheet of tissue simultaneous with jaw initiated

Varanus rosenbergi + V. panoptes	(1) pharyngeal slits closed simultaneous with three-segmented limb
Iguania	(1) limb ridge later than choroid fissure open*
Acrodonta	(1) allantois vesicle simultaneous with torsion completion+
Chamaeleonidae	(1) allantois contacts chorion later than limb ridge*
Agama + Calotes	(1) jaw initiated later than hemipenal buds form on cloacal lips*, (2) jaw initiated later than three-segmented limb*, (3) pharyngeal slits closed simultaneous with jaw initiated*
Pleurodonta excluding *Tropidurus*	(1) pharyngeal slits closed simultaneous with three-segmented limb*
Liolaemus	(1) jaw initiated simultaneous with three-segmented limb+, (2) pharyngeal slits closed earlier than jaw initiated

b) Morphological phylogeny

Clade	**Synapomorphies**
Iguania	(1) hyomandibular slit later than allantois bud*
Pleurodonta	(1) pharyngeal slits closed simultaneous with three-segmented limb*, (2) pharyngeal slits closed earlier than eyelid forms as thin ribbon-like sheet of tissue*
Liolaemus	(1) allantois bud earlier than otic placode, (2) secondary optic vesicle earlier than otic placode, (3) secondary optic vesicle simultaneous with allantois bud+, (4) hyomandibular slit simultaneous with torsion completion*, (5) hyomandibular slit later than secondary optic vesicle*, (6) choroid fissure open simultaneous with otic placode, (7) choroid fissure open earlier than allantois vesicle, (8) jaw initiated simultaneous with three-segmented limb+, (9) pharyngeal slits closed earlier than three-segmented limb*, (10)

	pharyngeal slits closed earlier than jaw initiated
Acrodonta	(1) allantois vesicle simultaneous with torsion completion+
Agama + Pogona	(1) eyelid forms as thin ribbon-like sheet of tissue simultaneous with jaw initiated*
Chamaeleonidae	(1) allantois contacts chorion later than limb ridge*
Scleroglossa	(1) torsion completion simultaneous with allantois bud*, (2) hyomandibular slit simultaneous with torsion completion
Gekkota except Diplodactylidae	(1) pharyngeal slits closed simultaneous with three-segmented limb*
Varanus rosenbergi + V. panoptes	(1) pharyngeal slits closed simultaneous with three-segmented limb
Serpentes	(1) pharyngeal slits closed earlier than eyelid forms as a thin ribbon-like sheet of tissue*
Scincomorpha	(1) allantois bud simultaneous with otic placode+, (2) secondary optic vesicle simultaneous with otic placode
Scincoidea (*Mabuya*)	(1) torsion completion later than allantois bud*, (2) secondary optic vesicle earlier than torsion completion*, (3) hyomandibular slit later than allantois bud*, (4) hyomandibular slit later than secondary optic vesicle*, (5) allantois vesicle earlier than torsion completion, (6) allantois vesicle earlier than hyomandibular slit, (7) allantois contacts chorion simultaneous with torsion completion, (8) allantois contacts chorion simultaneous with hyomandibular slit, (9) allantois contacts chorion earlier than choroid fissure open, (10) allantois contacts chorion earlier than limb ridge, (11) jaw initiated simultaneous with three-segmented limb+
Gymnophthalmidae	(1) jaw initiated simultaneous with maximum pharyngeal slits, (2) jaw initiated earlier than

	hemipenal buds form on cloacal lips, (3) jaw initiated earlier than three-segmented limb*, (4) pharyngeal slits closed simultaneous with hemipenal buds form on cloacal lips*, (5) pharyngeal slits closed simultaneous with three-segmented limb*, (6) jaw completion simultaneous with digits differentiated in limb paddle

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