

## The New Mode of Thought of Vertebrates' Evolution

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### Abstract

Molecular phylogeny of the reptiles does not accept the basal split of squamates into Iguania and Scleroglossa that is in conflict with morphological evidence. The classical phylogeny of living reptiles places turtles at the base of the tree. Analyses of mitochondrial DNA and nuclear genes join crocodylians with turtles and places squamates at the base of the tree. Alignment of the reptiles' ITS2s with the ITS2 of chordates has shown a high extent of their similarity in ancient conservative regions with Cephalochordate *Branchiostoma floridae*, and a less extent of similarity with two Tunicata, *Saussurea tunicate*, and *Rinodina tunicate*. We have performed also an alignment of ITS2 segments between the two break points coming into play in 5.8S rRNA maturation of *Branchiostoma floridae* in pairs with orthologs from different vertebrates where it was possible. A similarity for most taxons fluctuates between about 50 and 70%. This molecular analysis coupled with analysis of phylogenetic trees constructed on a basis of manual alignment, allows us to hypothesize that primitive chordates being the nearest relatives of simplest vertebrates represent the real base of the vertebrate phylogenetic tree.

**Keywords:** Vertebrates; Reptiles; Chordates; Evolutionary relations; Base of the phylogenetic trees

### Introduction

The standard view has been that lizard species split at the base of the phylogenetic tree into Iguania (iguanas, chameleons, and relatives) and Scleroglossa (all remaining lizards, including geckos, skinks, monitors, and snakes). In the past decade, molecular phylogenetic's analyses, have strongly contradicted this view [1]. They concluded that iguanians evolved more recently, locate in the lizard tree close to monitors and Anguimorpha and snakes, and that their supposedly ancestral characteristics arose as the result of re-evolution. Recently obtained morphological data set, analyzed with state-of-the-art phylogenetic methods, has not resolved contradictions between morphological and DNA-based studies [2]. So, we have a conundrum here. The molecular data suggest very limited knowledge of the functional link between structures and lifestyle. Conversely, morphology implies a pattern of molecular evolution that has yet to be explained.

Recently, we published the paper in which two different types of phylogenetic trees were constructed on the basis of the ITS2s primary structures alignment for almost 60 reptiles belonging to different orders, and some other animals [3]. All these ITS2s differ significantly by sizes, and primary structures of functionally neutral regions. So, we used (i) GeneBee Services program [4,5] which had made it possible to carry out synchronous total automated alignment of the group of sequences of any sizes with subsequent construction of the unrooted trees based on this alignment. (ii) The other phylogenetic tree was constructed on the basis of manual alignment with the help of the Bayesian inference method (the MrBayes program) [3].

In both cases the trees were generated by separate clades of Iguania (Iguanidae, Agamidae, Chameleonidae), suborder clades of Crocodylians+Testudines, and Snakes+Lacertidae lizards+Anguimorph lizards. The intermediate clades are formed by Scleroglossa (all remaining lizards, including geckos, and skinks). Analysis of ITS2-based phylogenetic trees coupled with molecular analysis, allows us to hypothesize that chordate *Branchiostoma floridae*, *Saussurea tunicate*, and *Rinodina tunicate* being the nearest relatives to vertebrates can be placed near the base of their total phylogenetic tree.

Phylogeny of the reptiles differs significantly in works of various authors. Morphological classification of Squamata lets to make a proposal about a split of Iguania and Scleroglossa in the late Triassic period [6], and places snakes close to the other limbless forms, Dibamidae and Amphisbaenia. In some other, more recent papers, snakes were considered as the sister taxon of varanids or placed into

the Anguimorpha clade, thereby controverting their separation as an individual clade from other Squamata [7-9].

Molecular phylogeny does not accept the basal split of squamates into Iguania and Scleroglossa that is in conflict with morphological evidence [10-13]. Phylogenetic analyses based on the molecular data from a number of nuclear protein coding genes places snakes to the same clade as lacertids and amphisbaenids [14]. Furthermore, recently snakes, anguimorphs, and iguanians were combined in a clade Toxicofera based on a presence of toxin secreting oral glands in their organisms [15]. The position of turtles among amniotes remains in dispute, with morphological and molecular comparisons giving different results [16]. The classical phylogeny of living reptiles places turtles at the base of the tree. Analyses of mitochondrial DNA and 22 nuclear genes join crocodylians with turtles and places squamates at the base of the tree [13,17,18].

Our phylogenetic trees constructed on the basis of the ITS2s primary structural alignments revealed a split between Iguania clade and Scleroglossa that is in agreement with morphological classification. True lizards and snakes showed sister relationships, as well as the two other reptilian orders, Crocodylia+Aves, and Testudines. In summary, our phylogenetic trees exhibit specific features deduced or, to the contrary, rejected earlier by other authors. We hypothesize that simplest organisms at the base of the vertebrates' phylogenetic tree were among simplest chordates.

### Results and Discussion

The ITS2 of *Branchiostoma floridae* contains four conservative segments (consensus sequences) similar to those found in all known vertebrates [19-21]. The very first 12 nucleotides of the ITS2 (consensus 'a') represent a *cis*-element for the U3 small nucleolar RNA. Five nucleotides at the 5'-end often incorporate taxon specific substitutions, whereas seven bases at the 3'-end form extremely conservative nucleotide block. The second consensus ('b') is the most conservative one, but its functions are unknown until now. The consensuses 'c' and

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'd' contain the regions providing the ITS2 specific cleavages for the 8S and 12S pre-rRNA forming during 5.8S rRNA maturation Table 1.

It is known that at least two conservative break points provide maturation of 5.8S rRNA. Their positions were detected experimentally for *X.laevis* 8S pre-rRNA as (C) 3-5 (N)1-3 AAG(N)3-4A^GA [19,20], and CGGCTGTC^TGTGGA for 12S pre-rRNA [21]. The corresponding sequence for Muridae is different (CGTCCG^TGCGCCGA) [21]. These sequences are present practically in all analyzed vertebrates' ITS2s, and contain very rare substitutions [3].

The consensus 'a' of *Branchiostoma floridae* ITS2 contains at its 3'-

end the same 5'-TCAATCG-3' sequence as all known vertebrates with the exception of a G→A substitution in one lizard, and one crocodile. At the same time Tunicata *Ciona intestinalis* contains at the 3'-end of the consensus 'a' three substitutions, and two deletions, and *Rinudina tunicata* has two orthologic substitutions, and one rare insertion upstream 5'-TCAATCG-3' sequence similar to those in *Gallus gallus* (Table 1). The consensus 'b' is practically identical in all vertebrates (5'-CGCGGCTGGGG-3') including *Branchiostoma floridae*. However, in *Ciona intestinalis*, it involves three substitutions and one deletion, whereas in *Rinudina tunicata* consensus 'b' has only one substitution. The consensus 'c' and 'd' are more variable. In the consensus 'c' 5'-

Species	a	b	c	d	
Callorhynchus_milli	--C-GA—TCAATCG	CGCGGTTGGGG	GCCCCAAGTGCAGA	CGT-CTG-CGGTTGT	Fishes
Alopias_pelagicus	-AC-GA—TCAATCG	CGCGGCTGGGG	CCCCCTAAATGCAGA	CGG-CTG-TCATCGG	
Centroscyllium_fabricii	-AA-AA—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTGCAGA	CGG-CTG-TCGTTGG	
Tor_khudree	CT--A—TCGATCG	CGCGGTTGGGG	CCTCCTAAGTGCAGA	CGG-CTG-CCGGTGG	Amphibia
Xenopus_laevis	GAC-G---TCCATCG	CGCGGCTGGG-	CCCCCAAGGCCAGA	CGG-CTG-TCTGTGG	
Xenopus_borealis	GAC-G---TCCATCG	CGCGGCTGGGG	CCCCCAAGGCCAGA	CGG-CCG-TCTGTGG	
<b>Branchiostoma floridae</b>	-TT-CA—TCAATCG	CGCGGCTGGGG	CCCCGAAGGCAGG	CGGCCTG-GCCATC	<b>Species under study</b>
<b>Ciona intestinalis</b>	GTG-AA--TTG--CA	TGCGGCCTCGG	CCGCGAAAATCAGT	CGGCTG-CAGAGCC	
Rinudina_tunicata	CAC--CCCTCAAGCA	CGCGGCGGGGG	TA-GC-AAGATTCT	AGC-CTC-TCGCGGC	Iguania
Iguana_iguana	GAC-GG—TCAATCG	CGCGGCTGGGG	CCCCGAAGCGCAGA	CGG-CTG-TCTGTGG	
Uta_stansburiana	GAC-GG—TCAATCG	CGCGGCTGGGG	CCACCTAAGCGAAGA	CGG-CTG-TCTGTGG	
Anolis_carolinensis	GAC-GG—TCAATCG	CGCGGCTGGGG	CCACCTAAGCGAAGA	CGG-CTG-TCTGTGG	Agamidae
Basiliscus_plumifrons	GAC-GG—TCAATCG	CGCGGCTGGGG	CCCCGAAGCGCAGA	CGG-CTG-TCTGTGG	
Laudakia_caucasia	GAC-GG—TCAATCG	CGCGGCTGGGG	CCCCGAAGTCCAGA	CGG-CTG-TCTGTGG	Chameleonidae
Chamaeleo_chamaeleon	GAC-GG—TCAATCG	CGCGGCTGGGG	CTCCCAAAGTCTAGA	CGG-CTG-TCCGTGG	
Calumma_parsonii	GAC-GG—TCAATCG	CGCGGCTGGGG	CTCCCAAAGTCTAGA	CGG-CTG-TCTGCGG	Gekkonidae
Eublepharis_macularius	CAG-CA—TCAATCG	CGCGGCTGGGG	CCCCCAAGGCAGA	CGG-CTG-TCTGTGG	
Hemitheconyx_caudicinctus	CAG-CA—TCAATCG	CGCGGCTGGGG	CCCCCAAGGCAGA	CGG-CTG-TCTGTGG	Scincidae
Eulamprus_murrayi	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCAAGGCCAGA	CGG-CTG-TCCGTGG	
Chalcides_ocellatus	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCC--AAGGCCAGA	CGG-CTG-TCCGTGG	
Tiliqua_scincoidea	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCAAGGCCAGA	CGG-CTG-TCCGTGG	Anguimorpha
Varanus_exanthematicus	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCAAGGCCAGA	CGG-CTG-TCTGTGG	
Varanus_prasinus	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCACAAGCGCAGA	CGG-CTG-TCTGTGG	
Dibamus_dehavengeri	ATTCGT—TCAATCG	CGCGGCTGGGG	CCCCCTAAGGTGAGA	CGG-CTG-TCTGTGG	Lizards
Anguis_fragilis	TAACGG—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTCCAGA	CGG-CTG-TCTGTGG	
Gallotia_galloti	GAA-GG—TCAATCA	CGCGGCTGGGG	CCCCCAAGTCCAGA	CGG-CTG-TCTGTGG	
Darevskia_armeniaca	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCAAGTCCAGA	CGG-CTG-TCTGTGG	Lizards
Darevskia_rostombekovi	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCAAGTCCAGA	CGG-CTG-TCTGTGG	
Lacerta_media	GAA-GG—TCAATCG	CGCAGCTGGGGG	CCCCCGAGTGCAGA	CGG-CTG-TCTGTGG	
Lacerta_agilis	GAA-GG—TCAATCG	CGCAGCTGGGGG	CCCCCGAGTGCAGA	CGG-CTG-TCTGTGG	Snakes
Natrix_natrix	GAC-GGATTCAATCG	CGCGGCTGGGG	CCCCCAAGTCCAGA	CGG-CTG-TCTGCGG	
Boa_constrictor	GAA-GG-TTCAATCG	CGCGGCTGGGG	CCTCCCAAGTCCAGA	CGG-CTG-TCTTCCG	
Naja_kaouthia	-AC-GG-TTCAATCG	CGCGGATGGGG	CCCCCAAGTCCAGA	CGG-CTG-TCTGCGG	Snakes
Vipera_renardi	GAC-GGATTCAATCG	CGCGGCTGGGG	CCCCCAAGTCCAGA	CGG-C-G-TCTGCGG	
Psammophis_lineolatus	--C-GGATTCAATCG	CGCGGCTGGGG	CCCCCAAGTCCAGA	CGG-CTG-TCTGCGG	Crocodylomorpha
Crocodylus_niloticus	GAC-GA—TCAATCG	TGCAGTAGGG	CCCCCTAAGGTGAGA	CGG-CTG-TCGGTGG	
Crocodylus_siamensis	GAA-GG—TCAATCA	TGCAGTAGGG	CCCCCTAAGGTGAGA	CGG-CTG-TCTCAGG	
Taeniopygia_guttata	GAC-GA—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTGCAGA	CGG-CTG-TCTGCGG	Birds
Gallus_gallus	---TGCCATCAATCG	CGCGGCTGGGG	CCCCCTAAGTGCAGA	CGGGCTG-CGGTGG	
Testudina_graeca	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTCCAGA	TGG-CTG-TCTGTGG	Testudines
Chrysemys_picta	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTCCAGA	CGG-CTG-TCTGTGG	
Emys_orbicularis	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTCCAGA	CGG-CTG-TCTGTGG	
Malacochersug_tornieri	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTCCAGA	GGG-CTG-TCTGTGG	
Platemys_platycephala	GAA-GG—TCAATCG	CGCAGCTGGGG	CCCCCTAAGTCCAGA	CGG-CTG-TCTGCGG	
Heospines_spinosa	GAA-GG—TCAATCG	CGCGGCTGGGG	CCCCCTAAGTCCAGA	CGT-CTG-TCTCCCT	

Table 1: ITS2 sequences.

<i>Callorhynchus milii</i>	55.2%	<i>Varanus exanematicus</i>	62.6%
<i>Tor khudree</i>	56.9%	<i>Varanus prasinus</i>	60.9%
<i>Xenopus laevis</i>	48.8%	<i>Dibamus dehavengeri</i>	61.2%
<i>Xenopus borealis</i>	47.6%	<i>Anguis fragilis</i>	61.7%
<i>Iguana iguana</i>	57.7%	<i>Darevskia rostombekovi</i>	68.3%
<i>Uta stansburiana</i>	53.6%	<i>Lacerta agilis</i>	62.6%
<i>Laudakia caucasia</i>	51.2%	<i>Boa constrictor</i>	65.0%
<i>Pogona vitticeps</i>	52.0%	<i>Naja kaouthia</i>	65.0%
<i>Chamaeleo chamaeleon</i>	56.1%	<i>Malpolon monspessulanum</i>	65.8%
<i>Calumma parsonii</i>	55.2%	<i>Crocodylus niloticus</i>	69.1%
<i>Eulamprus murrayi</i>	65.8%	<i>Crocodylus porosus</i>	65.8%
<i>Chalcides ocellatus</i>	55.2%	<i>Testudina graeca</i>	58.5%
<i>Eublepharis macularius</i>	65.8%	<i>Chrysemys picta</i>	53.6%
<i>Hemiteconyx caudicinctus</i>	67.4%		

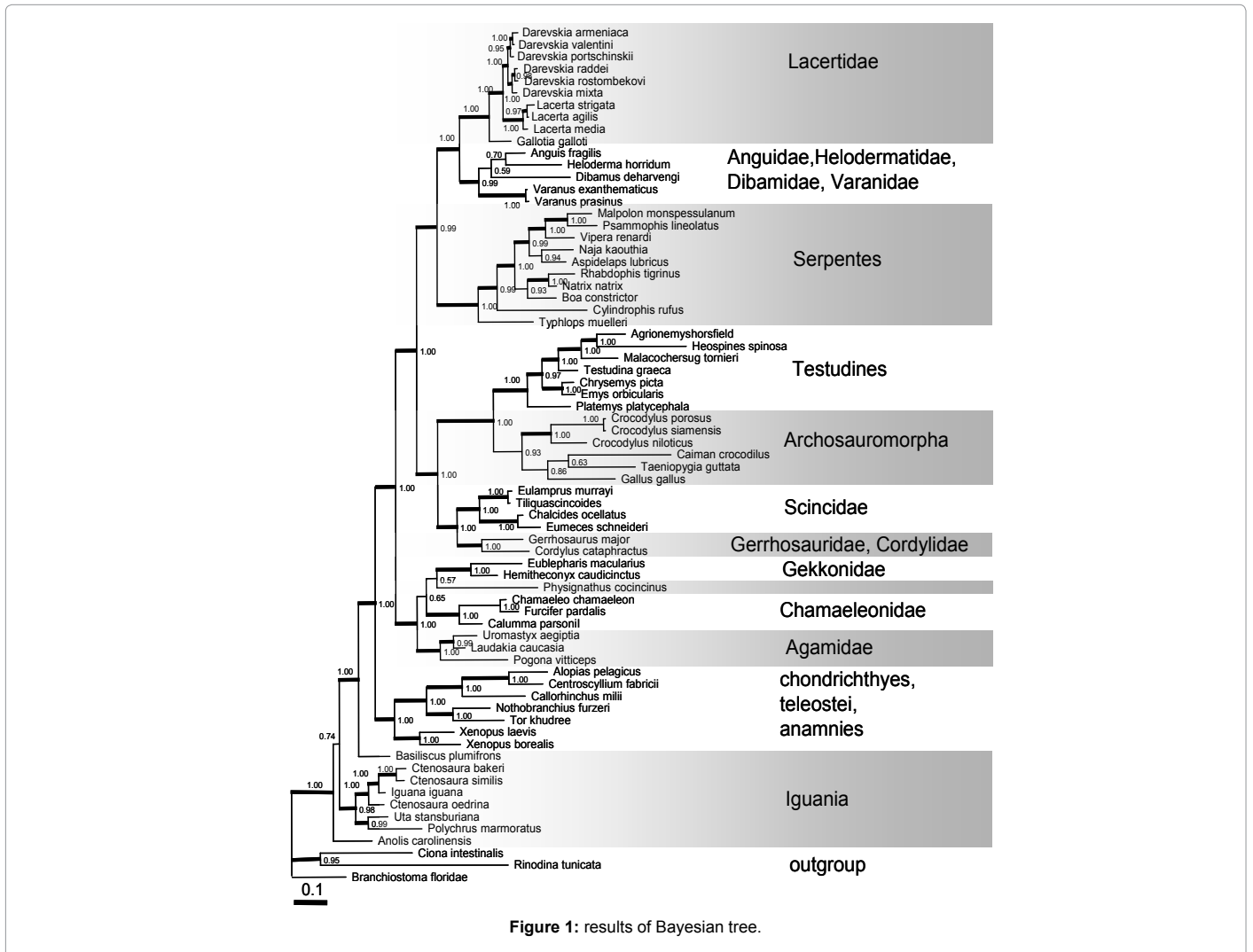
An extent of similarity between the ITS2 regions of *Branchiostoma floridae* including the break points of 5.8S rRNA maturation in pairs with orthologs from a number of different vertebrates taxons (%).

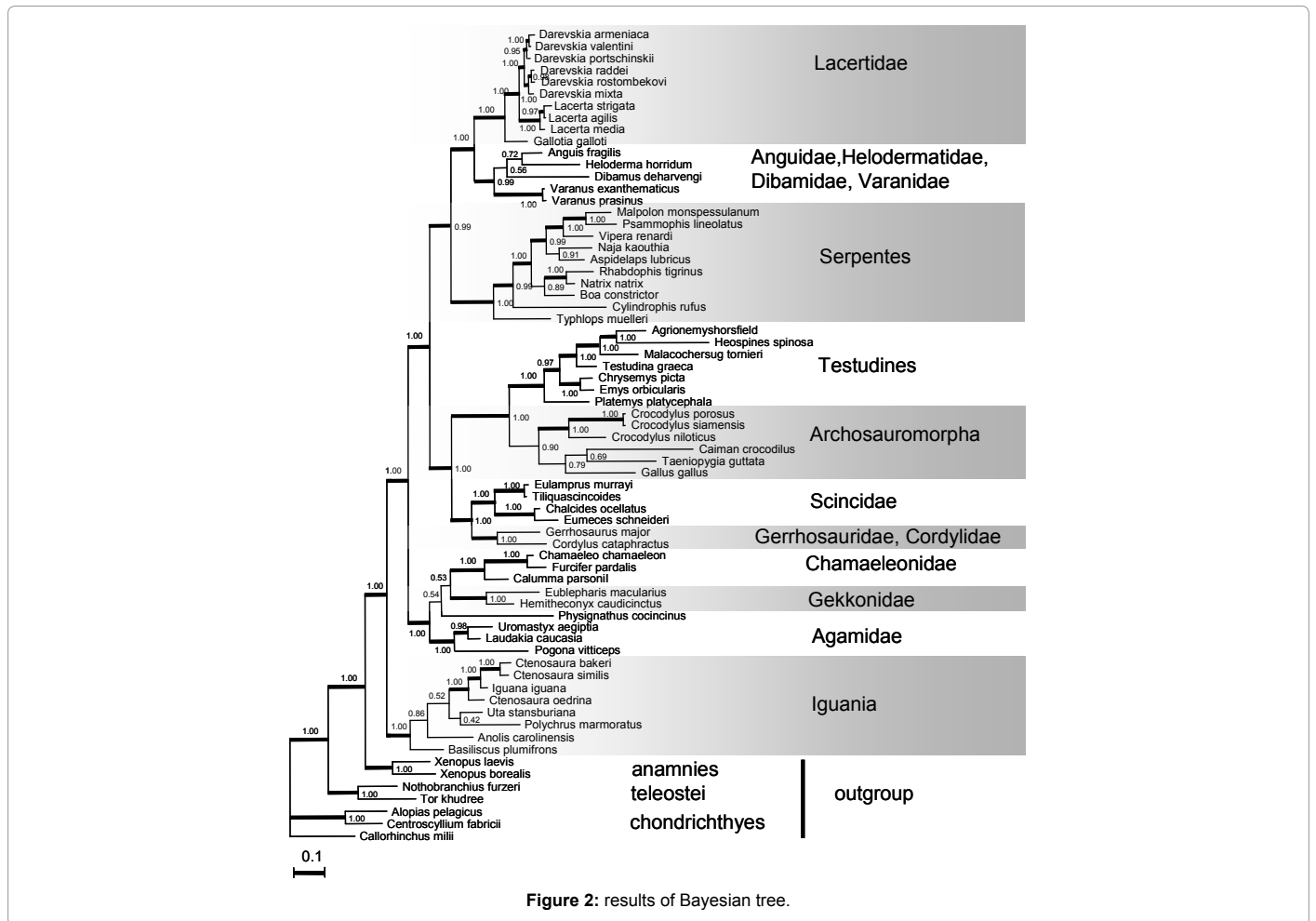
**Table 2:** An extent of similarity between the ITS2 regions of *Branchiostoma floridae* including the break points of 5.8S rRNA maturation in pairs with orthologs from a number of different vertebrates taxons (%).

poly C, central AAG, and 3'-AGA regions can be marked as the most constant, and, possibly, more important ones for 5.8S rRNA maturation, although these elements contain 2-6 substitutions in *Branchiostoma floridae*, and both Tunicata. In the consensus 'd' the most constant role can possibly play 5'-CGG-CTG-TCT-3' elements. It is necessary to note that consensus'd' is especially variable in Cephalochordata and Tunicata, where indicated constant element is practically absent Table 1.

Furthermore, we have performed an alignment of the ITS2 variable segment localized between 'c' and 'd', namely between the two break points coming into play in 5.8S rRNA maturation for *Branchiostoma floridae*, in pairs with its orthologs from different vertebrate classes (Supplement 1). For most taxons similarity is fixed at the level of about 50-70% Table 2. Corresponding part of the ITS2s of the two Tunicata reveals substantially less similarity with the region implicated in 5.8S rRNA maturation in vertebrates, and its position can be only roughly estimated (Table 1).

We have obtained unexpected result on comparison of the two Bayesian trees differing only by presence or absence of Cephalochordata and Tunicata ITS2 among sequences implicated in the Bayesian trees construction Figures 1 and 2. We see that clade Iguanidae changes its position on phylogenetic tree becoming tightly coupled with Cephalochordata, and Tunicata. Furthermore, fishes, and amphibians





occupy a middle position between Iguanidae and Acrodonts. The upper part of the tree stays unchanged in these conditions. It is difficult to explain this phenomenon with an exception of the only proposal that primary structures of Iguanidae, Cephalochordata, and Tunicata ITS2s are most similar to each other.

So, we hypothesize that “the base of the tree” is possibly misused termini, but all vertebrates’ classes started their evolution almost simultaneously from a group of primitive chordates. They could probably form hybrids in the beginning of radiation, but rather soon they have formed individual branches. A time of formation of discrete branches, and rate of evolution in different taxons could be significantly different. Higher rates of molecular evolution in iguanians and snakes [22] for an example suggest that the genes in these taxa are not evolving like those in other lizard lineages,

In a recent paper a phylogenomic dataset based on 248 nuclear genes for 16 vertebrate taxa including turtles, caimans, lizards, and a lungfish were obtained to resolve the origins of turtles. Maximum likelihood and Bayesian concatenation analyses and species tree approaches unambiguously support turtles as a sister group to birds and crocodiles [15]. It is intriguing that conclusions inferred from analysis of all our trees are in a full agreement with the conclusions made in this paper. Besides of it our results show that a branch leading to mammals’ line starts among turtles in spite of their vigorous subsequent diversification. It is not appropriate now to discuss this result without of supplementary data.

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