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Sequence variation in mitochondrial Cytochrome b gene in *Boa constrictor* across its range with phylogeographical and ecological implications

Variabilita sekvencí mitochondriálního genu pro Cytochrom b u *Boa constrictor* s fylogeografickým a ekologickým vyvozením

IVANA HYNKOVÁ

MASTER THESIS

magisterská diplomová práce

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Prague 2007

I declare that I have composed this thesis myself and I have used the sources listed in references.

Prague August 29th, 2007 …….

Ivana Hynková

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Motto:
Cause is hidden, Result is generally known
Ovidius

LIST OF APPENDICES

1. INTRODUCTION

Boa constrictor is a heavy-bodied species and generally considered to be polytypic (Price & Russo 1991, Henderson & Hedges 1995). It represents one of the widest ranging snakes in the world (Langhammer 1983). Its massive range begins in northern Mexico in Central America including adjacent islands and extends to a latitude of about 35 degrees South in Argentina in South America (Vergner 1985, Boback 2005, Quick et al. 2005).

The phylogenetic relationships within *Boa constrictor* subspecies are not very clear and basicly not known. The closely related species are considered to be genus *Epicrates* occuring in Central and South America or Malagasy boine snakes such as *Sanzinia* and *Acrantophis*. However, there have always been disputes over *Boa constrictor* affinities and these are still not sufficiently proved (e.g. Austin 2000). Currently genus *Boa* is considered to be sister taxon to the clade comprising Neotropic genera *Corallus*, *Eunectes* and *Epicrates.* The closest relative of this whole group (i.e., including *Boa*) is considered to be Pacific genus *Candoia* (Campbell 1997, Austin 2000, Noonan & Chippindale 2006a) although some uncertainties still remain.

The aim of this work is to clarify the phylogenetic relationships among *Boa constrictor* subspecies with the assistance of molecular characters and unique amount of samples as there is no evidence that such a study would be ever done on these species.

As a molecular marker for this work I used the entire mitochondrial protein-coding gene cytochrome b (cytb) which is traditionally used for studies that occupy phylogeny of species including snakes (e.g. Campbell 1997, Puorto et al. 2001, Slowinski & Lawson 2002, Noonan & Chippindale 2006).

The suggested phylogenetic relationships within the studied group of snakes could serve as a foundation or support for further morphological or other molecular studies.

Boa constrictor is a favourite species in private collections and one of the most heavily exploited reptile species in the world (the Argentinian subspecies *B. c. occidentalis* is included in Appendix I of the **C**onvention of **I**nternational **T**rade in **E**ndangered **S**pecies of Wild Fauna and Flora, the others are listed in Appendix II, Chiaroviglio et al. 2003, Rivera et al. 2005). For example from 1977 to 1983 over 113,000 heads were imported to the USA (Dodd 1986 ex Boback 2005). It was frequently imported to Europe in the

previous decades as well. And for this reason I could carry out this research. Most of the samples come from Czech breeders but German, Spanish and French are no less important.

1.1. Quick view on the recent neotropical history

The splendid diversity of the Neotropics has always attracted humans since being discovered. For all that, we still do not seem to understand fully all of its complexity. There are many forces that helped to create such a stunning place and we need more phylogeographic and geological data to comprehend it all. For better understanding of the following text you may see geological terminology given in Appendix 9.

As the most important biogeographical factors driving the present distribution of the Neotropical species we could name (1) "the uplift of the Andes", (2) creation of Panama land bridge and (3) climatic fluctuations during Pliocene and Pleistocene (Stuart 1957, Montoya-Burgos 2003, Wüster et al.2005).

The first two events are dated to the Late Tertiary approximately 3.5-3 million years ago (MYA) (Webb 1991, Gregory-Wodzicki 2000) when the Andes reached a peak of more than 4000m (Zamudio & Greene 1997).

Creation of Panama Isthmus enabled the northern hemisphere species to reach South America easily and vice versa. But the North-South American fauna exchange took place even before the Panama Portal closure and is dated to Cretaceous or Cenozoic approximately 65 – 45 MYA when even the first orogeny of the Andes occured (e.g. Bush 1994, Zamudio & Greene 1997). This connection was intermediated by a system of volcanic plateaus (Hedges 1996, Cadle 1985). The first forms using the Panama Isthmus reached the northern part of South America about 2.9 MYA (Hooghiemstra 1989 ex Bush 1994). These are mainly plants but animals in larger numbers surely followed soon after.

The third most important event is dated to Plio-Pleistocene when the climate became very arid and cooler (Hooghiemstra 1989 ex Bush 1994) and caused a fragmentation of Amazonian rainforest (Haffer 1969, Haffer 1997, Costa 2003, Wüster et al.2005, Bonaccorso et al. 2006) that continued till Quaternary (Cracraft & Prum 1988, Bush 1994). After this drying event the Amazonian range became inhabitable for xerophilous species (such as rattlesnakes) but the rainforest refuge enabled the tropical species to survive. This climatic change followed by fragmentation seem to be an important part of South American

speciation (e.g. Pennington et al. 2000, Ribas et al. 2005, Rull 2006). In mid-Quaternary the conditions got humid again and so the rainforest refuge expanded and very possibly overlapped. It is more than probable that in the overlapping zones the hybridization occured (Bush 1994). It should be noted that the rainforest refuge remained in highlands (along the Peruvian Andes, Atlantic coastline in South America and in the Central Brazil) where the conditions were still moist enough (Haffer 1969).

1.2. Comparable species

Processes leading to formation of present day Central American fauna may serve as a unique model in evolutionary ecology. One of my interests was to testify or refute the suggested differences between Central and South American populations (e.g. Rosen 1975, Savage 1982, Candle 1985, Zamudio & Greene 1997). Consequently, for comparative purposes I searched for animal species having both Central and South American distribution like *Boa constrictor*. These are for example rattlesnakes, snapping turtles, treeboas, bushmasters and some kinds of rodents, primates, birds, fish and insects as well. Unfortunately, there are only few papers dealing with the phylogeography of these groups and most of them, unlike boas, are those originated in North America (e.g. Phillips et al. 1996, Walker et al. 1997, Riddle et al. 2000, Rodríguez-Robles et al. 2001).

Zamudio & Greene (1997) explored the phylogeography of *Lachesis muta* that currently occurs in the whole Neotropics but its place of origin involves North America. During Miocene bushmasters reached Central American region that possibly served them as a preparatory area for tropic environment that awaited in South America. Zamudio & Greene (1997) found a significant sequence divergence between South and Central America (more than 9% regarding to 18-6.5 MYA). Among South American clades they found 2% of sequence divergence (regards to 300,000-800,000 years ago) and there are two subspecies dwelling in Costa Rica but on opposite sides of Cordillera de Talamanca that differ in 5.3% of sequence divergence. This presented significant sequence divergence between Central and South America is rather congruent with my data for *Boa constrictor* and their values are summarized in Table 1. However, as will be mentioned further, I found no such variation in Costa Rican populations.

It should be mentioned that while estimating the time span Zamudio & Greene (1997) used different molecular clocks that "vary from 0.47 to 1.32% per million years". The molecular clock using 2%/million years are mainly for mammals but I will use this for the further calculation.

There is no doubt that the uplift of Andes played an important role in the Neotropics speciation as their affect on atmospheric circulation is undeniable. The time estimates for *Lachesis muta* differention are consistent with the commencement of Andes elevation and means that bushmasters have considerably ancient lineage.

Wüster et al. (2005) were another ones who explored the phylogeographic relationships of other viperous snake *Crotalus durissus* that is originally dwelling in northern Mexico savannahs but currently is spread in Neotropics. During the Plio-Pleistocene dry periods rattlesnakes started their way to South America carrying on further South. Their progression from the Central America to the north South America (Venezuela) happened approximately 1.85 MYA (the early Pleistocene), then they moved eastwards (Guayana) about 1.54 MYA and crossed the Amazon Basin towards Argentina around 1.08MYA (the middle Pleistocene, these are penalized likelihood ages). The southeastward movements of the rattlesnakes had to be accompanied by several adaptations as the South American environment was still tropicly humid compared to the Mexican and Central American dry savannahs during the early Quaternary (Eberhard & Bermingham 2004, Wüster et al.2005).

It should be mentioned that the movements of the Central (North) American biota towards South was, on the other hand, asymmetricly balanced by the penetration of the South American species further north and west during the mid-Quaternary (Bush 1994). This is for example a case of the neotropical parrots *Amazona ochrocephala* (Eberhard & Bermingham 2004).

Besides, exchange of savannah vertebrates was followed by an exchange of forest animals. This may be the case of treeboas but the accurate data are still missing. There is only one available evidence of treeboas movement across the Amazon Basin but this time we are dealing with the horizontal Peruvian-Brazilian-Guayana Shield interchange. In the meantime, I am not aware of any evidence that treeboas would have ever reached North America and also their phylogeography across Central America is not well documented.

Nevertheless, Vidal et al. (2005) found significant differences between Peruvian and other South American haplotypes of *Corallus caninus*. The Peruvian group appears to be the most divergent with the striking sequence divergence of 16.2% from the remnant South American and is considered to be a basal lineage. This notable divergence would suggest some significant vicariant event in South America that had to occur during Tertiary (Bush 1994).

Taxon	**Central/South Am.**	**Within South Am.**	**Within Central Am.**
Boa	5.7-7.3 %	1.9 %	2 %
Epicrates	4-5 %	3.7 %	not available
Eunectes	not available	5 %	not available
Corallus	13.5 %	1 %	1 %
Lachesis	9.1 %	1-2 %	5.3 %
Crotalus	1-11 %	1.3 %	6-11 %

Taxon	**Argentina/South Am.**	**Peru/South Am.**	**Peru/Central Am.**
Boa	3.5 %	2 %	6.8 %
Epicrates	10 %	1.3 %	3.5 %
Eunectes	11.2 %	not available	not available
Corallus	not available	16 %	15 %
Lachesis	not available	not available	not available
Crotalus	1 % (Mato Gosso,Brazil X Venezuela)	not available	not available

Taxon	Argentina/Central Am.
Boa	5.7 %
Epicrates	9.4 %
Eunectes	not available
Corallus	not available
Lachesis	not available
Crotalus	0.6-11% (Mato Gosso,Brazil X Venezuela)

Table 1: Uncorrected (p) distances between and within the important South and Central American places. *Boa* and *Epicrates* are unpublished data the rest of the sequences were gained from GenBank and literature. These are only approximate values.

1.3. Taxonomy and phylogeny

Boa constrictor belongs to superfamily Booidea, family Boidae, subfamily Boinae. Morphological variation of boa constrictor representatives is truly extensive and currently we can recognize 11 subspecies (Langhammer 1983, Price & Russo 1991 state only 8-10 subspecies, Russo 2002, Chiaraviglio et al. 2003). It can be found in open formations, in forests or in edge-situations of savannas and forests.

The family Boidae is worldwide and used to comprise three subfamilies:

1) Boinae (from Neotropics,Madagascar and Pacific Islands)

2) Pythoninae (from Australia to Africa)

3) Erycinae (from western North America, Africa, southeastern Europe, southwestern Asia and India) (Noonan & Chippindale 2006a).

The real pythons are currently assumed to be a separate family Pythonidae, distinct from Boidae (Mc Dowell 1979, Campbell 1997, Slowinski & Lawson 2002, Wilcox et al. 2002, Lawson et al. 2004).

The present studies working on Boidae use this term for Boinae and Erycinae (Burbrink 2005, Noonan & Chippindale 2006a). These are live-bearing snakes and contain 7 genera and 28 species (Burbrink 2005).

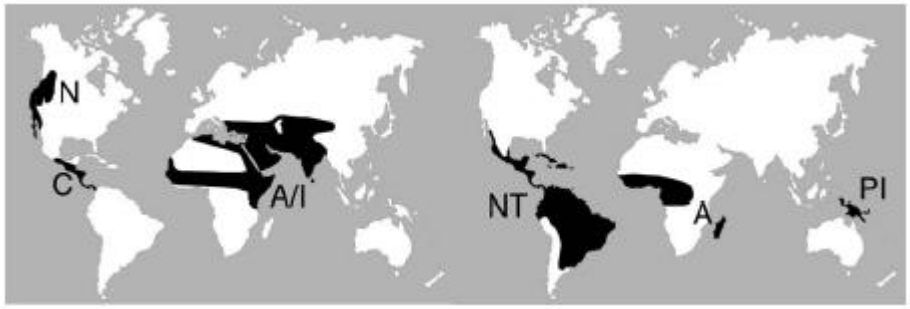

Fig. 1: Geographic distribution of the Boidae: Abbreviations and the black marking show the distribution of currently recognized clades: NT-Neotropical (*Boa, Corallus, Epicrates, Eunectes*) A-African (*Calabaria, Sanzinia, Acrantophis*), PI-Pacific Island (*Candoia*), N-North American (*Lichanura, Charina*), C-Central American (*Exciliboa and Ungaliophis*), A/I-Afro-Indian (*Eryx*) (from Noonan & Chippindale 2006a)

1.4. Review of relationships within Boinae

In 1991, Kluge (ex Burbrink 2005) made one of the first analysis of Boidae based on morphological characters that was soon after widely accepted. His analysis suggested the Neotropical boines to be polyphyletic and genus *Boa* was considered to be a sister taxon to *Acrantophis* and *Sanzinia* from Madagascar. According to his finding clade *Boa, Acrantophis* and *Sanzinia* was closely related to the New World clade consisting of *Epicrates* and *Eunectes* and these two clades were stated as sister's to the New World *Corallus*. The proximity of *Boa* and *Sanzinia* seemed to Kluge so significant that he suggested a replacement of the name *Boa mandritra* for *Sanzinia madagascariensis*. However, this suggestion was later negated by many authors such as Austin (2000) or Vences et al. (2001). Kluge himself found a unique karyotyp of *Sanzinia* and *Acrantophis* (2n=34 compared to 2n=36 of *Boa, Epicrates, Eunectes*) which is not excessively phylogeneticly informative, nevertheless, it paradoxically confirms the differences between the Madagascan and New World boas. This difference was also mentioned by Campbell (1997) who suggested that the Madagascan genera should have had their own family (based on molecular data – cytochrome b) and explains the morphological similarity of these two groups of boas as a convergency.

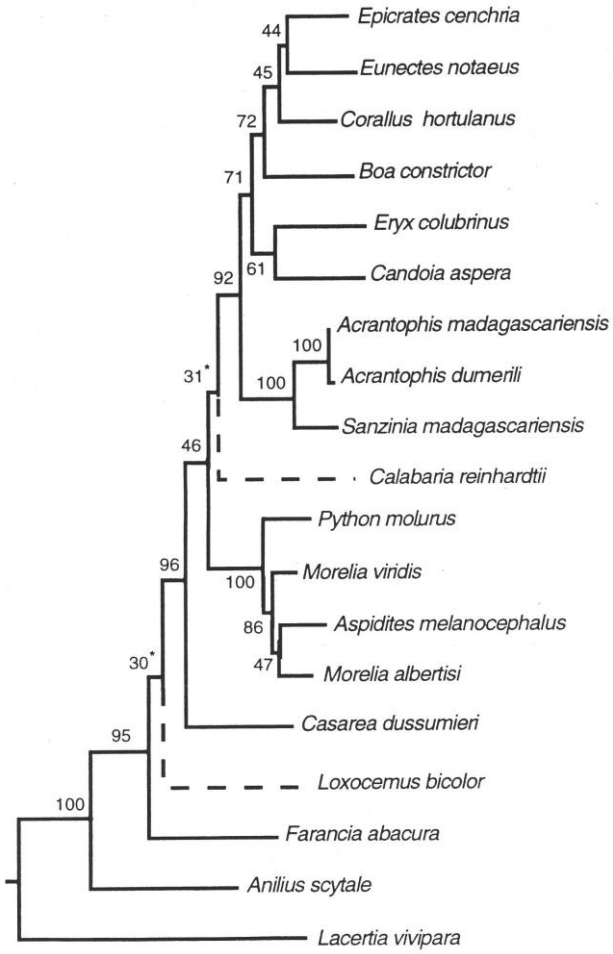

Fig. 2: Phylogeny for the Booidea based on Kimura transversional distances (from Campbell 1997).

Morphological characters may be prone to homoplasy in snakes and so Burbrink (2005) was another one who used a molecular marker (cytochrome b) to re-examine the relationships within Boinae. The results of his analysis based on the mitochondrial gene rejected Kluge's hypotheses and proposed *Boa constrictor* to be a sister taxon to the New World *Epicrates-Eunectes-Corallus* clade, which is consistent with Campbell's discovery, see Figure 2. He also suggests the monophyly of all New World boines and paraphyly of the Boidae (but see Campbell 1997).

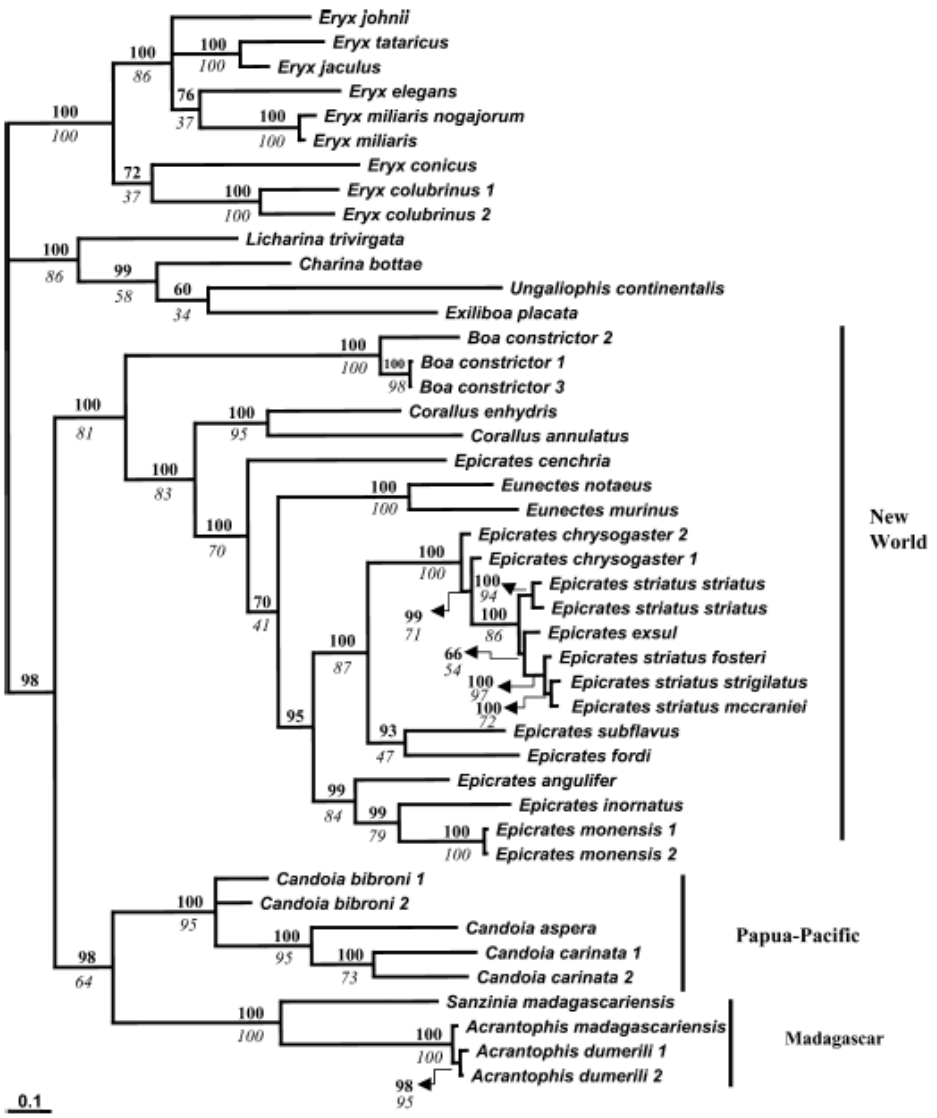

Fig. 3: Phylogeny of Boinae using Bayesian inference of cytochrome b. Numbers above the branches in bold indicate support summarized from the posterior probability distribution. Numbers below the branches represent maximum likelihood nonparametric bootstrap (from Burbrink 2005).

His Bayesian and maximum likelihood analysis indicate that *Boa-Corallus-Epicrates-Eunectes* clade is monophyletic and that the Madagascan boines are more related to the Papuan-Pacific monophyletic *Candoia* (but with low nonparametric bootstrap support). Slowinski & Lawson (2002) also found the same proximity of *Candoia* and *Sanzinia-Acrantophis* based on cytb data (however c-mos gene data placed *Candoia* close to *Boa-Lichanura-Charina* clade in the same paper).

In addition, Burbrink made an analysis of combined data using Kluge's morphological characters with the similar conclusion as previous, placing *Boa* out of the Madagascan genera.

Another survey on Boidae was made by Noonan & Chippindale (2006a) who presented very similar results to Burbrink's. The only difference is a close relationship of *Candoia* and *Eryx* (also see Campbell 1997 in Figure 2).

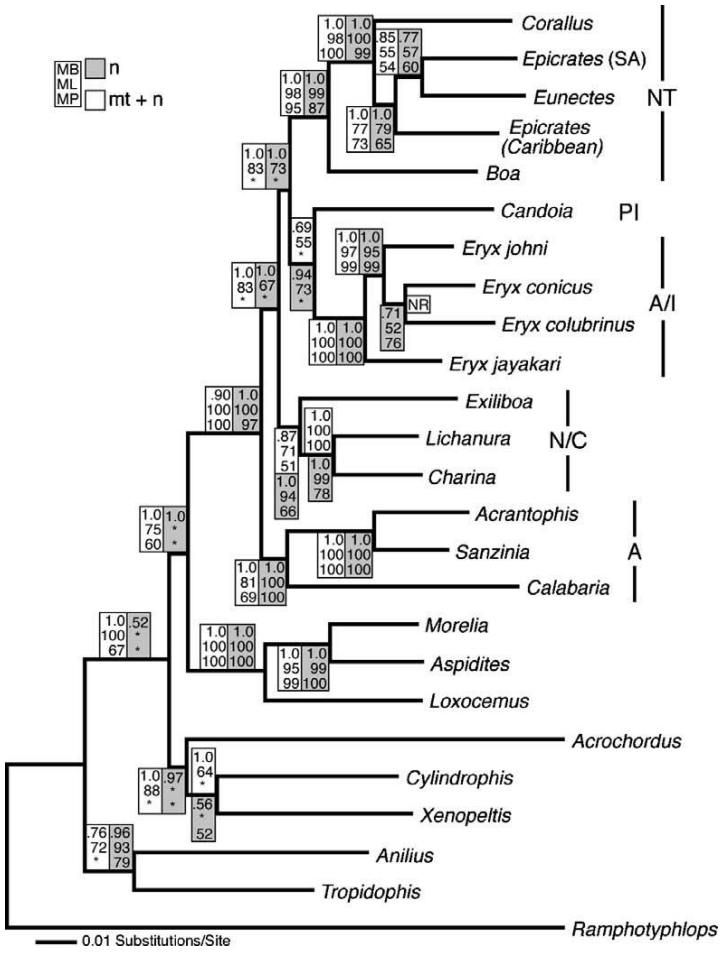

Fig. 4: Bayesian phylogeny of Booidea according to Noonan & Chippindale (2006a). This analysis was carried out using combined data (mitochondrial and nuclear gene). The white boxes along the branches represent bootstrap support for nuclear data, the gray boxes then for combined data. The number order is Bayesian posterior probabilities, ML and MP bootstraps.

The last suggestion, that in my mind should be presented here, is Austin's phylogenetic analysis. In contrast with Kluge's finding Austin (2000) does not find a

relation between *Boa* and *Sanzinia* (based on cytb sequences) and rather see proximity between *Sanzinia* and *Candoia* (as Burbrink 2005). He inferred that *Boa* is just a marginal group, doubted the monophyly of Madagascan boids and suggested that the timing for separation between *Candoia* and New World boids is 40 million years or more. There was also previous molecular evidence supporting this idea of divergence (e.g. Johns & Avise 1998). Austin presented the uncorrected p-distances between *Candoia* and other boids, *Sanzinia*, *Corallus, Epicrates* and *Boa* to be 0,19, 0,23, 0,24, and 0,25 respectively.

1.5. Palaeontological implications for boids distribution in South America

This section and 1.6. section rather deals with ancient events affecting the whole family Boidae and is not fully relevant in the narrow sense of the *Boa constrictor* distribution. Yet, it may serve as an idea that helps to imagine the forces affecting *Boa constrictor* ancestors. In addition, *Boa constrictor* itself is generally assumed to be rather ancient species although this notion will not be confirmed by the results of this work.

The fossil recording and field surveys are still very infrequent in South America (Albino 1993) and very often unsuccessful due to humid conditions. The same limitations can be found in African and Australian fossils records where further more is very hard to distinguish whether the fossil issue belongs to Boidae or Pythonidae (Austin 2000).

The Gondwanan versus Laurasian origin of Neotropical species including Boidae has been discussed for decades. The Gondwanan origin of the Boidae was supported by Albino (1993) whose research took place in Argentina and who suggested that the basic diversification of Boidae took place during the Cretaceous and Paleocene. She concluded that the most ancient boids came from Cerro Guadrado, Río Negro province, Argentina. Furthermore, there is another suggestion about the timing of diversification of Neotropical fauna based on fossil records dated back to the late and mid Miocene (Salazar-Bravo 2001, Montoya-Burgos 2003).

Rage (1986) hypothesized that the first fossil snake distributed in Gondwana landmass belonging to the ancient family Madtsoiidae (120 milion years old) might be a primary member of the Booidea and its genus *Dinilysia* to be closely related to Boidae. Albino (1993) remade Madtsoiinae to be a subfamily of family Boidae represented by genus *Madtsoia sp.*

Madtsoiid snakes are mainly found in Gondwanan continents but some of them were also found in Spain, France and Romania. However, these are considered to be immigrants from African continent (Late Cretaceous, Gheerbrant & Rage 2006).

Albino's results implicate that boid snakes were abundant in South America during the Late Paleocene and Early Eocene. These are fossils of the genus *Madtsoia sp., Chubutophis, Alamitophis* and *Patagoniophis* (Albino 1993, Albino 1986 and 1994 ex Albino 2000). The last two snakes were also found in the Early Eocene in Australia and their distribution actually confirms a connection between Antarctica, Australia and South America during the Late Mesozoic and Tertiary. This is going to be discussed in the following section 1.6.

It should be noted that an important role in an early evolution of snakes played a presence of warm-blooded animals representing their prey. We can find the representatives of all boid currently recognized subfamilies in Paleocene as well as the differentiation of main currently recognized mammal groups in South America. It is more than probable that boid snakes represented very important predators in this region.

1.6. Main events driving the Boidae distribution

The subsequent radiation of Gondwanan pre-boas and the current biogeographic distribution of the Boidae have always been problematical. Dispersal and vicariance were suggested by many authors (Rosen 1975, Wilson 1991, Morrone & Crisci 1995, Noonan & Chippindale 2006b) and most of the times were treated separately. Vicariance has been the main explanation in the last decades of the previous century (de Queiroz 2005) and dispersal was treated as an "irrelevant noise" (McGlone 2005). However, currently they are considered to be non-exclusive and even more, dispersal is treated as a primary process driving the current distribution (McDowall 2004). Furthermore, vicariance is considered to be an explanation for old history events and dispersal is considered to be a suitable explanation for recent events (de Queiroz 2005, McGlone 2005). Additionally, it should be reminded that the vicariance model does not consider the ecological backgroung and interactions that might be no less important (Wilson 1991).

The role of the oceanic dispersal should not be underestimated as there are several signs that animals (even those that we would not supposed such device to be used, Schrago & Russo 2003) use this way quite often (Campbell 1997, Honda et al. 2003) Also this way of

transport is not random as some thought before (Albino 1993, McDowall 2004, McGlone 2005) but for example water currents may make this way much easier (Stuart 1957, Sanmartín & Ronquist 2004).

Several authors (Austin 2000, Burbrink 2005, Vences et al.2001) supported the idea of Boidae distribution coming from Gondwanan breakup (which took place about 150 million years ago, Austin 2000, this hypothesis may be found in a literature as plate tectonics) and subsequently underwent an oceanic dispersal.

One thing has been emphasized lately and this is a relationship between South American and Madagascan fauna that is lacking in Africa (Rage 2003). Southern and northern routes were suggested for explaining these relationships. Both are dated to the Late Cretaceous . The southern route consisted of South America-Antarctica-Madagascar-India-Australia (e.g. Krause 2001). Antarctica and Madagascar-India were connected through so-called Kerguelen Plateau about 80 MYA (Crisci et al.1991, Hay et al. 1999, Rage 2003, McDowall 2004). An alternative of Kerguelen Plateau is the Gunnerus Ridge described in Case 2002 (ex Rage 2003). This connection would suggest a closer relationship between *Candoia* and New World boines. This pattern has been revealed before in the molecular analysis by Austin (2000) and Noonan & Chippindale (2006a). Additionally, this coalescence would also suggest a close relationship between South American and Madagascan boines and other species like pelomedusid turtles or iguanas, that have the same disjunct distribution as Boinae (Georges et al. 1999, Schulte et al. 1998). Another part of this connection included Australian continent. Knapp et al. (2005) put out that about 65-35 MYA there was very tight bonding of South America and Australia which could be demonstrated by similarity of *Corallus caninus* (South America) and *Morelia viridis* (Australia) (but we may deal with convergency).

The northern route consisted of South America, North America and Europe. The groups that were coming from South America used the land bridge to reach North America and from there they reached Europe. This all took place in the Late Cretaceous and is supported by fossils (Rage 1988). It has been suggested that this fauna passed Africa and reached Madagascar through Eurasia (Krause 2001, Gheerbrant & Rage 2006). The reversed direction of faunal movements is known for pitvipers (Zamudio & Greene 1997).

Rage (1988) stated that South America and South Africa were in the Triassic part of so-called southern Gondwanan province which could show a closer relationship of their fauna. Northern Africa might overlap with Laurasia and this would prove the previous suggestion about madtsoiid immigrants to the southern and eastern Europe (section 1.5.). The similarity of Gondwanan and Laurasian fauna is subsequently occuring in the early Jurassic (Rage 1988).

Both Gondwanan and Laurasian origin of Neotropical vertebrates was proposed by Rosen (1975, also see Underwood & Stimson 1990) as well. He was one of the first to consider the vicariance and dispersal hypothesis and suggested four main trakcs causing the current distribution of Neotropical fauna.

1) South American-Caribbean track (SACT)
2) North American-Caribbean track (NACT)
3) Eastern Pacific-Caribbean track (EPCT)
4) Eastern Atlantic (or West African) Caribbean track (EACT)

He put forward that the southern ancestor of Netropical biota had Gondwanan origin and the other, northern, had Laurasian origin as was implied before. He also implied a possible dispersal that might happen in Late Mesozoic and carried on from Middle to Late Cenozoic when some vicariant event occured (Rosen 1975, Hedges 1982) which is consistent with Rage (1988).

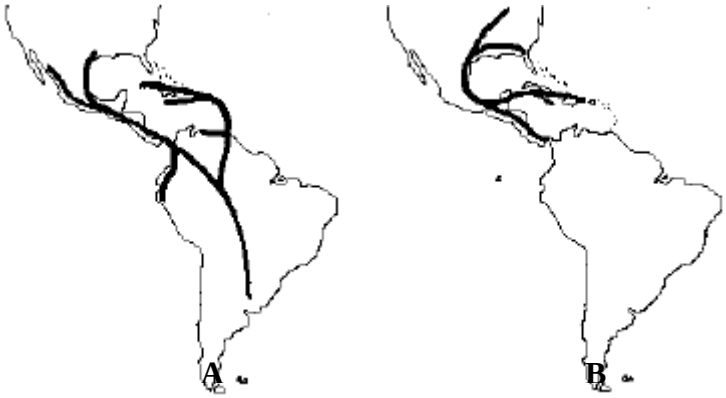

Fig. 5: A: the South American-Caribbean tracks represented by selected species **B:** the North American-Caribbean tracks (adopted from Rosen 1975). It is obvious that A and B overlapped in Central American region.

The last possible ways that could cause the South American and African biota close relationship and that I would like to present, are trans Pacific and trans Atlantic routes.

Trans Pacific way was used by Indian and consequently Australian species, trans Atlantic route was used by Indian and African species (e.g. *Mabuya* Honda et al. 2003, african primates Schrago & Russo 2003). The trans Atlantic route seems to be relevant since Palaeocene till Oligocene, when little islands in Atlantic ocean occured - Walvis and Rio Grande Rises - and also water currents and winds were favourable to these movements (Rage 1986, Schrago & Russo 2003). Gheerbrant & Rage (2006) see Rises Ceara and Sierra Leone that occured during Eocene and Oligocene to be more important than Walvis a Rio Grande Rises. No matter what islands we consider more important the truth is that some land-like connection existed that enabled the African-South American fauna exchange since the end of Mesozoic till mid-Cenozoic.

It should be noted that all of this modeling and suggestions depend on the known taxonomy and precondition that all studied groups are monophyletic, so the results are only as good as that.

1.7. List of *Boa constrictor* subspecies

Boa constrictor constrictor Linnaeus, 1758 – Red-Tailed Boa

This nominotypic subspecies has the largest area of distribution living from the northern part of South America and adjacent islands Trinidad and Tobago, the eastern Ecuador and to a latitude of 13° South in Brazil (Vergner 1985). It used to be labeled as *Boa constrictor rubricauda* on the pet market (Langhammer 1983).

Other names (www.reptiles-database.org)

Constrictor auspex Laurenti, 1768

Constrictor constrictor constrictor (Linnaeus, 1758)

Constrictor diviniloquus Laurenti, 1768

Constrictor formosissimus Laurenti, 1768

Constrictor rex serpentum Laurenti, 1768

Boa constrictor imperator Daudin, 1803 – Common Boa constrictor

This subspecies lives mainly in Central America and adjacent islands, from the northest part of Mexico called Sonora and Tamaulipas to the south-eastern South America, along the coast of Pacific and its range reaches the southern Ecuador (Vergner 1985). Hog Island Boa is included within this subspecies.

Other names (www.reptiles-database.org)

Boa constrictor mexicana Jan, 1863
Boa constrictor sigma (Smith, 1943)
Boa constrictor var. *isthimica* Garman, 1883
Boa diviniloquax var. *mexicana* Jan, 1863
Boa eques Eydoux & Souleyet, 1842
Boa imperator Daudin, 1803
Constrictor constrictor imperator (Daudin, 1803)
Constrictor constrictor mexicanus (Jan, 1863)
Constrictor constrictor sigma Smith, 1943

Boa constrictor occidentalis Philippi, 1873– Argentine Boa

This subspecies can be found in the southern part of Paraguay and in the forests of Argentina between the Andes and the river Paraná. In Argentina it reaches the province Córdoba, San Luis and Mendoza.

Other names (www.reptiles-database.org)
Boa occidentalis Philippi, 1873
Constrictor constrictor occidentalis (Philippi, 1873)

Boa constrictor amarali Stull, 1932 – Short-tailed Boa

Distribution of this subspecies reaches the eastern Bolivia across the provinces Mato Grosso and Goiás to the Brazilian province Sao Paulo and also to the southern part of Paraguay.

Other names (www.reptiles-database.org)
Constrictor constrictor amarali (Stull, 1932)
Boa constrictor amarili Forcart 1951

Boa constrictor ortonii Cope, 1878– Peruvian Boa

This species dwells on a little area of the north-western Peruvian Andes and its habitat are savannas and highlands. It reaches the altitude of 2000m. I will not deal with this subspecies as it is not accessible.

Other names (www.reptiles-database.org)
Boa ortonii Cope, 1878
Constrictor constrictor ortonii (Cope, 1878)

Boa constrictor melanogaster Langhammer, 1983- Black-bellied Boa

Only a little area of Andes in the eastern Ecuador is resided by this subspecies.
I will not deal with this subspecies as it is not accessible.

Boa constrictor longicauda Priece & Russo, 1991– Long-tailed Boa

This subspecies was found in Tumbes Province in the northern Peru in 1991.

All the other subspecies (including *B. c. orophias, B. c. sigma, B. c. nebulosa* and *B .c. sabogae*) are endemists of West Indies and Pearls Islands and are susceptible to extinction (Boback 2005). Only *B .c. sabogae* inhabiting Saboga Island, Pearls Islands, Panama was included in this study. The other subspecies were not included in this study because they are not accessible.

Boa constrictor orophias Linnaeus, 1758 – St.Lucia Boa

Endemic subspecies of one of the Carribbean island St.Lucia

Other names (www.reptiles-database.org)

Boa ophryas Shaw, 1802

Boa orophias Linnaeus, 1758

Constrictor constrictor orophias (Linnaeus, 1758)

Constrictor orophias (Linnaeus, 1758)

Boa constrictor sigma Smith, 1943

This subspecies dwell on the Pacific island Tres Marias, not far from Mexico

Boa constrictor nebulosa Lazell, 1964 – Clouded Boa

This subspecies can only be found on Dominice Island, Lesser Antilles.

Other names (www.reptiles-database.org)

Constrictor constrictor nebulosus (Lazell, 1964)

Boa constrictor sabogae Barbour, 1906 – Pearl Island Boa

This subspecies inhabits the Pearl Islands not far from Panama especially Saboga Island. T

Other names (www.reptiles-database.org)

Constrictor constrictor sabogae (Barbour, 1906)

Epicrates sabogae Barbour, 1906

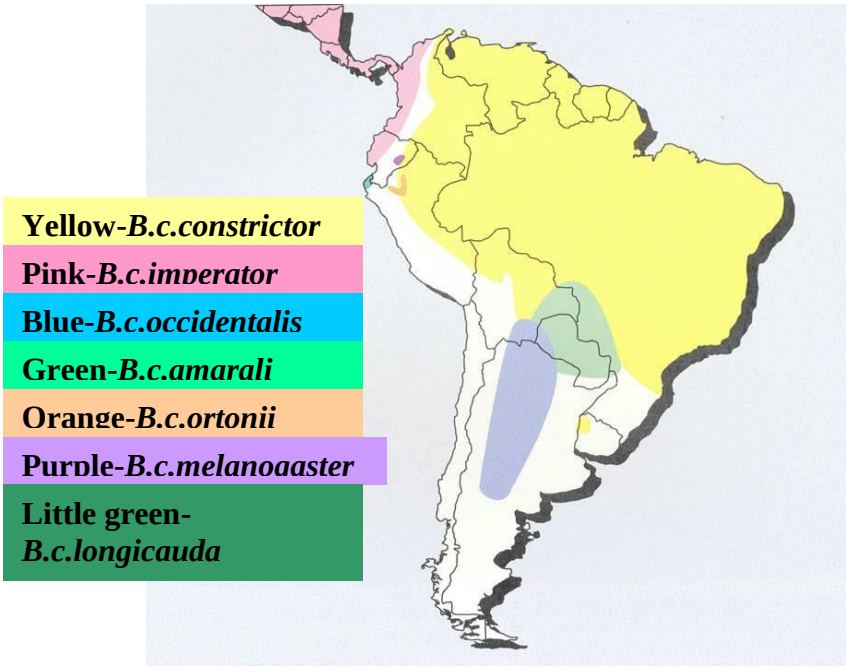

Fig. 6: Map showing range of *B.constrictor* subspecies in the mainland South America and part of its Central American range (adopted from Walls 1998)

2. AIMS OF THIS STUDY

- to study the genetic variability of genus *Boa constrictor* based on cytochrome b sequences
- to estabilsh phylogenetic relationships within the *Boa constrictor* subspecies
- to specify the taxonomy of studied species – to find populations that differ on species or subspecies level, or revise the subspecies/species boundaries
- to suggest a possible geographic scenario and time-span of diversification

3. MATERIALS AND METHODS

3. 1. Materials

124 samples of boa constrictor subspecies were collected, 115 were eventually used for further analysis. These represent 5 subspecies of *Boa constrictor* from the mainland and one from Saboga Island. The rest of mainland and other island subspecies were not gained. The list of analysed samples of boa constrictor subspecies with their places of origin and abbreviations can be found in Appendix 2. Some of the samples were the direct imports from the natural locality years ago, some of them were the offsprings of these imports. All the samples were collected with the kindly help of breeders whose names are listed in the acknowledgements.

As outgroups (Watrous & Wheeler 1981) I used other Boinae snakes. These are: *Eunectes* (South America), *Corallus* (South America), *Epicrates cenchria* (Central and South America), *Epicrates angulifer* (Cuba) and *Candoia* (Polynesia). The sequences of *E. cenchria* and *E. angulifer* were our own samples the other sequences were obtained from the GenBank, NCBI (see www.ncbi.nlm.nih.gov).

All available nucleotide sequences of boa constrictors were also downloaded from the GenBank, NCBI including the whole genome sequences where only required gene was extracted. Appendix 1 contains a list of the access numbers of GenBank sequences used.

3.2. Gene used

Mitochondrial gene for cytochrome b (see Figure 7) was employed as a molecular marker. No insertions/deletions were present. Slowinski & Lawson (2002) verified that the entire cytochrome b sequence does not contain nuclear pseudogene as no stop codons were find inside. This was also rechecked by translation of DNA sequences into amino acids using different reading frames and MEGA 2.1 software, Kumar et al. 2001). The sequences began with the ATG methionine codon (see e.g. Burbrink et al. 2000). The snakes cytochrome b gene varies between 1101 nad 1131 bp (Campbell 1997, Lawson et al. 2005). The termination of translation may be a "post-transcriptionally polyadenylated

thymine" in Boidae (Campbell 1997) and according to this finding I used the first 1114 bp for the following analysis.

Cytochrome b is widely used gene for tracing evolutinary history of lineages in snakes (Russo et al. 1996 assume that it is one of the best) and other animal groups as well (e.g. Sullivan et al. 1997 and 2000). Its advatage is a relatively slow evolution (although this was disputed by Brown et al. 1979) and a conserved structure. The absence of the indels had been mentioned above and is a reason for an easy alignment of cytb (Campbell 1997).

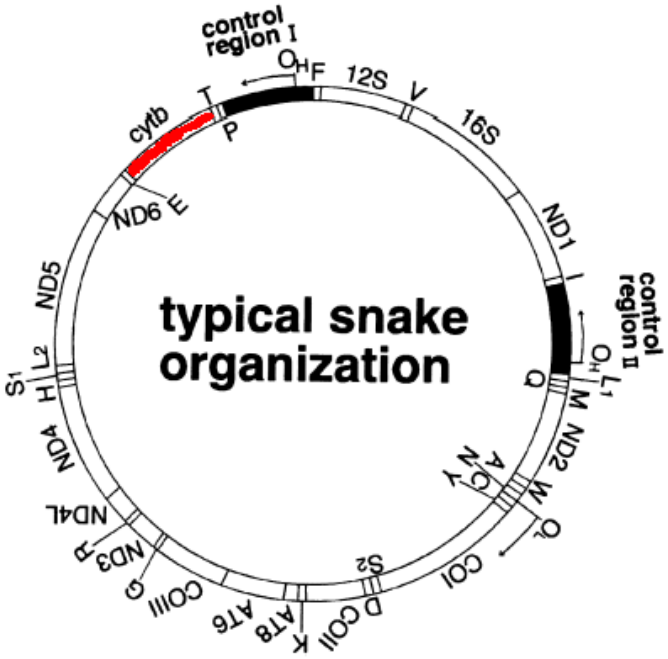

Fig. 7: Gene organizations of vertebrate mtDNA. The organization found for six snakes including *Boa constrictor* (adopted from Dong & Kumazawa 2005). The red colour shows a position of cytochrome b which is used in this study as a phylogenetic marker

3.3. Molecular methods and laboratory protocols

3.3.1. Collecting and isolation

For collecting the samples we used the non-destructive method of taking buccal swabs (Pidancier et al. 2003). This was made using the sterile cotton swabs. The end of each swab with the required sample was cut and placed into a test tube filled with pure ethanol (96%) and stored in the refrigerator in 4°C for a short time (few days).

DNA extraction was performed with NucleoSpin Tissue kit (Macherey-Nagel) according to manufacturer's protocol for buccal swab isolation. The intensity of isolated DNA was visualised on a 1% agarose gel (0,4g agarose in 40ml of 0,5M TBE buffer with 1,2µl ethidium bromide stain - EtBr), at 100V, for 50 minutes. The gel-applied mix consisted of 9µl of extracted DNA and 5µl of marking buffer.
Extracted DNA was stored at –18°C until used as a template for Polymerase Chain Reaction (PCR, Sambrook et al. 1989).

3.3.2. Amplification

The PCR method was used for the amplification. The entire 1114 bp long mitochondrial gene for cytochrome b was amplified.
The thermo-cycler PTC-200 (MJ Research) was used for the amplification of all PCR-products. One pair of oligonucleotide primers was used for the amplification of cytochrome b gene. These were designed by Burbrink et al. 2000 and are as following:

L14910 5' – GAC CTG TGA TMT GAA AAC CAY CGT TGT - 3'
H16064 5' – CTT TGG TTT ACA AGA ACA ATG CTT TA - 3'

30 µl of the reaction mix (Mastermix) used for PCR-amplification contained the following set-up (these values are only for one reaction/sample):

10x buffer with $MgCL_2$ (2mM)……3µl
dNTPs (2mM)……...3µl
forward primer 0,01mM (L14910)……1,2 µl
revers primer 0,01mM (H16064)……1,2µl
Taq polymerase (5U/µl)……..…..0,15µl
PCR H_2O…….14,47µl
isolated DNA……..…..7µl

The temperatures and times of PCR program shows Table 3.

step	time	temperature	repeat
1	7 min	94°C	1x
2	40 sec	94°C	step 2,3,4 40x
3	30 sec	46°C	
4	1 min	72°C	
5	7 min°	72°C	1x
6	for ever	4°C	END

Table 3: The PCR program used for amplification of cytochrome b (according to Burbrink et al. 2000)

The PCR products were controlled by electrophoresis applying the entire volume (30 µl plus 10µl of marking buffer was added) on the 2% agarose gel (1g agarose in 50ml of 0,5M TBE buffer with 1,5µl EtBr), and with 5µl of the ladder standard (GeneRulerTM 100bp DNA Ladder Plus) and with the voltage of 95V.

After about 70 minutes the products were checked and cut out of the gel and put into the clean 1,5 ml microtubes. Each of them was weighed before and after the cutting so the weight of the gel itself was gained. This information was required for another step.

The PCR products were purified using the QIAquick® PCR Extraction Kit (QiaGen) according to manufacturer's instructions. The acquired cytochrome b products have not always been completely pure and there were some difficulties to sequence them. Thus alternatively I changed the after-PCR-steps described previously. I applyed only 10µl of the mix (consisting of 5µl of the Mastermix and 5µl of the marking buffer) on the 2% agarose gel with 3µl of the ladder standard, at 95V. After about 70 minutes I checked the intensity of the product which is needed for a final step of the purification. The rest of the Mastermix (remaining 25µl of PCR products) I used for the purification with the QIAquick® PCR Purification Kit (QiaGen) according to the manual instructions.

3.3.3. Sequencing

The PCR products were sequenced using the same primers L14910 and H16064 and the sequencing kit ABI PRISM® BigDye™ Terminator v 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The sequencing PCR mix of 14µl contained:

0,4 µl of primer

1-5 µl of the purified PCR product

PCR H_2O – the volume of it depends on the volume of purified PCR product + primer and must be topped up to 14µl

After the sequencing PCR mix is done, 4µl of the Sequencing Buffer and 2µl of the Cycle Sequencing Mix were added. Thus the volume of the whole mixture is 20µl.

When the sequencing PCR is finished the marked products need to be removed into a 0,5 ml microtubes and precipitated by the following instructions:

1) Add 2µl of 3M NaAc and with 50µl of pure 96 % ethanol to the PCR product

2) Vortex and let the microtube stand in the room temperature for 20 minutes

3) Centrifuge in 13000 rpm for 15 minutes in 4°C

4) Remove the supernatant with caution, the product may appear as a white pellet on the bottom

of the microtube.

5) Add 180µl of 70% ethanol.

6) Centrifuge in 13000 rpm for 5 minutes in 15°C

7) Remove the supernatant with caution

8) Repeat step 5,6 and 7 once more

8) Let the microtubes stand until it is dryed thoroughly

9) Add 20ul of the formamide, let it stand for 20 minutes to resuspend the pellet

10) Heat the product on 95 °C for 2 minutes, after this denaturation cool it rapidly

11) The samples are ready for sequencing

The nucleotide sequences were assessed on an automatic sequencer ABI PRISM® 3100 Avant Genetic Analyzer in the Department of Parasitology, Faculty of Science, Charles University, Prague. Scheme of the sequencing method you can see in Figure 8.

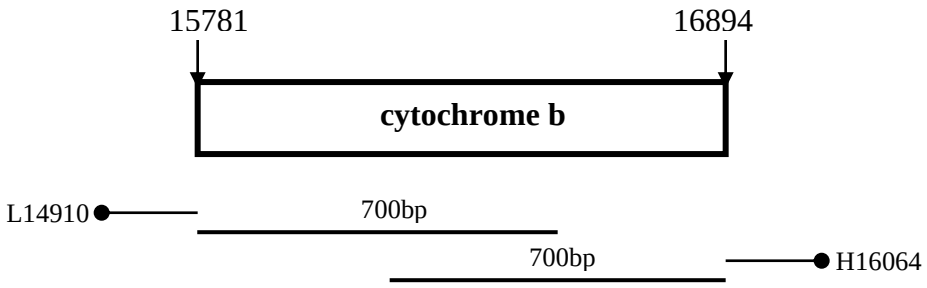

Fig. 8: The PCR fragments and primers used for cytochrome b sequencing and a position of cytochrome b within the mitochondrial genome

3.4. Sequence analysis

3.4.1. DNA alignment

The cytochrome b was sequenced from the forward and reverse primers separately . The reverse sequence from the H16064 primer was inverted in Chromaslite (http://www.technelysium.com.au/chromas.html) and then the both DNA strands obtained for each sequence were checked by eye and connected together using BioEdit v.5.0.6. (Hall 1999).

All the obtained sequences were double checked and aligned in ClustalX (Thompson et al. 1997) using the default settings.

The cytochrome b sequences resulted in about 1130 nucleotides. I only used the first 1114 bp for the following analyses for the reasons mentioned in the section 3.2.

During the alignment analysis the haplotype sequences were determined. Haplotype sequence represents a unique sequence in the dataset. Most of the haplotypes are represented by more than one sequence/sample. The alignment file used for further analysis consists of only one representative of each haplotype. After all sequences were checked the alignment file was transfered to the NEXUS format which is required for most of the phylogenetic programs.

3.4.2. Dataset

All phylogenetic analyses were performed for two datasets that differed in the number of outgroups included. First dataset contained *Epicrates angulifer, Epicrates cenchria, Eunectes notaeus, Corallus caninus* and *Candoia carinata* as the outgroups (*Boa*-with-*Candoia* dataset), the other did not contain *Candoia carinata* (*Boa*-no-*Candoia* dataset). The basic alignment contained 115 boa constrictor sequences, six of them were obtained from the GenBank, NCBI (accession numbers are in Appendix 1). For phylogenetic reconstruction I only used unique sequences (haplotypes), so the final *Boa*-with-*Candoia* dataset is composed of 72 haplotypes and the final *Boa*-no-*Candoia* dataset of 71 haplotypes. It should be pointed out that a few interesting samples are still on their way but because of the time limitation they could not be included in this study.

3.5. Phylogenetic methods

For alignment processing I used distance method Neighbor-joining (NJ) and couple of character methods such as Maximum parsimony (MP), Maximum likelyhood (ML), and Bayesian Method (BM).

The analysing softwares included PAUP 4b10 (Swofford 2002) and MrBayes 3.1 (Ronquist & Huelsenbeck 2003). PAUP software was used for NJ, MP and ML analysis. The input file was created in ClustalX using its NEXUS saving options.

PAUP 4b10 was also used for searching for the phylogenetic information in the sequences (skewness of tree length distribution, command *randtrees*, Huelsenbeck 1991, Hillis & Huelsenbeck 1992). The g1 values are listed in Table 4 for each dataset including one extra dataset which was not used for further analysis. This last dataset consists of only boa constrictor haplotypes with no outgoups.

The gained phylogenetic trees were examined using TreeView 1.6.6 program (Page 1996), Corel X3 was used for graphic layout.

3.5.1. Neighbor-joining (NJ)

NJ method (Saitou & Nei 1987) is based on determined algorithm. The direct way of getting a single resulted tree makes this method extremely fast. For distance calculation I used the uncorrected (p) distances and corrected Jukes-Cantors distances. The resulted

topology was supported by non-parametric bootstraps with 10,000 replicates (Felsenstein 1985).

3.5.2. Maximum parsimony (MP)

This method is based on Occam's Razor principle and searches for a tree with as little evolutionary changes as possible. First the parsimony informative places need to be determined. The heuristic search was used while searching for the most parsimonious tree with random addition of sequences, investigating the trees area using TBR branch swapping (Tree Bisection/Reconnection) and 100 replications (command: *hsearch addseq=random nrep=100 swap=TBR*).

The resulted nodes positions were supported by nonparametric bootstrap analysis using 100 bootstrap replicates (command: *bootstrap search=heuristic nrep=100*).

The resulted cladogram was constructed using the majority rule (50% is default setting, command: *contree all/majrule=yes*).

Two transition/transversion weighting approaches were used for MP trees when all three codon positions were included. These approaches weight transversions 4 and 10 times more than transitions.

To eliminate the saturation impact, the third codon position was excluded from the *Boa*-with-*Candoia* dataset in one MP approach. Transversions were weighted 4 times more than transitions, again.

3.5.3. Maximum likelihood (ML)

For using this method it is essential to determine substititional evolutinary model suitable for the data. ML then counts the likelihoods (hierarchical Likelihood Ratio Test – hLRT, Huelsenbeck & Crandall 1997) for generated phylogenetic trees and takes into acount tree with the hightest likelihood value.

The suitable evolutionary model for my data was chosen by Modeltest, version 3.7(Posada & Crandall 1998). Modeltest3.7 uses hLRT as well as Akaike criterion while searching for the most suitable model. The advantages of this approach have been explained lately in Posada & Buckley (2004).

Parametres of chosen evolutionary model were used for further analyses using PAUP 4b10 (Swofford 2002) and MrBayes 3.1(Ronquist & Huelsenbeck 2003).

The bootstrap support for the best tree topology is extremely computationaly demanding for ML analysis and because my datasets are unusually large, it was not possible to estimate these within the required time-span and so I provide the bootstrap supports (only 10 replications) for only *Boa*-no-*Candoia* dataset. Additionally, I estimated the likelihood distances based on the chosen evolutionary model parametres and the tree topology was scored by Fitch-Margoliash method. These were estimated for only *Boa*-with-*Candoia* dataset and are assigned as a comparative output with the results of the other methods.

3.5.4. Bayesian inference

This approach (proposed by Yang & Rannala 1997) basicly uses Markov chain Monte Carlo (MCMC*) algorithm (e.g. Lanave et al. 1984) to count the posterior probabilities of trees (Huelsenbeck & Ronquist 2001). The posterior probabilities are as much useful as bootstrap supports and inform us about the support for a certain node. The posterior probabilities are not counted as pseudoreplicates (typical for bootstrap) but a certain tree with a certain topology is chosen at the beginning. Every node at this tree has its probability. During the analysis the new trees are created along the way and new probabilities are estimated for their nodes. If these values are higher than the previous ones, the new topology is accepted for futher analysing (Zima et al. 2004). For constructing trees according to Bayesian method I used the MrBayes 3.1 program (Ronquist & Huelsenbeck 2003).

Bayesian approach apriori needs parametres of the evolutionary model. These parametres were also estimated by Modeltest.

Using this approach the datasets were divided into three partitions, representing first, second and third codon positions. In this approach all 1114bp were used. The Bayes analysis of the *Boa*-with-*Candoia* dataset ran for 7,000,000 generations and the chains were sampled every 1000th generation.

The *Boa*-no-*Candoia* dataset ran for 6,000,000 generations and the sampling frequency was every 100th generation. The analysis performed two simultaneous independent runs for

every dataset, as is the default setting. Each of them started from different randomly chosen trees.

After 6 and 7 milions of generations respectively the average standard deviation was approaching zero. The stationarity of parametres were checked by plotting log-likelihood scores (LnL) against generation numbers (Gen). Trees that did not reach the stationarity were discarded using two approaches again. First, I burned the conservative 25% of trees as is advised by the program authors so in the *Boa*-with-*Candoia* dataset 1,750 trees were burned. Second, I plotted LnL against Gen and checked the plateau by eye which can save some trees and so I only burned 100 trees.

In the *Boa*-no-*Candoia* dataset I burned 15,000 trees according to the conservative approach, and 6,000 trees according to the plotted values.

***** MCMC algorithm got its name after the famous casino in Monaco where the winning activities are random and repetetive as the functions included in the algorithm

4. RESULTS

The aim of this work was to revise the genetic pool of *Boa constrictor* across its range. Another goal was to establish phylogenetic pattern within its subspecies and to find out whether the currently recognized subspecies "deserve" their subspecies status or whether they should be promoted to species status. It is generally known that morphological characters are very different in *Boa constrictor* representatives and can vary significantly among individuals even from the same litter.

All the outcomes have biogeographic implications in the context of understanding the environmental changes that drove their radiation across South and Central America. These are mentioned in discussion in more details.

4.1. Data analysis

4.1.1. The input dataset

The cytb sequences were emloyed to determine the molecular phylogeny of boa constrictors. The alignment comprises 1114bp. Not every sample was amplified correctly for this length and so some of them have a few additional N bp at their 3' or 5' ends just to reach the desired length. This particular length was used because the GenBank sequences that were added to this study are 1114bp long (although they are incorrectly stated as 1113bp in GenBank) and I did not intend to adapt these. The GenBank sequences with their accession numbers are in Appendix 1.

The cytb sequences commenced with the methionine start codon ATG which seems to be universal for squamates (e.g. Campbell 1997, Slowinski & Lawson 2002).

Tree length distribution was slightly left skewed ($P \leq 0{,}01$ according to Hillis & Huelsenbeck 1992) in all data matrices and so it indicates phylogenetic signal in both datasets. The g1 values were estimated from 100,000 random trees and are listed in Table 4.

Dataset	g1 value	Constant characters	Parsimony uninformative characters	Parsimony informative characters
Boa* no *Candoia	-0,276404	714	121	279
Boa* with *Candoia	-0,292412	689	121	304
***Boa* no outgroup**	-0,274453	909	55	150

Table 4: g1 values with other features of the MP data matrices.

Maximum likelihood and Bayesian method used model gained by Modeltest 3.7 (Posada & Crandall 1998) incorporating likelihood and Akaike criterion that are based on hierarchical hypothesis testing of alternative models. For both datasets of cytb sequences the likelihood criterion utilized TVM+I+G model and TrN+I+G model was chosen by the Akaike criterion. These models are comparable with GTR (general time-reversible model Yang 1994) incorporating rate variation parameter (G) and invariable sites parameter (I). The list of models and their parametres for both datasets are summarized in Fig. 9 below.

Boa no *Candoia* dataset

Likelihood criterion

Model selected: TVM+I+G

-lnL = 5528.3779

K = 9

Base frequencies:

freqA = 0.3727

freqC = 0.3025

freqG = 0.0982

freqT = 0.2266

Substitution model/rate

Rate matrix

R(a) [A-C] = 1.4570

Akaike criterion

Model selected: TrN+I+G

-lnL = 5521.3296

K = 7

AIC = 11056.6592

Base frequencies:

freqA = 0.3657

freqC = 0.3161

freqG = 0.0813

freqT = 0.2369

Substitution model/rate

Rate matrix

R(a) [A-C] = 1.0000

R(b) [A-G] =15.1138
R(c) [A-T] = 1.1851
R(d) [C-G] = 0.5129
R(e) [C-T] = 15.1138
R(f) [G-T] = 1.0000

Proportion of invariable sites (I) 0.4804
Gamma distribution shape parameter 0.736

R(b) [A-G] = 21.3514
R(c) [A-T] = 1.0000
R(d) [C-G] = 1.0000
R(e) [C-T] = 9.7746
R(f) [G-T] = 1.0000

Proportion of invariable sites (I) 0.5398
Gamma distribution shape parameter 1.1143

Boa with *Candoia* dataset

Likelihood criterion

Model selected: TVM+I+G
-lnL = 6043.3325
K = 9

Base frequencies:
freqA = 0.3799
freqC = 0.3088
freqG = 0.0959
freqT = 0.2154

Substitution model/rate
Rate matrix
R(a) [A-C] = 1.1413
R(b) [A-G] =11.4906
R(c) [A-T] = 0.9015
R(d) [C-G] = 0.3602
R(e) [C-T] = 11.4906
R(f) [G-T] = 1.0000

Proportion of invariable sites (I) 0.5115
Gamma distribution shape parameter 0.8954

Akaike criterion

Model selected: TrN+I+G
-lnL = 6037.2510
K = 7
AIC = 12088.5020

Base frequencies:
freqA = 0.3736
freqC = 0.3220
freqG = 0.0798
freqT = 0.2246

Substitution model/rate
Rate matrix
R(a) [A-C] = 1.0000
R(b) [A-G] = 20.5475
R(c) [A-T] = 1.0000
R(d) [C-G] = 1.0000
R(e) [C-T] = 9.7472
R(f) [G-T] = 1.0000

Proportion of invariable sites (I) 0.5490
Gamma distribution shape parameter 1.2546

Fig. 9: Summary of evolutionary models parametres for both datasets used for ML and Bayesian analysis

Several approaches of maximum parsimony were used. First approach treats transitions and transversions equally, second approach performed different weighting schemes of transversions against transitions for both datasets. These schemes were: ts/tv 1/4 and 1/10. Both approaches resulted in the same trees with slightly different bootstrap values (see Appendix 4 and 5).

These maximum parsimony analyses resulted in the 112 most parsimonious trees (mpts). Descriptive statistics of these mpts are summarized in Table 5.

Dataset	**CI**	**RI**	**Tree lenght**
Boa* with *Candoia	0.6123	0.8665	962
Boa* no *Candoia	0.6471	0.8910	819

Table 5: Parameters describing the MP trees with no weighting schemes included.

Another approach was used when considering the affect of saturation in the third codon position. This position is the one where most changes in cytb occurs and these can undervalue the "phylogenetic information" (Xia et al. 2003). The sequence divergence between *Boa constrictor* sequences and especially *Candoia* outgroup sequence are not trivial (they also inhabit geographicly distant places) and so I excluded the third position for constructing a maximum parsimony tree just to avoid the saturation in the *Boa*-with-*Candoia* dataset. Additionally, the transitions were four times down-weighted in this analysis.

Nevertheless while excluding the 3rd position, a considerable amount of substitutions can be lost and so the resulted tree is based on changes that could affect the gene function. This MP analysis resulted in the 6 mpts. For tree illustration see Appendix 6.

Two kinds of distances were used while estimating the NJ trees. These were uncorrected p-distances and corrected Jukes-Cantor distances. In either case the bootstrap support was carried out and both approaches resulted in the same tree topology with similar bootstrap supports (see Fig. 13 and Appendix 3).

4.1.2. Support of tree topology and approaches used

In most of the analysis the topology of gained trees was supported by appropriate resampling method. This is usually bootstrap support for NJ, MP or ML method, in Bayesian approach the posterior probabilities are estimated. Because of the enormous size of my datasets all the analysis were more or less computationally demanding and time-consuming (except NJ). This was the main reason why *Boa*-with-*Candoia* ML tree does not contain the bootstrap support and *Boa*-no-*Candoia* ML tree includes bootstrap support for only 10 replicates. In addition, ML distances were estimated using the designed evolutionary model parameters and the tree topology was scored by Fitch-Margoliash method.

The bootstrap supports for the main clades are fairly high in all analysis and are marked along the branches in another section.

4.2. Tree section

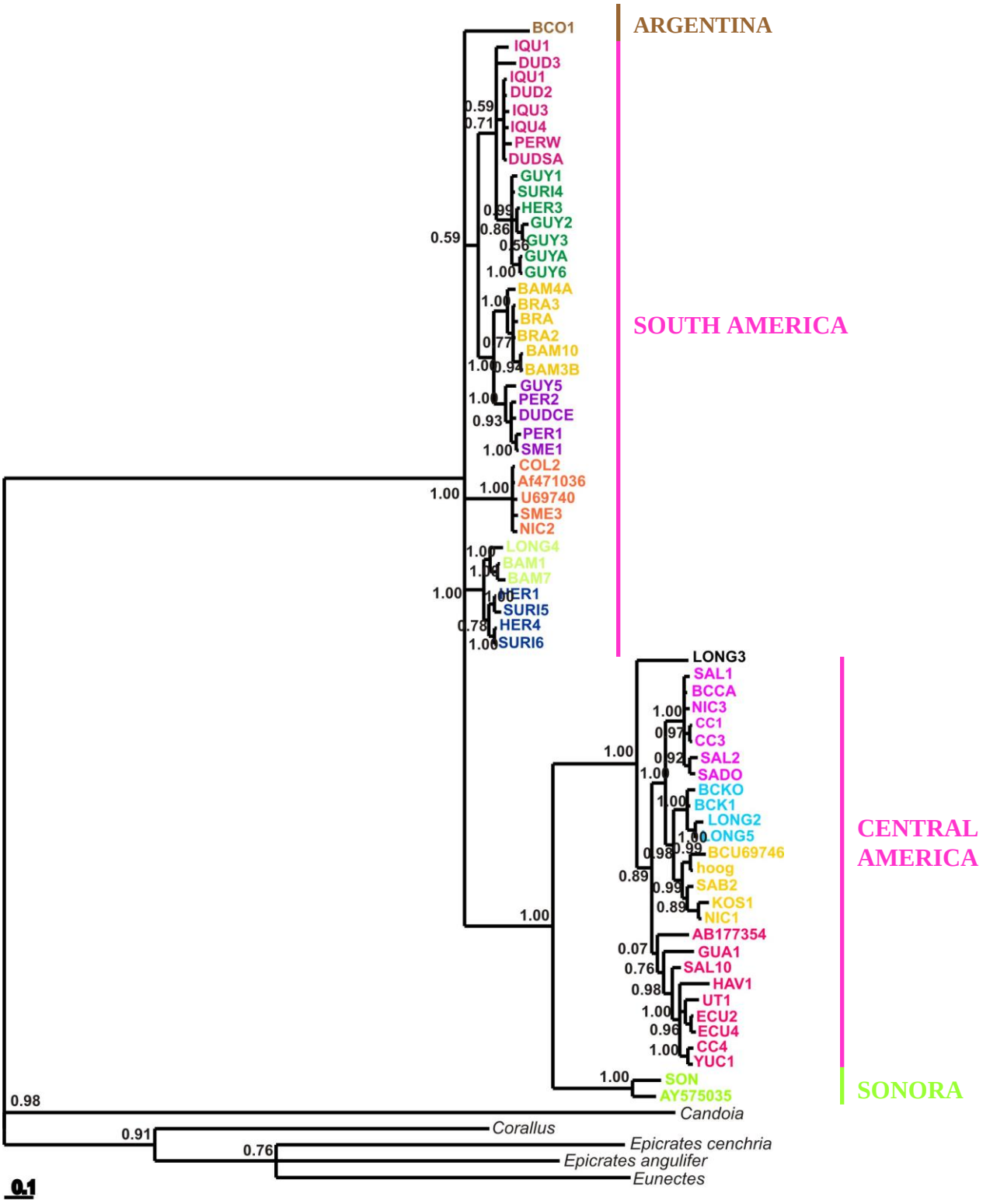

Fig. 10: The consensual Bayesian tree based on 1114 bp of cytochrome b and 72 haplotypes including outgroups (*Boa*-with-*Candoia* dataset). The analysis ran for 7,000,000 generations, 5,250 trees were used. The numbers on the nodes indicate the posterior probability values.

Fig. 11: Map showing the major clades incorporating in the Bayesian analysis across Central and South America. The coloured spots are only illustrative in some cases indicating just the country of origin of the samples obtained as the exact localities are not always known (the map outline from www.aquarius-geomar.de)

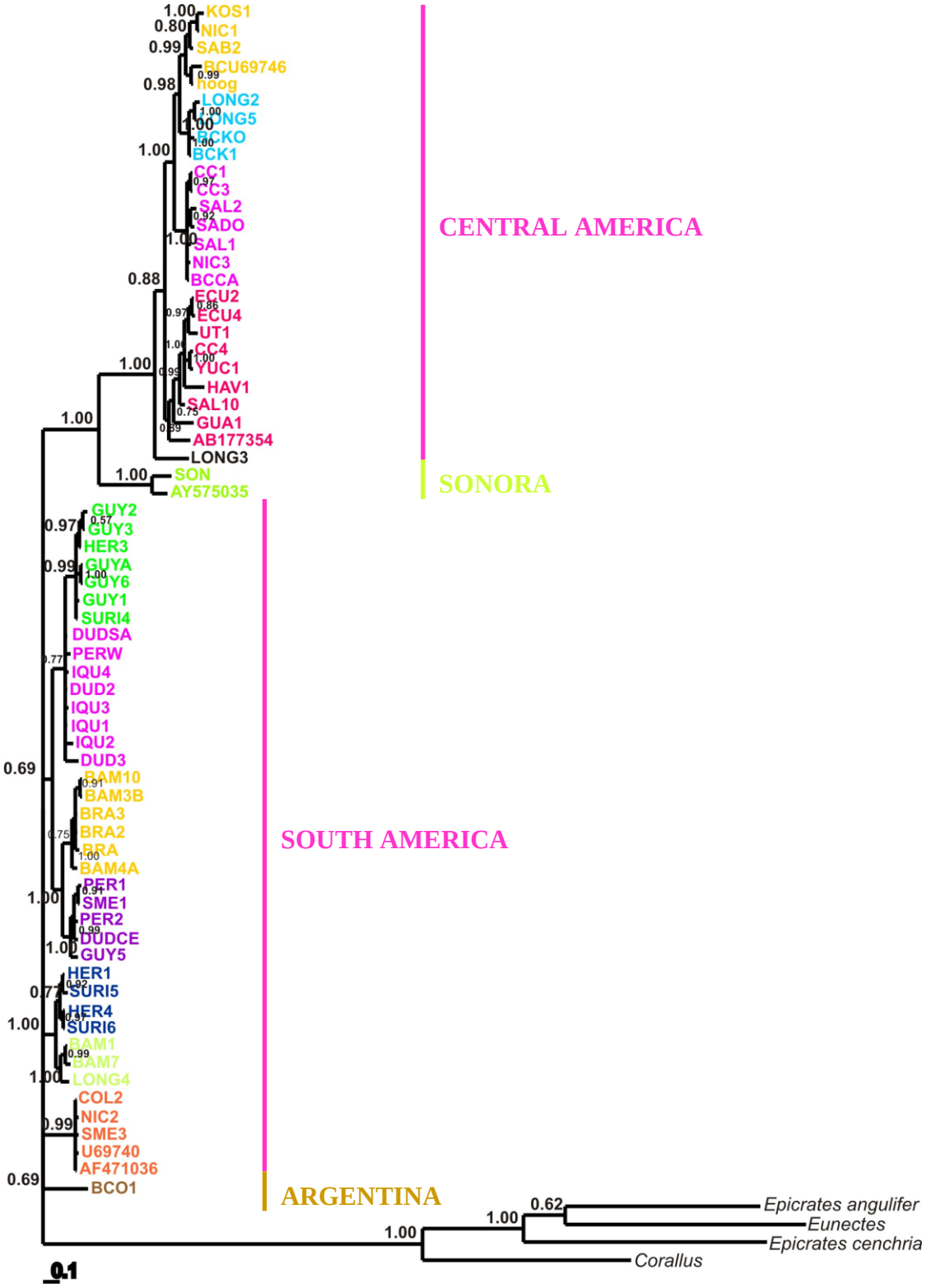

Fig. 12: The consensual Bayesian tree based on 1114 bp of cytochrome b and 71 haplotypes including outgroups (*Boa*-no-*Candoia* dataset). The analysis ran for 6,000,000 generations, 45,000 trees were used further. The posterior probability values are indicated on nodes. The positions of single clades are marked on the map on the previous page.

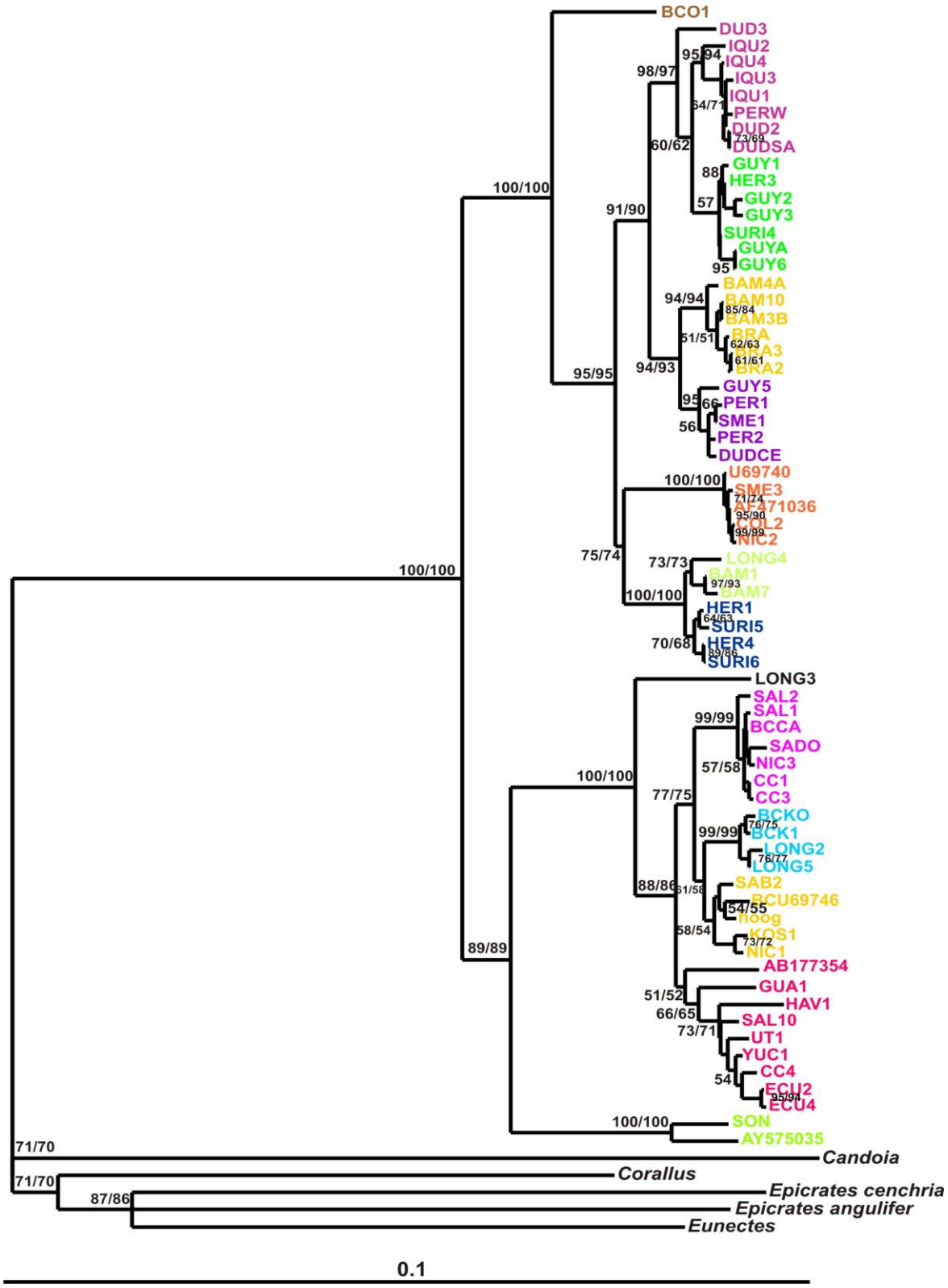

Fig. 13: NJ analysis applied on the *Boa*-with-*Candoia* dataset, the branches lenghts are included. The numbers along the branches show the bootstrap supports. The first number represents the bootstrap value using uncorrected distances with 10,000 pseudoreplicates, right of slash there are the bootstraps values using Jukes-Cantor distances with 10,000 pseudoreplicates. Branches without bootstraps were not supported in ≥ 50% of the 10,000 replicates.

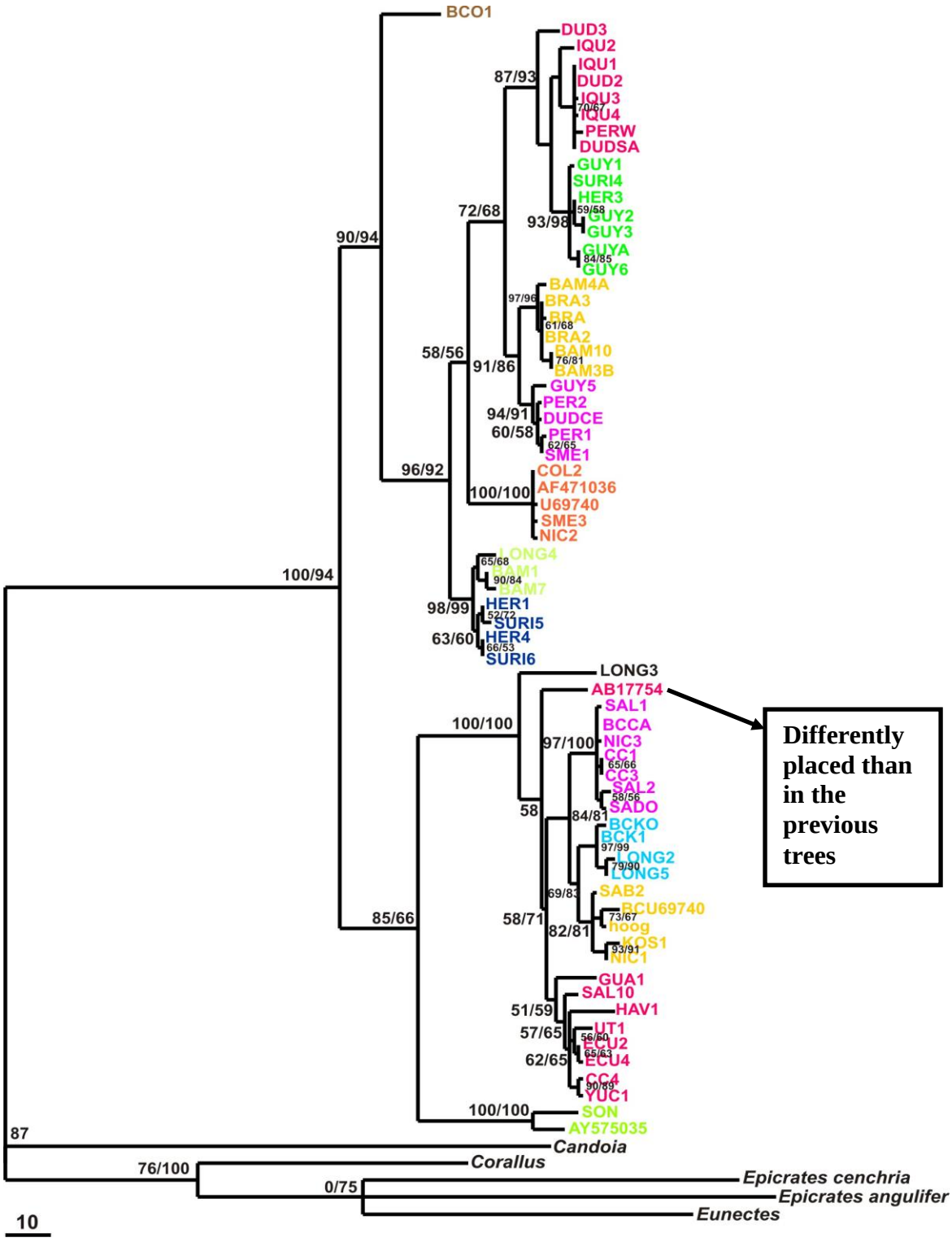

Fig. 14: MP tree for *Boa*-with-*Candoia* dataset using heuristic search with 100 replications and TBR branch swapping algorithm. The tree lenght is 962 steps, based on 425 variable characters, 304 of which are parsimony informative. Bootstrap proportions (%) are indicated on nodes, the first numbers are for *Boa*-with-*Candoia* dataset, right of slash are the bootstraps for *Boa*-no-*Candoia* dataset. Branches with no bootstrap values shown were present in <50% of the replicates.

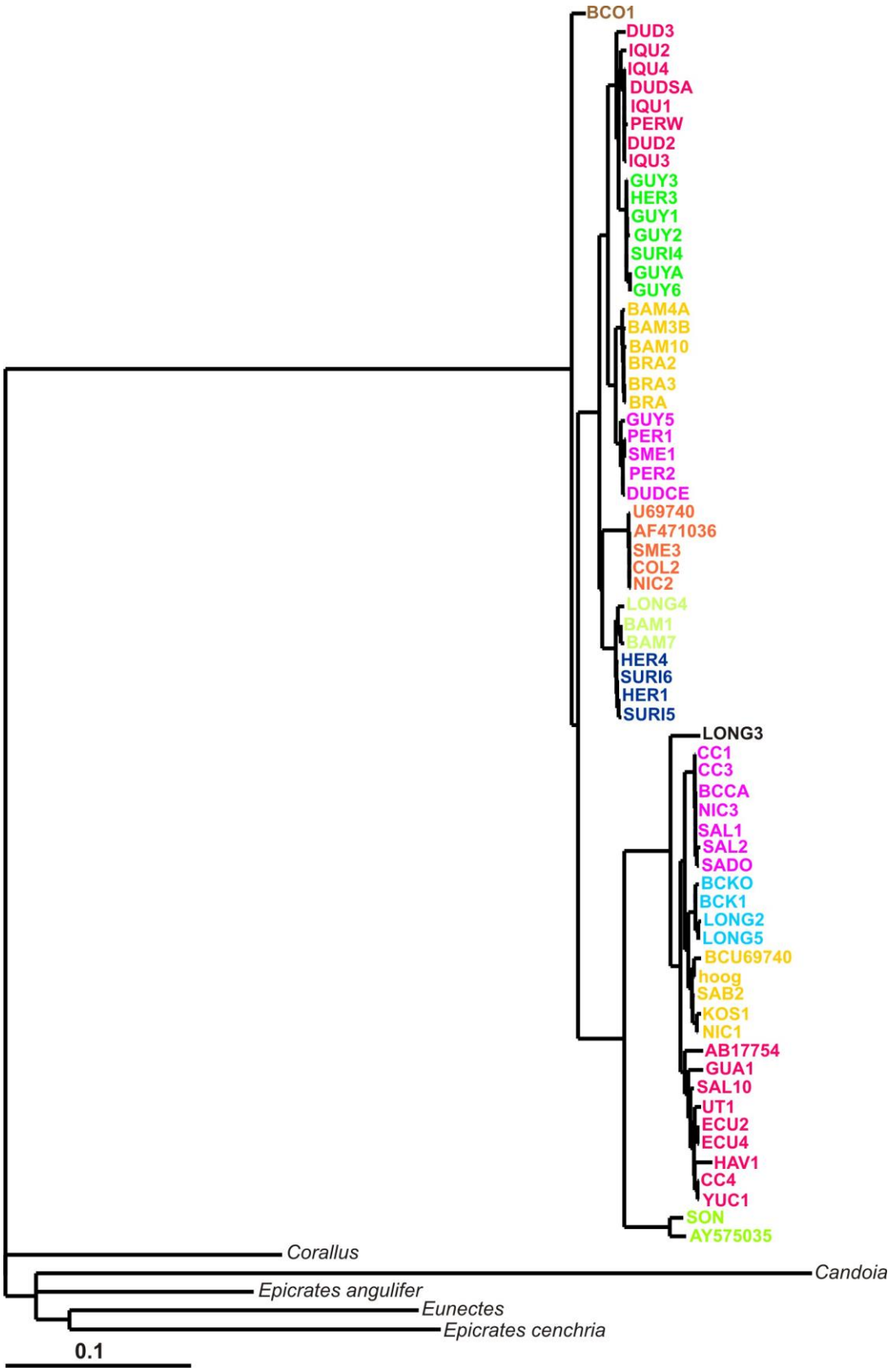

Fig. 15: ML distance tree based on the TVM+I+G model parametres with heuristic search and 100 replicates. The topology was scored using Fitch-Margoliash method.

4.3. Phylogenetic patterns

In all trees we can track a common pattern represented by South and Central American clades that are clearly separated. This separation is highly supported in all analyses (94-100% except ML analysis). South America could be divided into several bigger clades: the first consists of Peru + Guayana (DUDSA, PERW, IQU4, DUD2, IQU3, IQU1, IQU2, DUD3 + GUY2, GUY3, HER3, GUYA, GUY6, GUY1, SURI4), the second consists of Brazil + Guayana + Peru (BAM10, BAM3B, BRA3, BRA2, BRA, BAM4A + PER1, SME1, PER2, DUDCE + GUY5), the third is mixed South/Central American clade consisting of Colombia + Peru + Nicaragua (COL2, AF471036, U69740, SME3, NIC2), the fourth clade includes Ecuador + Brazil (LONG4 + BAM1, BAM7), the fifth Guayana + Surinam clade (HER1, HER4 + SURI5, SURI6) and the last clade including only Argentina (BCO1) that is sister to the rest of South American clades. BCO1 has in ML and Bayesian trees unsolved relationships and basicly does not belong to either South or Central America. In all NJ and MP trees is BCO1 clearly placed as a basal lineage to the rest of South American clades.

Central America has the following groups division: the first clade involves northern Mexico (SON, AY575035) and is sister to the rest of Central American clades, the second clade contains Ecuador (LONG3) which is, next to the Sonora clade, sister the the other Central American groups, the third partition connects Salvador + Nicaragua + Crawl Cay (Belize) (SAL2, SAL1, SADO + NIC3 + BCCA, CC1, CC3), the fourth group inlcudes Colombia + Ecuador + Costa Rica + Nicaragua + Hog and Saboga Islands (BCKO, BCK1, LONG2, LONG5 + BCU69746, KOS1 + NIC1 + hoog, SAB2) and the last fifth group consists of Guatemala + Mexico (Chiapas) + Salvador + Utila Island (Honduras) + Yucatán peninsula + Crawl Cay (Belize) + Ecuador (AB17754 + GUA1 + HAV1 + SAL10 + UT1 + YUC1 + CC4 + ECU2, ECU4).

There are very little differences in the described scheme across the all analyses. These are: the close relationship of ECU2,ECU4 + UT1 in Bayesian, MP and ML distance analyses versus ECU2, ECU4 + CC4 relationship in NJ tree, and AB17754 is not nested within the fifth Central American clade in MP analysis but is sister and more basal to them.

Surprisingly, LONG4+BAM1, BAM7 clade combines *B. c. amarali* and *longicauda* although I rather see membership of *B. c. longicauda* to Central America in close relationship with *B. c. imperator*. This could be caused by mistaken assessment of LONG4 sample or can implicate that *B. c. longicauda* is a hybrid species of *B. c. constrictor* or *B. c. imperator*. Another similar pattern could be tracked in all analyses in

the extra little clade composed of COL2, AF471036, U69740, SME3, NIC2 nested within South American groups where the possible mistake in country-of-origin assessment has to be considered or (and it cannot be ruled out) the introgression of some *B. c. constrictor* genetic features into *B. c. imperator* genome is possible. For this clade the relationships among involved samples remained unresolved except for NJ analysis.

B. c. occidentalis was the most divergent in MP and NJ analyses (employing the *Boa*-with-*Candoia* dataset) among South American groups and was a sister group to them. The Sonora clade was also significantly divergent from the rest of Central American clades in all analyses and is sister to them as well.

B. c. occidentalis clade is supposed to be a basal South American lineage (MP and NJ analyses) and the same could be proposed for the northmexican clade within Central America. In ML and Bayesian analyses there we can find *B. c. occidentalis* to be a sister taxon to both South and Central American clades. In NJ tree we can find a monophyly for the South American clade. In addition, we can find monophyly for the Central American clade in NJ and MP analysis.

Considering the sequence divergence it should be noted that there is 5.7-7.3 % between South and Central America, 6.1 % between *B. c. occidentalis* and Ecuador haplotype, 5.5 % between Sonora and others Central American haplotypes and among the rest of South and Central American clades there are low levels of sequence divergence (2 %). These are all approximate uncorrected distances values, the exact values can be found on the CD ROM attached (Appendix 10).

It may seem noticeable that the *B. c. occidentalis* clade is represented by only one haplotype. Six samples of this subspecies were gained, however, all belong to the same haplotype even when provided by several independent sources. This result was also found by Rivera et al. (2005), who studied two populations in Argentina and found only minor protein polymorphism between them. They concluded that abundant gene flow occurs or these populations have been separated only recently.

I was able to gain six samples labeled as coming from Sonora region. Four of the samples share the same haplotype as representative sample SON, the other two share their haplotype with sample called SAL10 (Salvador). Sample SON together with GenBank sample AY575035 create the divergent Sonora clade. AY575035 is localized in Mexican region called Michoacán which position is on the Pacific coast several hundred kilometres from Sonora on the same side of Mexican mountain chain Sierra Madre Occidental. This Sonora haplotypes variation is possible because Sonora is often used as a trademark for small forms of boas coming from N Mexico. Additionally, the

Sonora haplotypes variation could also be caused by the possible snakes movements. After they reached the Sonora region they might turn and head back towards South America as there is no sign that they continued further north. This maybe rather "strange" explanation but it was revealed in the giant anteater movements as well (Webb 1991).

4.4. Summary of tree trends:

- clear diversification between South and Central American groups
- deep divergence between *B. c. occidentalis* and other South American clades (except ML and Bayesian tree)
- deep divergence between Sonora clade and other Central American clades
- high bootstrap and posterior probabilities values for all important nodes in all analyses (except ML where bootstraps are not present)
- the uncorrected sequence divergence between *Boa constrictor* haplotypes and outgroups ranges from 16 to 19 % (time divergence roughly 8-9.5 MYA).
- no significant differences were found between trees constructed with either dataset (*Boa*-no-*Candoia* versus *Boa*-with-*Candoia*)

5. DISCUSSION

5.1. Cytochrome b and its rate of evolution

Mitochondrial gene for cytochrome b was used as an "yardstick" (Johns & Avise 1998) according to the knowledge that the mitochondrial DNA (mtDNA) is supposed to be the appropriate tool for phylogeographic analysis (Avise et al. 1987). The suitability of the nuclear genes had been argued before as their evolutionary rate is basicly much slower and so the assessment of the divergence time could be incorrectly determined (Bermingham & Moritz 1998). Even though some may argue the suitability of cytochrome b because it sometimes provide weak bootstrap support (Vidal et al. 1997).

In vertebrates the mtDNA evolves extensively fast, 5-10x faster than nuclear DNA. This is mainly because of the quick mutation (0.02 substitution per million years) and fixation rate. The weak repairing mechanisms were also revealed in mtDNA (Brown et al. 1979). Brown et al. (1979) considered the mtDNA to be eligible for species that demonstrate the divergence about 5 MYA.

However the estimation of appropriate rates of evolution in DNA sequences has always been problematical as it depends on quality fossil records (Near & Sanderson 2004) or some important events such as mountain range uplift . The rate of evolution is also affected by other factors such as body size or metabolic rate (Bronham 2002). In the meantime, I am not aware of any molecular clock calibration provided for Boidae and so the time-divergence estimation will be based on widely used molecular clocks that seem to me as a suitable tool for reflecting geological events fitting with the results of this study. These are 2 % sequence divergence per million years (Brown et al. 1979) even though these are widely used for mammals or avian groups (Riddle et al. 2000, Ribas et al. 2005). Zamudio & Greene (1997) used 0.47-1.32 % per million years for *Lachesis muta* occuring in the same region as boa constrictors but this is not taken into account in this study.

Johns & Avise (1998) found that cytb reveals greater genetic differences among reptilian families (0-25%) than among avian or mammalian ones (0-15%).

5.2. Biogeographic implications I

Going back to the very history of Central and South American connection we can track that these two places were connected for relatively long period (12 MYA) during Paleocene. This contact was interrupted from Eocene to Pliocene when the region of current Nicaragua-Colombia was inundated (Savage 1966, Zamudio & Greene 1997). During this time the separate evolution on both sides of the flooded area seems

to occur (Stuart 1957). This affected for example the populations of *Ameiva* and the mainland anoles (Savage 1966).

Central America is not actually a land in its true meaning but a mass of oceanic sediments. Based on this knowledge it is not hard to understand that the flooded region became silted again. In Oligocene the volcanic activities coupled with orogenic processes caused the first island-like landmasses to emerge in the channel and in Early Pliocene the renovated connection between Central and South America occured once again (Savage 1966, Cadle 1985).

During the Late Pliocene the Central American highlands were rised to their present elevation. Additionally, during Pliocene-Pleistocene the uplift of Sierra Madre mountain range in Mexico was in progress.

Since the Early Pliocene there is no evidence that this conjunction would have been ever interrupted. Plistocene is therefore a key period when the extensive fauna exchange between those two continents started to take place. Central American fauna invasion was for some reason significantly more frequent and succsessful than the penetration of the South American species (Savage 1966, Webb 1991). This would support the Savage's suggestion that *Boa constrictor* is originally Central American species that invaded South America using the Pliocene connection (the same was assumed for Iguanidae).

5.3. Biogeographic implications II

The uplift of Andes, Panama Isthmus creation, climatic oscillations and several other forces affecting the Neotropical region has been discussed for ages and are indisputable. Each of them affected the Neotropical populations in different ways and it is difficult to determine which had been the main cause of speciation for either population (Costa 2003). The speciation model assumes to be rather "species-specific" (Bush 1994). The speciation process commencement is dated to 10 MYA but the crucial time is supposed to be around 2 MYA (Rull 2006). The pre-Quaternary vicariant events taking place in Amazonian region were supposed to be one of the main forces for the species splitting there but in the meantime the climate/glacial oscillations seem to be more probable (e.g. Pennington et al. 2000, Ribas et al. 2005, Rull 2006).

One of the most discussed question arises when it comes to determination of the southern versus central (northern) American origin of Neotropical species. Expectedly every scenario has its representatives.

Wüster et al. (2005) proposed the North American origin for *Crotalus*, which is supported by sequence divergence within Central American clades (6-11%) and within South American clades (1.3%). The same was assumed for *Lachesis* (Zamudio & Greene 1997) where the sequence divergencies among Central American clades were 5.3% and South American 1.2% and whose Eurasian and consequently North American origin had been suggested.

My data do not clearly support either Central or South American origin of *Boa constrictor*. However, I rather see South American origin more probable. I consider that the possible source population dwelled in southern Colombia and was consequently divided into north and south Colombian population by Colombian Andes rising. These populations could communicate for some time after splitting because some places with lower elevation still remained. This could explain the two mixed clades that were presented before (LONG4, BAM1, BAM7 and COL2, AF471036, U69740, SME3, NIC2) although I emphasise the need to treat them with a high degree of causion (also see the unusual appearence of NIC2 on the CD ROM attached). The similar scenario was revealed for anoles lizards by Glor et al. (2001) and in *Thamnophis* by De Queiroz et al. (2002), as well. South American ancestor of Neotropical Boinae was suggested by Albino (1993), however, the Sonora clade origin remains difficult for reasonable explanation. But the divergence of Sonora clade does not seem so unusual because the divergent north Mexican population was also found in *Ctenosaura* lizards whose South American origin was also proposed (Hasbún et al. 2005). The South American origin was also proposed for other Neotropical animals (Eberhard & Bermingham 2004, Henderson 2004). Taking into account Rosen's suggestions (1975) about the southern and northern routes that collided in Central American region the complicated explanation for current *Boa constrictor* distribution, according to the results of this study, remains.

The main splitting on South and Central American clades is confirmed by all Neotropical species whose phylogeography was studied, as far as I am concerned. Although the Central American region had been occupied ever before, the Panama Portal uplift (3.5 MYA) seems to be the reason for massive biota exchange between Central and South America (Savage 1966, Webb 1991, Eberhard & Bermingham 2004), as has been mentioned before. This event is dated to the Pliocene which is congruent with our data as the sequence divergence is approximately 7 % between South and Central America.

The rising of any mountain range always affects fauna and flora nearby and the Andes orogeny in any part of South America is no exception. Their affect on atmospheric circulation and subsequent changes in precipitations, temperatures and vegetation composition is undeniable (Pennington et al. 2000).

The commencement of Andes orogeny is, by some sources, dated back to the Cretaceous (Zamudio & Greene 1997) when the elevation did not reach more than 1000 metres above the sea level. Another rising process continued through Cenozoic. South American Cordillera comprised an important barrier for western (Peruvian, Ecuadorian) and amazonian species in this period (Bush 1994). This affect was also mentioned by Vidal et al. (2005) whose research envolved one of the boine snakes, *Corallus caninus*. They found a significant divergence of Peruvian group from the rest of South American groups (approx. 16 % of sequence divergence). The same scenario was supposed for our data as well but this finding is not eventually confirmed by our data.

It is worth mentioning that Colombian region is another important part of Neotropical speciation. Not only that right here the Central and South America collided but its tectonic activity and sea level fluctuations during Tertiary had a definite influence on the ancestors of present populations. It has been assumed that the unsettled sea level and mountain uprise affected the northern Colombian coast causing the separation of western and eastern biota in this region (Cracraft & Prum 1988). The whole process is dated to Eocene when Cordillera Occidental (Colombia) reached the elevation of 5000m. Different time-scale (Pleistocene) for this area was published before (Haffer 1969), however Cracraft & Prum (1988) concluded that in Pleistocene only "culmination" of Colombian Andes orogeny occured. Another time-scale is proposed for Mérida Andes in Colombia that is dated to Miocene (about 8 MYA, Hoorn 1993). According to this data it becomes obvious that several centres for Colombian Andes orogenesis occured.

5.4. Data relationship analysis

The clear division of Central and South America in our data indicate one important fact that currently recognized *B. c. imperator* is not, in the event, subspecies of *B. c. constrictor* but rather distinct species. This fact is supported by 7% of sequence divergence between Central and South American group. Additionally, the high bootstrap values supporting Central and South American groups implicate their monophyly. Long branches indicate the deep divergence between Sonora clade and the rest of Mezoamerican haplotypes and the same assumption for Argentinean *B. c.*

occidentalis and the rest of South American group rather indicate ancient relationships and ancestral state of *B. c. occidentalis* and northmexican *B. c. imperator*. The uncorrected distance between these two considerably divergent species is 6.2 % which would imply the separation approx. 3 MYA in case that they both came from the same ancestral population.

In addition, the 5% sequence divergence is generally considered to be the "magic" boundary between species and subspecies status. Based on this presumption both *B. c. occidentalis* and northmexican *B. c. imperator* are so significantly different from the remaining clades that their subspecies status should be revised.

Harris (2002) investigated the differences in the species status inside several herpetofaunal generas and found that it varies between 0 and 25% (*Emoia, Geochelone, Uma, Thamnophis* ect). This implies that our discoveries are not trivial.

Short branches (or small sequence differences) within Mezo and South American haplotypes indicate that these regions were colonized quickly and very recently. The same scenario was suggested for other Neotropical animals (Savage 1982, Bermingham & Martin 1998, Cortéz-Ortiz et al. 2003, Eberhard & Bermingham 2004, Wüster et al. 2005) and is dated to about 1.1 MYA.

Both South and Central America are very well covered by our samples. Every clade is represented by several haplotypes which country of origin can be found in Appendix 2 but the exact localities are only rarely known.

All Mezoamerican samples come from either Atlantic or Pacific coast and it seems that occasional highlands occuring in this region do not represent a critical barrier for their movements. This is in contrast with result of Zamudio & Greene (1997) for two populations of *Lachesis* from Costa Rica where the highland seems to be causing their sequence detachment (5.3 %). This finding could point the undemanding character of *Boa constrictor* individuals on the environmental structure and their adaptation ability (Langhammer 1983). Nevertheless, this was rejected for *B. c. occidentalis* who seems to be dependent on the certain habitat (Rivera et al. 2005).

Additionally, South American samples also cover the whole region, in certain parts more or less abundantly. It is obvious that South American region is rich of water sources such as the Amazon, Paraná or Orinoco rivers. This may seem to be a significant limitation for some species distribution but this does not appear to be the case for Boinae snakes. One of the *Boa constrictor* relatives-the anacondas-populated this kind of habitat and boa constrictors themselves seem to have no difficulties overcoming the water sources.

5.5. Biogeographic hypothesis for *Boa constrictor* distribution

To sum it all up, several hypotheses for *Boa constrictor* distribution could be pronounced:

1) These snakes originated in South America and expanded to Central America soon after the Panama Isthmus closure. Some population got into Sonora (Mexico) region where they evolved amazingly fast. This is not so unexpected considering the desert-like Sonora region where any species had to undergo certain adaptations to allow its survival. Also the rich partitioning among South American clades can only confirms boa constrictors South American origin.

   The 5-6% sequence divergence between Sonora and other Central American clades can also indicate that the northmexican population was isolated from the other Central American populations for the next 2.5-3 million years. Those individuals that were part of the north Colombian population could possibly be one of the first to reach Central America. This relationship could be confirmed by a placement of our Colombian samples among Central American clades (BCKO, BCK1). In addition, Peruvian coast seems to be colonized via W Equador and W Colombia i.e., by populations belonging to the same clade as those that reached Central America after the Panama Portal closure.

   Peruvian Andes could represent a significant barrier for *Boa constrictor* penetration and had to be bypassed through the alternative Central American/Colombian way.

   On the other hand, the Peruvian Andes did not have to play that important role in boas distribution as could be expected and that certain way across them could existed. I would assume that Maraiión valley along the Marañón River not far from the Napo refuge (see Figure 16) could be that certain crossing place. It was discovered that right this place was used by several avian groups as a bridge enabling them to reach Peru and Ecuador on both sides of Andes (Haffer 1969). Our LONG4,BAM1,BAM7 and COL2, AF471036, U69740, SME3, NIC2 clades are rather consistent with such explanation.

2) This scenario takes into account the possible oceanic currents flowing along the Atlantic coast of South America towards the Carribean region and further north during the Tertiary (Hower & Hedges 2003, Wüster et al.2005). These currents might have transported some of the South American species including *Boa*

constrictor that got to Mexico and evolved separately for some time. After the Panama Isthmus creation northmexican boa constrictors started their way back to South America and mixed with the populations coming from South America itself in the Panama-Colombian area. Glor et al. 2001 suggested similar scenario for *Anolis* lizards, however they did not explain in details the way these lizards occured in Central America. Currents help was proposed for example for *Ameiva* lizards distribution going from South America towards West Indies (Hower & Hedges 2003).

3) This presumption takes into consideration more-or-less ecological requirements and also the first hypothesis of South American origin of boa consrictors. About 3-2.5 MYA the assymetrical "Great American Biotic Interchange" began (Webb 1991) and mainly North and Central American animals started their way towards South America using especially eastern Panamanian lowlands for their tracks (Savage 1966). Ten species out of seven rodents families and some other animals reached South America during this time (capybaras, porcupines, armadilloes, sloths and several others including cricetid rodents, Webb 1991). The wide range of *Boa constrictor* prey (from shrimps to monkeys) has been documented lately (Quick et al. 2005). This rich food spectrum would not be so surprising considering the extensive size of these snakes but this is true for only adults. It is generally known among breeders that the crucial time for any species growing is their "baby" stage. And this is when the penetration of the little rodents gains its importance. The baby rodents may have been a valueable and easy prey for baby boas that took this advantage for rapid and enormous breeding boom (about 1-2 MYA). In other words, distribution and fairly rapid evolution might have only reflected the murid rodents invasion. It was assumed before that the favoured prey of *Boa constrictors* are just rodents (Henderson 2004).

4) The last hypothesis that I would like to present here is Andean refuge origin of boas populations in South America. During the last million years in Pliocene the South American environment was affected by glacial cycles that caused a penetration of Andes species into Amazon basin (Noonan & Gaucher 2005). I consider that Boa constrictor is one of the species that originally dwelled in the higher elevation in Peruvian Andes and during the repetetive glacial cycles it

spread into the other parts of South American continent. I found a particular similarity between the placement of my clades on the map and Haffer's (1969) map illustration of refuge in South America during Pleistocene (Figure 16). I assume that the Napo refuge (number 5 on Haffer's map) could be the source from which they spread.

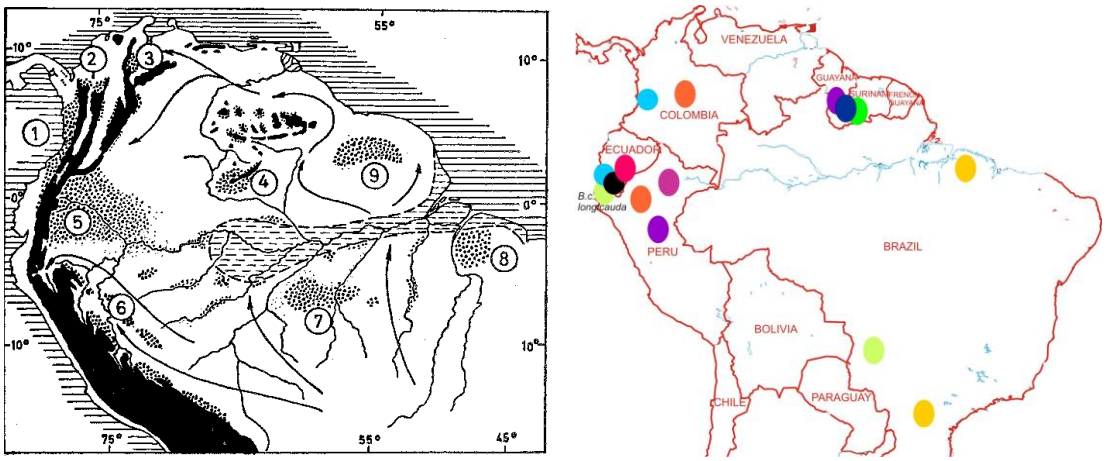

Fig. 16: Comparison of Haffer's (1969) refuge map and my South American clade-illsutrative map. The numbers on Haffer's map indicate following: 1-Chocó refuge 2-Nechí refuge 3-Catatumbo refuge 4-Imerí refuge 5-Napo refuge 6-East Peruvian refuge 7-Madeira-Tapajós refuge 8-Belém refuge 9-Guiana refuge

5.6. Taxonomy and conservation implications

The current taxonomy rather overestimates species diversity of *Boa constrictor* as there are seven currently distinguished mainland subspecies and four insular.

Russo (2002) doubted the existence of *B. c. melanogaster* from Ecuador that was discribed for the first time in 1983 by Langhammer and rather believes that it represents a different colour morph of *B. c. constrictor* from Peru. Although I lack *B. c. melanogaster* in my samples, I rather agree with Russo (2002) suggestion.

On the other hand, I am not in agreement with Price & Russo (1991) that discribed *B. c. longicauda* from Peruvian Tumbes province. This "subspecies" shares parapatric range with *B. c. imperator* and so, according to the results of this study, I would suggest that so-called *B. c. longicauda* is in fact another population of *B. c. imperator* that just used the Pacific coast lowlands and eventually reached a higher altitude (3000m) in Ecuador. This finding is further supported by the very close relationship of Chocó (Colombia) and Ecuadorian snakes in my samples. The proximal relationship of Chocó region and

Central America has been supported in birds (Cracraft & Prum 1988) and our discoveries are congruent with it.

The subspecies status for *B. c. ortonii* is, in my point of view, doubtful as well. Although I do not possess samples of it, I tend to assume it to be an isolated population of *B. c. constrictor* or *B. c. imperator* (suggested by Price & Russo 1991).

The subspecies statuses for all the insular boa constrictors are also controversial. I only hold samples of *B. c. sabogae*, however, it is clearly nested in one of the *B. c. imperator* clades sharing it with Hog Island boas. This finding rather confirms the weak difference between the mainland and island boa constrictors and so I suggest, according to Price & Russo (1991), that *B. c. sigma* and *B. c. sabogae* should be considered as *B. c. imperator* fragments and *B. c. orophias* and *B. c. nebulosa* to be derivatives of *B. c. constrictor.*

The last subject that I would like to raise is a substantially different appearence of Crawl Cay boas (Belize). Their dwarfed size, different head shape and their ability to change colour seemed to Boback (2005) so nontrivial that he hypothesized it to be a new subspecies. However, my molecular data nest it clearly in a clade with other Central American "*imperator*" samples.

To sum up, the results of this study support a suggestion that the name *B. c. constrictor* should be set up for all South American individuals including adjacent islands except *B. c. occidentalis*. Additionally, the species status should be set up for *imperator* (i.e., *Boa imperator*) and *occidentalis* (i.e., *Boa occidentalis*) as their sequence divergence is considerebly behind the subspecies status (Henderson & Hedges 1995). Also at least subspecies status should be considered for Sonora representatives. However, I realize the certain need for additional work on northmexican populations of *B. constrictor* and other groups of animals coming from the same region just to confirm their significant difference.

It deserves mentioning that Langhammer (1983) shyly suggested that *B. c. imperator* crossed Peruvian Andes one day and became a source subspecies for later estabilished *B. c. occidentalis* and *B. c. amarali.*

Our datasets contained 67 *Boa constrictor* haplotypes from 115 samples used. This indicate a rich genetic pool within their populations, however, other findings provided in this study could improve the conservation management of these species as there is a continuous evidence that the population sizes are more and more affected by human cause (Boback 2005).

Because the discoveries of this study are not worn and only handful of studies deal with specific phylogeographic hypothesis for species having both Central and South American distribution I would suggest more detailed revision of these species to help us understand the complex Neotropical history. These could be for example *Kinosternon, Rhinoclemys,* mainland *Anolises, Basciliscus, Iguana, Gonatodes, Liotyphlops, Clelia, Dryadophis, Drymarchon, Enulius, Imantodes, Lampropeltis, Micrurus, Bothrops, Caiman, Crocodylus*.

6. CONCLUSION

- so far I am not aware of any published study focusing on the phylogenetic relations among the *Boa constrictor* subspecies in this dimension
- clear divergence between Central and South American groups indicating that *B. c. imperator* should be elevated to full species *B. imperator*
- I suggest that the different lineages are clearly divided and I just emphasise the need to treat them as new species. All South American boa constrictors should be known as *B. c. constrictor* (except *B. c. occidentalis*) and *B. occidentalis* should be used for Argentinian boa. All the Central American boas should be named *B. imperator* (possibly except Sonora clade) and subspecies status should be considered for northern Mexico (Sonora) representatives
- explanation for placement of LONG4 and NIC2 among South American clades should be treated with special care, we may deal with erroneous assessment or mistake in line of descent and for different interpretation would be essential to discovery the same phenomenon repeatedly
- several hypothesis on *Boa constrictor* distribution have been proposed but it remains uncertain to assess which of them reflect the real history and also all of them together could be nonexclusive
- because there is certain rationality and logic in the samples ditribution I assume that this could serve as the feedback that the samples come from credible sources
- there is a lack of the ancestral link between *Boa constrictor* and the outgroups used because I think there had to be a closer ancestor
- we are dealing with a particularly young species extremely adaptable whose invasions we even witnessed in the previous decades – Aruba Island invasion (Quick et al. 2005), Cozumel Island invasion (Martínez-Morales & Cuarón 1999) and it is more than probable that another ones will follow
- quick and recent radiation of *Boa constrictor* if we employ 2% sequence divergence/MY $^{-1}$ molecular clocks

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8. APPENDIX SECTION

TAXON NAME	ACCESSION NUMBER	REFERENCES	NOTE
unspecified *Boa constrictor*	BCU69740	BN Campbell 1997	
Boa c.imperator	BCU69746	BN Campbell 1997	Costa Rica
unspecified *Boa constrictor*	AB177354	Dong S & Kumazawa Y 2005	Same haplotype as NC007398
	AF471036	Lawson R et al.2005	
unspecified *Boa constrictor*	AY575035	Lawson R - unpublished	Mexico, Michoacán
unspecified *Boa constrictor*	NC007398	Dong S & Kumazawa Y 2005	Same haplotype as AB177354
Corallus caninus	CEU69770	BN Campbell 1997	
Eunectes notaeus	ENU69810	BN Campbell 1997	
Candoia carinata	CCU69754	BN Campbell 1997	

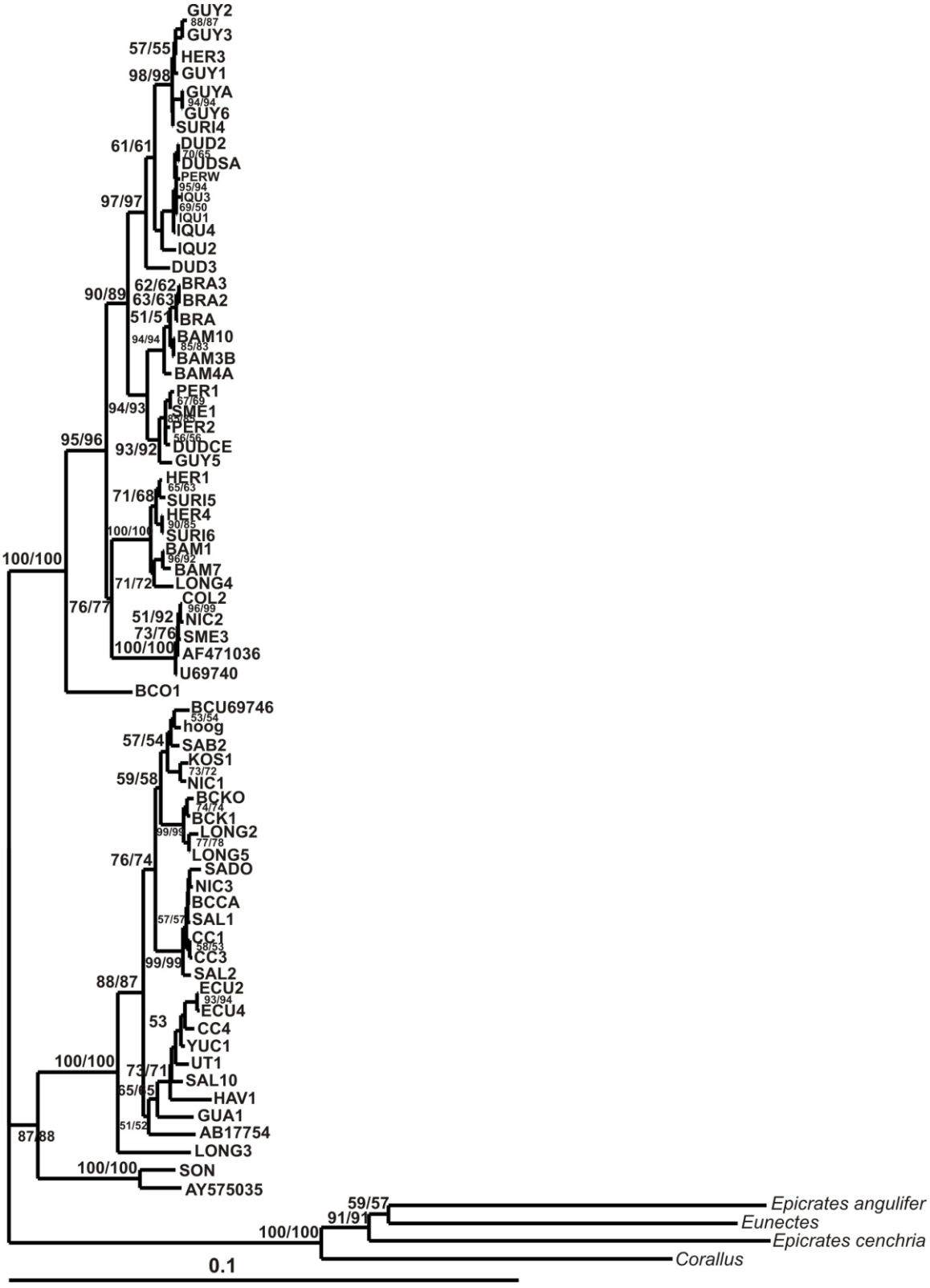
Appendix 1: GenBank accession numbers of used sequences

Sample name	Subspecies	Country/Locality
BCO1	*B.c.occidentalis*	Argentina
BCOS	*B.c.occidentalis*	Argentina
BCO2	*B.c.occidentalis*	Argentina
BCOFRI	*B.c.occidentalis*	Argentina
BCO3	*B.c.occidentalis*	Argentina
BCO5	*B.c.occidentalis*	Argentina
BCCA	*B.c.imperator*	Belize, Crawl Cay
BCCB	*B.c.imperator*	Belize, Crawl Cay
CC1	*B.c.imperator*	Belize, Crawl Cay
CC3	*B.c.imperator*	Belize, Crawl Cay
CC4	*B.c.imperator*	Belize, Crawl Cay
CC5	*B.c.imperator*	Belize, Crawl Cay
BAM6	*B.c.amarali*	Bolivia
BAM1	*B.c.constrictor*	Brazilia
BAM2	*B.c.constrictor*	Brazilia
BAM7	*B.c.amarali*	Brazilia
BAM8	*B.c.amarali*	Brazilia
BRA	*B.c.constrictor*	Brazilia
BRA2	*B.c.constrictor*	Brazilia, Marajó
BRA3	*B.c.constrictor*	Brazilia
COL1	*B.c.imperator*	Colombia
COL3	*B.c.imperator*	Colombia
COL4	*B.c.imperator*	Colombia
COL2	*B.c.imperator*	Colombia
BCK0	*B.c.imperator*	Colombia, Bei Choco
BCK1	*B.c.imperator*	Colombia, Bei Choco
KOST	*B.c.imperator*	Costa Rica
KOS1	*B.c.imperator*	Costa Rica
KOS2	*B.c.imperator*	Costa Rica
KOS3	*B.c.imperator*	Costa Rica
KOS7	*B.c.imperator*	Costa Rica
COS4	*B.c.imperator*	Costa Rica, Canuita
ECU2	*B.c.imperator*	Ecuador
ECU4	*B.c.imperator*	Ecuador
GUA2	*B.c.imperator*	Guatemala
GUA3	*B.c.imperator*	Guatemala
GUA1	*B.c.imperator*	Guatemala, Escuintla
HER1	*B.c.constrictor*	Guayana
HER2	*B.c.constrictor*	Guayana
HER3	*B.c.constrictor*	Guayana
HER4	*B.c.constrictor*	Guayana
HER5	*B.c.constrictor*	Guayana

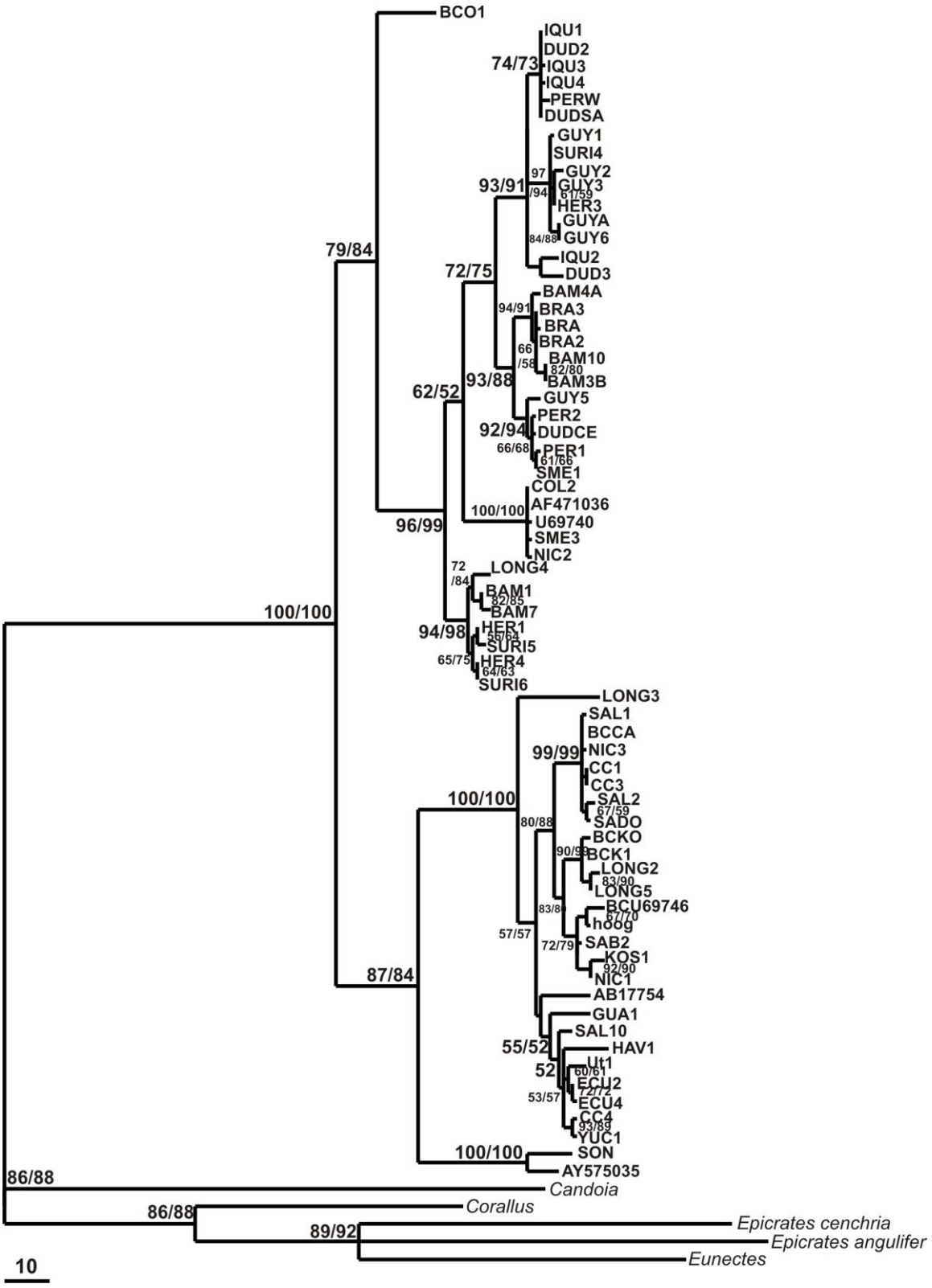
HER6	*B.c.constrictor*	Guayana
GUYA	*B.c.imperator*	Guayana
GUY1	*B.c.imperator*	Guayana
GUY2	*B.c.imperator*	Guayana
GUY3	*B.c.imperator*	Guayana
GUY4	*B.c.imperator*	Guayana
GUY5	*B.c.imperator*	Guayana
GUY6	*B.c.imperator*	Guayana
GUY7	*B.c.imperator*	Guayana
GUY8	*B.c.constrictor*	Guayana
JAHO	*B.c.imperator*	Honduras
HOOG	*B.c.imperator*	Honduras, Hog Island
HOG2	*B.c.imperator*	Honduras, Hog Island
HOG3	*B.c.imperator*	Honduras, Hog Island
UT1	*B.c.imperator*	Honduras, Utila
OBYC	*B.c.imperator*	hybrid
MECA1	*B.c.imperator*	Mexico, Cancún
HAV3	*B.c.imperator*	Mexico, Chiapas
HAV4	*B.c.imperator*	Mexico, Chiapas
JAK5	*B.c.imperator*	Mexico, Chiapas
HAV2	*B.c.imperator*	Mexico, Chiapas
HAV1	*B.c.imperator*	Mexico, Chiapas
SON1	*B.c.imperator*	Mexico, Sonora
SON2	*B.c.imperator*	Mexico, Sonora
SON3	*B.c.imperator*	Mexico, Sonora
SON4	*B.c.imperator*	Mexico, Sonora
SON5	*B.c.imperator*	Mexico, Sonora
SON	*B.c.imperator*	N Mexico, Sonora
NIC1	*B.c.imperator*	Nicaragua
NIC2	*B.c.imperator*	Nicaragua
NIC3	*B.c.imperator*	Nicaragua
NIC4	*B.c.imperator*	Nicaragua
LONG2	*B.c.longicauda*	not known
LONG3	*B.c.longicauda*	not known
LONG4	*B.c.longicauda*	not known
LONG6	*B.c.longicauda*	not known
LONG5	*B.c.longicauda*	not known
SAB2	*B.c.sabogae*	Panama, Saboga Island
SME1	*B.c.constrictor*	Peru
SME2	*B.c.constrictor*	Peru
SME3	*B.c.constrictor*	Peru
PER1	*B.c.constrictor*	Peru
PER2	*B.c.constrictor*	Peru

PER3	*B.c.constrictor*	Peru
IQU1	*B.c.constrictor*	Peru, Iquitos
IQU2	*B.c.constrictor*	Peru, Iquitos
IQU3	*B.c.constrictor*	Peru, Iquitos
IQU4	*B.c.constrictor*	Peru, Iquitos
DUDX	*B.c.constrictor*	Peru, Iquitos
DUDTM	*B.c.constrictor*	Peru, Iquitos
DUDCE	*B.c.constrictor*	Peru, Iquitos
DUDSA	*B.c.constrictor*	Peru, Iquitos
DUD2	*B.c.constrictor*	Peru, Iquitos
DUD3	*B.c.constrictor*	Peru, Iquitos
PERW	*B.c.constrictor*	Peru, Tarapoto, Amazonia
BAM3B	*B.c.amarali*	S Brazilia
BAM4A	*B.c.amarali*	S Brazilia
BAM10	*B.c.amarali*	S Brazilia
SAL1	*B.c.imperator*	Salvador
SAL2	*B.c.imperator*	Salvador
SAL10	*B.c.imperator*	Salvador
SADO	*B.c.imperator*	Salvador
SURI1	*B.c.imperator*	Surinam
BAM5	*B.c.amarali*	Surinam
BC1	*B.c.imperator*	Surinam
SURI2	*B.c.imperator*	Surinam
SURI3	*B.c.imperator*	Surinam
SURI4	*B.c.imperator*	Surinam
SURI5	*B.c.imperator*	Surinam
SURI6	*B.c.imperator*	Surinam
SURI7	*B.c.imperator*	Surinam
YUC1	*B.c.imperator*	Yucatan, Cancún,Tulum
ML20	*B.c.constrictor*	Hybrid, not known

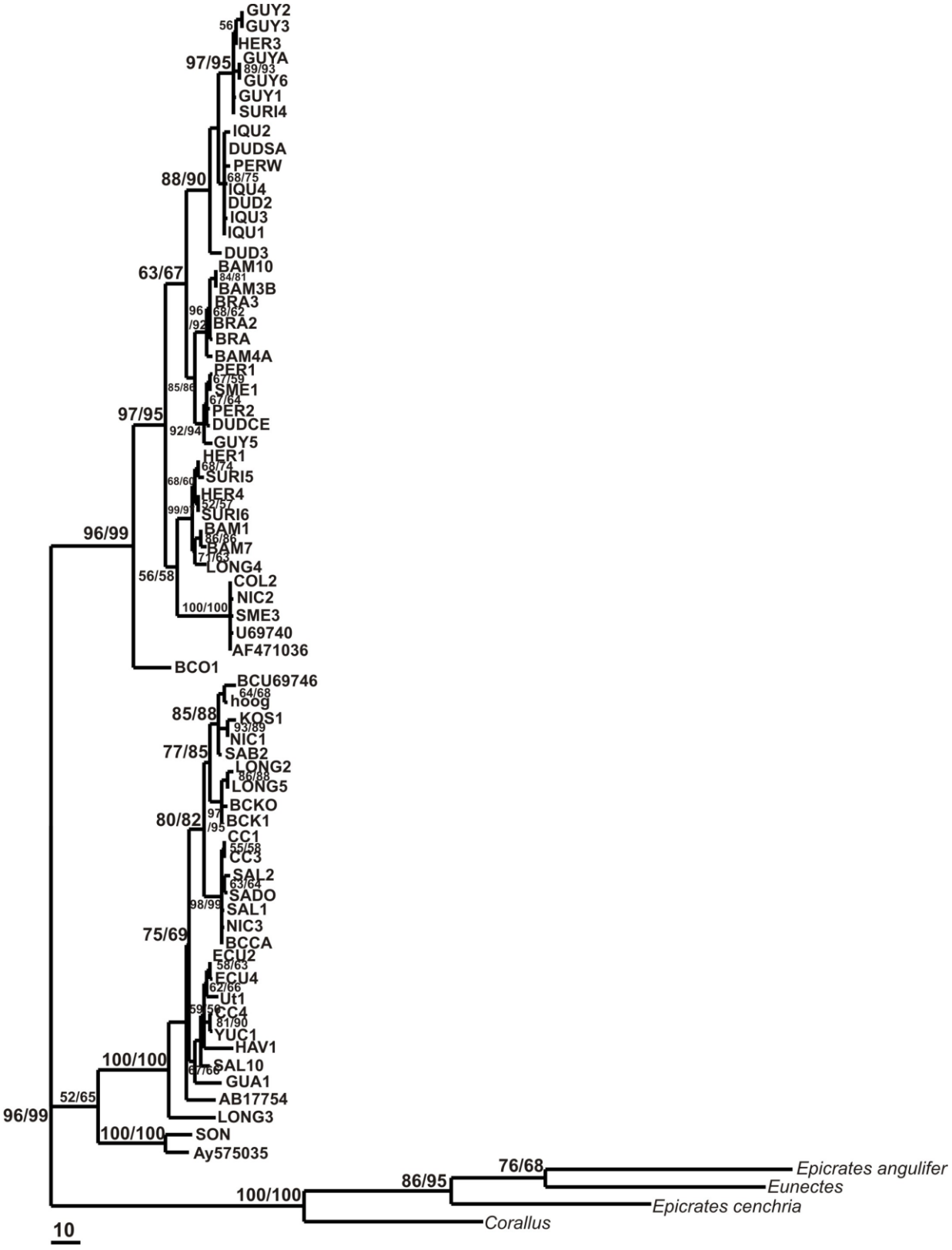
Appendix 2: List of samples abbreviations with their places of origin and the subspecies status they represent. The red colour labels samples used as haplotypes.



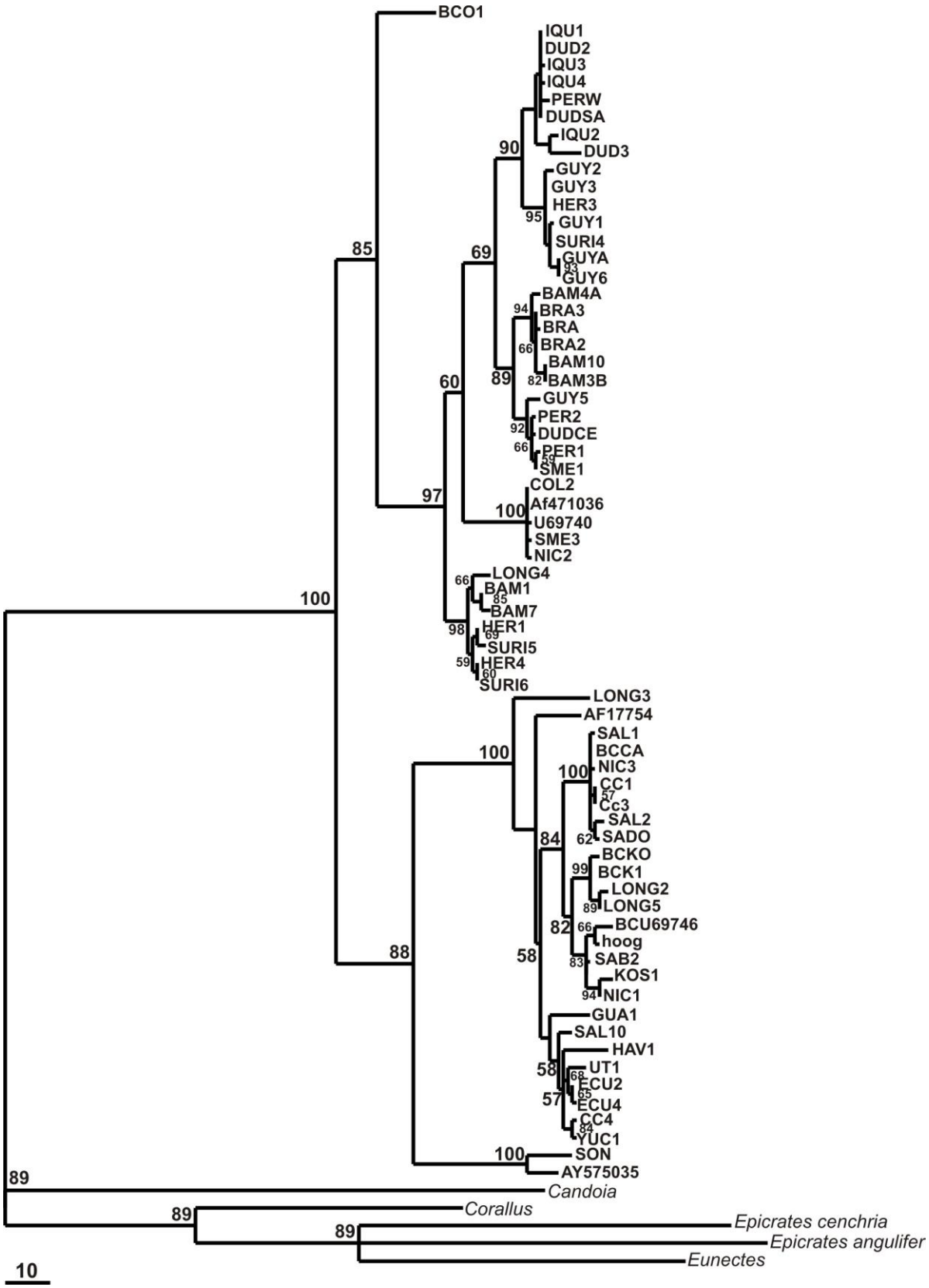
Appendix 3: NJ tree based on the *Boa*-no-*Candoia* dataset, the branches lenghts are included. The numbers along the branches show the bootstrap supports. The first number represents the bootstrap value using uncorrected distances with 10,000 pseudoreplicates, right of slash there are the bootstraps values using Jukes-Cantor distances with 10,000 pseudoreplicates. Values below 50% are not shown.



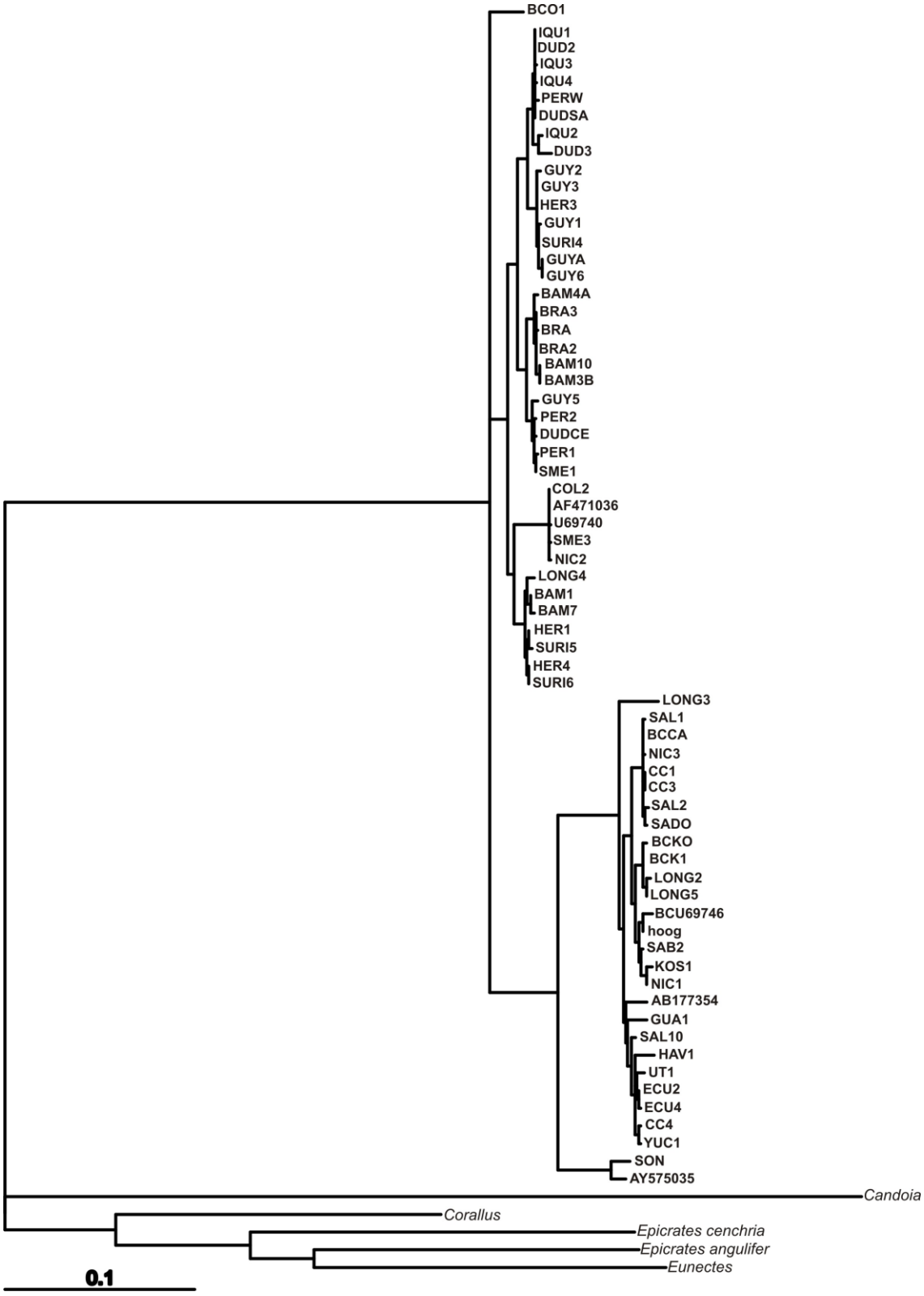
Appendix 4: MP tree with the different weighting schemes based on *Boa*-with-*Candoia* dataset. Bootstrap values (%) are shown along the nodes. The first number represents the bootstraps when transitions are down-weighted 10 times, right of slash when 4 times. The lenght of the tree is 958 steps, based on 425 variable characters, 304 of which are parsimony-informative. The consistency index is 0.6148 and the retention index is 0.8679.



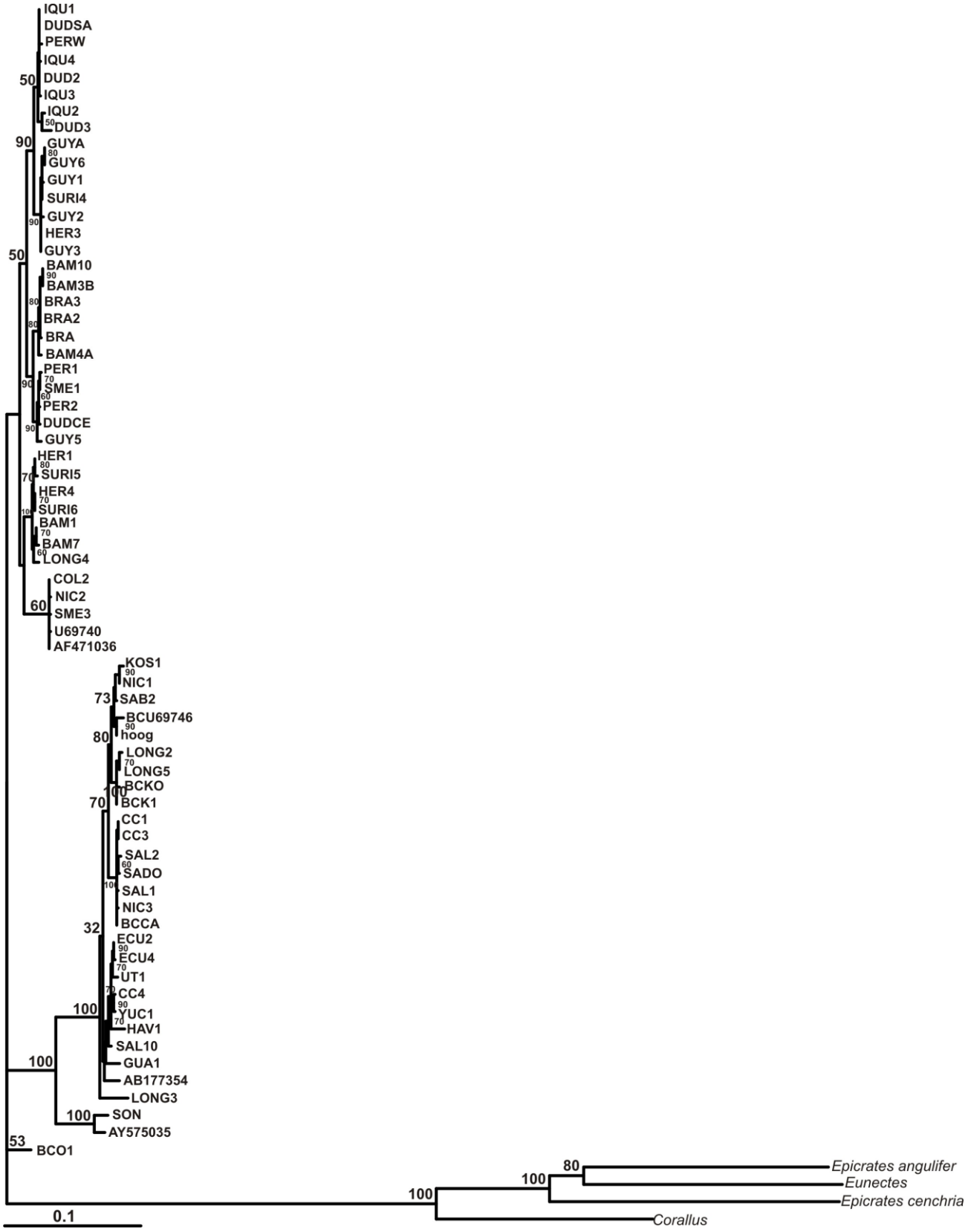
Appendix 5: MP tree with the different weighting schemes based on *Boa*-no-*Candoia* dataset. Bootstrap values (%) are shown along the nodes. The first number represents the bootstraps when transitions are down-weighted 10 times, right of slash when 4 times. The lenght of the tree is 819 steps, based on 400 variable characters, 279 of which are parsimony-informative. The consistency index is 0.6471 and the retention index is 0.8910.



Appendix 6: MP tree with the third codon position excluded based on *Boa*-with-*Candoia* dataset. Bootstrap values (%) are shown above the branches. The lenght of the tree is 252 steps, based on 743 variable characters, 90 of which are parsimony-informative. The consistency index is 0.5735 and the retention index is 0.8743.



Appendix 7: Maximum likelihood tree based on 1114 bp of cytochrome b and using *Boa*-with-*Candoia* dataset.



Appendix 8: Maximum likelihood tree based on 1114 bp of cytochrome b and using *Boa*-no-*Candoia* dataset. The numbers along the branches represent the bootstrap values for 10 replicates.

PERIOD/EPOCH	DATING
PALEOZOIC	
Cambrian	543 - 490 MYA
Ordovician	490 – 443 MYA
Silurician	443 – 417 MYA
Devonian	417 – 354 MYA
Carbonian	354 – 290 MYA
Permian	290 – 248 MYA
MESOZOIC	
Triassic	248 – 206 MYA
Jurassic	206 – 144 MYA
Cretaceous	144 – 65 MYA
TERTIARY	
Cenozoic era	65 – 23.8 MYA
Paleocene	65 – 54.8 MYA
Eocene	54.8 – 33.7 MYA
Oligocene	33.7 – 23.8 MYA
Neogene era	23.8 – 1.8 MYA
Miocene	23.8 – 5.3 MYA
Pliocene	5.3 – 1.8 MYA
QUATERNARY	
Pleistocene	1.8 MYA - 10.3 thousand
Holocene	10.3 thousand – present

Appendix 9: List of geological periods and epochs (from www.geol.umd.edu modified for the purposes of this work so only thematicly relevant information is included)

Appendix 10: CD ROM with samples photos and uncorrected p-distances table