A multilocus phylogeny of the cobra clade elapids

Anthony von Plettenberg Laing

Supervisor: Dr Wolfgang Wüster

PRIFYSGOL
BANGOR
UNIVERSITY

A thesis submitted to Bangor University for the degree of Master of Science by Research

Biological Sciences

February 2018

Declaration and Consent

Details of the Work

I hereby agree to deposit the following item in the digital repository maintained by Bangor University and/or in any other repository authorized for use by Bangor University.

Author Name: Anthony von Plettenberg Laing
Title: A multilocus phylogeny of the cobra clade elapids
Supervisor/Department: Dr Wolfgang Wüster, School of Biological Sciences
Funding body (if any): None

Qualification/Degree obtained: Master of Science by Research
This item is a product of my own research endeavours and is covered by the agreement below in which the item is referred to as "the Work". It is identical in content to that deposited in the Library, subject to point 4 below.

Non-exclusive Rights

Rights granted to the digital repository through this agreement are entirely non-exclusive. I am free to publish the Work in its present version or future versions elsewhere.
I agree that Bangor University may electronically store, copy or translate the Work to any approved medium or format for the purpose of future preservation and accessibility. Bangor University is not under any obligation to reproduce or display the Work in the same formats or resolutions in which it was originally deposited.

Bangor University Digital Repository

I understand that work deposited in the digital repository will be accessible to a wide variety of people and institutions, including automated agents and search engines via the World Wide Web.
I understand that once the Work is deposited, the item and its metadata may be incorporated into public access catalogues or services, national databases of electronic theses and dissertations such as the British Library's EThOS or any service provided by the National Library of Wales.
I understand that the Work may be made available via the National Library of Wales Online Electronic Theses Service under the declared terms and conditions of use (http://www.llgc.org.uk/index.php?id=4676). I agree that as part of this service the National Library of Wales may electronically store, copy or convert the Work to any approved medium or format for the purpose of future preservation and accessibility. The National Library of Wales is not under any obligation to reproduce or display the Work in the same formats or resolutions in which it was originally deposited.

Statement 1:

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree unless as agreed by the University for approved dual awards.

Signed …….. (candidate)

Date ……..

Statement 2:

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

All other sources are acknowledged by footnotes and/or a bibliography.

Signed ……. (candidate)

Date …….

Statement 3:

I hereby give consent for my thesis, if accepted, to be available for photocopying, for inter-library loan and for electronic storage (subject to any constraints as defined in statement 4), and for the title and summary to be made available to outside organisations.

Signed ……. (candidate)

Date …….

NB: Candidates on whose behalf a bar on access has been approved by the Academic Registry should use the following version of **Statement 3:**

Statement 3 (bar):

I hereby give consent for my thesis, if accepted, to be available for photocopying, for inter-library loans and for electronic storage (subject to any constraints as defined in statement 4), after expiry of a bar on access.

Signed …… (candidate)

Date ……

Statement 4:

Choose **one** of the following options

a) I agree to deposit an electronic copy of my thesis (the Work) in the Bangor University (BU) Institutional Digital Repository, the British Library ETHOS system, and/or in any other repository authorized for use by Bangor University and where necessary have gained the required permissions for the use of third party material.	
b) I agree to deposit an electronic copy of my thesis (the Work) in the Bangor University (BU) Institutional Digital Repository, the British Library ETHOS system, and/or in any other repository authorized for use by Bangor University when the approved **bar on access** has been lifted.	
c) I agree to submit my thesis (the Work) electronically via Bangor University's e-submission system, however I **opt-out** of the electronic deposit to the Bangor University (BU) Institutional Digital Repository, the British Library ETHOS system, and/or in any other repository authorized for use by Bangor University, due to lack of permissions for use of third party material.	

Options B should only be used if a bar on access has been approved by the University.

In addition to the above I also agree to the following:

1. That I am the author or have the authority of the author(s) to make this agreement and do hereby give Bangor University the right to make available the Work in the way described above.

2. That the electronic copy of the Work deposited in the digital repository and covered by this agreement, is identical in content to the paper copy of the Work deposited in the Bangor University Library, subject to point 4 below.

3. That I have exercised reasonable care to ensure that the Work is original and, to the best of my knowledge, does not breach any laws – including those relating to defamation, libel and copyright.

4. That I have, in instances where the intellectual property of other authors or copyright holders is included in the Work, and where appropriate, gained explicit permission for the inclusion of that material in the Work, and in the electronic form of the Work as accessed through the open access digital repository, *or* that I have identified and removed that material for which adequate and appropriate permission has not been obtained and which will be inaccessible via the digital repository.

5. That Bangor University does not hold any obligation to take legal action on behalf of the Depositor, or other rights holders, in the event of a breach of intellectual property rights, or any other right, in the material deposited.

6. That I will indemnify and keep indemnified Bangor University and the National Library of Wales from and against any loss, liability, claim or damage, including without limitation any related legal fees and court costs (on a full indemnity bases), related to any breach by myself of any term of this agreement.

Signature: …… Date : …….

Abstract

The extant medically and socially important cobras have been the subject to several comparative taxonomic studies since the 1940s, but still lack an inclusive and thorough phylogenetic tree. With recent major advancements in phylogenetic analysis, it is now common to use multiple independent loci for studying the phylogenetic relationships within groups. For the first time, 27 from the 29 identified *Naja* species, alongside 5 putative new or elevated species had 4426 base pairs across 1701 sequences of mitochondrial and nuclear DNA sequence data analysed. The results continue to support the monophyletic core cobra clade encompassing the genera *Walterinnesia, Aspidelaps, Hemachatus, Pseudohaje* and *Naja* (1.0 Bayesian posterior probability (BPP)), in addition to the grouping of four monophyletic subgenera within *Naja*. The group of African spitting cobras, *Afronaja,* is positioned as the sister group to the rest of the genus. Moderate support (0.8 BPP) is found for the grouping of the Asian cobras, *Naja,* with the African non-spitting cobras, *Ureaus.* The closest relative to the genus *Naja* is *Pseudohaje goldii*, a genus and species never before included in phylogenetic analysis, followed by the sister taxa *Hemachatus haemachatus*. The king cobra continues to be positioned outside the core cobra group, sister to *Hemibungarus calligaster.* The results support the hypothesis of three independent origins of spitting, once in the monotypic *Hemachatus haemachatus,* once within the subgenus *Afronaja*, and the final origin within the Asian cobras, subgenus *Naja*. The relationships found were broadly consistent with previous studies, with the additional inclusion of more species creating the most comprehensive cobra phylogeny to date. Further molecular analysis, specifically species delimitation, must be undertaken to ascertain the position of the 5 putative new species included in this study.

Table of Contents: A multilocus phylogeny of the cobra clade elapids

Acknowledgements **6**

1.0 Introduction **7**

1.1 Systematics: taxonomy and phylogenetics **7**

1.2 The Study Group: Cobras **9**

1.2.1 Systematics of cobras 9

1.2.2 Gaps in our knowledge 9

1.3 Genus *Naja* Laurenti, 1768 ..... **12**

1.3.1 Subgenus *Naja* Laurenti, 1768 12

1.3.2 Subgenus *Uraeus* Wagler, 1830 12

1.3.3 Subgenus *Boulengerina* Dollo, 1886 13

1.3.4 Subgenus *Afronaja* Wallach, Wüster, & Broadley, 2009 13

1.4 Other Cobras **14**

1.4.1 Genus *Aspidelaps* Fitzinger, 1843 14

1.4.2 Genus *Hemachatus* Fleming, 1822 14

1.4.3 Genus *Pseudohaje* Günther, 1858 14

1.4.4 Genus *Walterinnesia* Lataste, 1887 14

1.4.5 Genus *Ophiophagus* Günther, 1864 15

1.5 Defensive adaptations **15**

1.6 Aims and Objectives **17**

2.0 Methods **18**

2.1 Taxon Sampling and Laboratory procedures **18**

2.2 Sequence Data Preparation **20**

2.3 Multilocus analysis **20**

3.0 Results **22**

3.1 Sequence Data **22**

3.2 Multilocus phylogeny **22**

3.3 Individual gene trees **24**

4.0 Discussion **25**

4.1 Phylogeny of the core cobra clade **25**

4.2 Phylogeny of *Naja* **26**

4.3 Gene Trees **27**

4.4 Defensive Adaptions **28**

4.5 Assessing methods and analytical techniques used **29**

4.6 Conclusion **30**

References: **31**

Appendix **39**

01 Appendix. Sample list **39**

02 Appendix. PCR Mastermix composition **48**

03 Appendix. Cycling conditions **48**

04 Appendix. Primer identification **50**

05 Appendix. *Naja naja* discrepancy **51**

06 Appendix. Supplementary Gene Trees **53**

07 Appendix. Ancestral State Reconstruction **60**

Acknowledgements

This piece of work has been my biggest academic achievement to date, for which I would like to thank those who have either helped during the project directly or supported me throughout the 17 months.

Firstly, I must thank my supervisor Wolfgang Wüster for helping with the designing and preliminary research behind this project. His own specialist knowledge and experience of cobras has proven vital throughout the study. This has been the fourth year of having him as either a personal tutor or academic supervisor, and I am grateful for his generally-positive influence on me and all the great experiences we have had – be it in academic settings, herping in the field, or at conferences!

Despite my family often expressing concerns about pursuing an academic career, I must thank my parents, grandmother and the many uncles, aunts, and cousins who have supported my choices and helped whenever I have needed it. I'm sure my many stories from my studies and expeditions have proved both entertaining and unusual.

Lab work can at times be dull and repetitive, so I am glad I spent much of my time working alongside Edward Gilbert, Joshuah Hayes, Chloe McLaughlin, and Harry Rimmer. Together we helped each other with analytical and lab related problems, but more important was the stimulating conversation which was always refreshing, often original and certainly helped towards 'character building'. Appreciation must be emphasised for the help and understanding from Chloe McLaughlin – we often worked long hours together in frustrating times, and I am often less than empathetic.

Our lab manager Wendy Grail helped tremendously with setting us up in the laboratory and integrating us in to the 3rd floor of ECW, whilst Dr Giulia Zancolli introduced me to various new lab techniques that increased efficiency and thus saved valuable time and funds.

This study would not have been possible without access to the vast sample archive of Wolfgang Wüster, of which the key or vast samples used in this study came from the following people or institutes: Axel Barlow, Joe Beraducci, Donald Broadley, Laurent Chirio, Thomas Eimermacher, Bryan Grieg Fry, Iwan Hendrikx, Kate Jackson, Aleš Lubej, Mario Lutz, Mark O'Shea, Tony Phelps, Kate Sanders, Roger Thorpe, Marcel Witberg, Catharine Wüster, Wolfgang Wüster, Matthew Yuyek, Bio-Ken, Liverpool School of Tropical Medicine, and London Zoo.

1.0 Introduction

1.1 Systematics: taxonomy and phylogenetics

A vital discipline in biology is systematics, which deals with the classification of organisms and investigates their evolutionary relationships. The scientific process behind the naming of species was facilitated in the 18th century with the advent of Linnaean nomenclature, introducing a binomial two-part format. Species are a basic unit of classification, where although the definition is still unclear, it is generally accepted to describe genetically isolated interbreeding populations (Wheeler & Norman, 2000). This format was adopted by all biologists worldwide, and thus increased the accuracy and understanding when communicating across languages, as the binomial name is the same in every language. Species are then classified according to their evolutionary relationships with other taxa, forming a branching tree, called a phylogeny. Phylogenies are inferred from gathered data from the subject taxa, with the advancements in molecular techniques allowing for integrative approaches through the comparison of species' genetic, ecological and morphological characters. The inclusion of features shared by closely related species could indicate their shared ancestral history (if homologous), and establishing ancestral character states reflects evolutionary relationships (Padial *et al.* 2010; Reese *et al.* 2011).

For more than 250 years, the comparison of morphological characteristics formed the basis of taxonomy, the classification and discovery of species. Convergent evolution resulted in the adaption of analogous characteristics, causing doubt in the species taxonomy (Reese *et al.* 2011). The latter half of the 20th century brought along the increased development and usage of molecular techniques to be used in the investigation of systematic problems (Hillis, 1987). The application of morphological methods is still crucial when examining preserved specimens, especially for older samples. Closely related species, especially those who originated through rapid divergent selection, can express compelling morphological differences whilst showing weak reproductive isolation and limited genetic differentiation (Presgraves, 2010). Examples include the Darwin's finches and certain cichlid fishes, which demonstrate the continued requirement for the inclusion of a certain level of morphological analysis in taxonomic work. Molecular methods allow researchers to see beneath morphologically similar individuals to identify cryptic and candidate species, putatively perceived to be single species (e.g. Bickford *et al.* 2007; Fouquet *et al.* 2007). The use of molecular techniques in taxonomy has been widely complimented (e.g. Dayrat, 2005), however some have expressed scepticism (Valdecasas *et al.* 2008).

Reviewing the phylogeny of squamates, recent hypotheses have contradicted each other, especially when comparing trees generated from morphological (Conrad, 2008) and molecular data (Townsend *et al*. 2004; Wiens *et al.* 2012). The publication of a broad squamate phylogenetic study was suggested to attempt to reveal and clarify the major conflicts between morphological and molecular trees (Losos *et al.* 2012), with Pyron *et al.* (2013) completing a large-scale (4161 spp.) phylogenetic estimate for the group Squamata.

The increased importance of molecular phylogenetics in various biological disciples has accelerated the development and advancement of analytical techniques and software. Traditional approaches would include multiple separate gene trees, often focussed on the rapidly evolving mitochondrial genes (Brown *et al.* 1979). Alongside their evolutionary variability, mitochondrial genes were found to easily amplify via PCR (Kocher *et al.* 1989), facilitating molecular studies and thus advancing systematics (Moritz *et al.* 1987) due to nuclear trees often having low resolution. Soon after, the ability to simultaneously analyse

multiple mitochondrial genes was found and loci sequences were simply combined to increase sequence length, with the intention of increasing accuracy.

Frequently however, mitochondrial DNA is solely used for phylogenetic analysis (Beheregaray, 2008), which results in questionable support due to matrilineal mode of inheritance (Avise, 2004) and the relatively fast mutation rate when compared to nuclear DNA (Eo & DeWoody, 2010). The possibility of male cobras having larger home ranges, or searching for mates, could result in the bias of male dispersal not being detected in mtDNA (eg. Gibbons & Semlitsch, 1987; Gregory, Macartney & Larsen, Bonnet, Naulleau & Shine, 1999; Keogh *et al.* 2007). To avoid any male bias, and assuming a balanced sex ratio in the populations, it is best to include nuclear loci, as they carry twice the amount of genetic information when compared to mitochondrial DNA representing four times the effective population size.

Within the last decade, nuclear genes started to be included in molecular studies, with the addition of both nuclear and mitochondrial genes gaining the term multilocus analysis. Similar to the concatenation method of combining different mitochondrial genes, nuclear gene sequences were also added, resulting in a taxon being represented by one long genetic sequence, referred to as a "Super-gene" (Degnan & Rosenberg, 2009). This method was thought to be accurate with increased reliability due to an increase in sequence length and thus sample size. More recently however, it was suggested that there was a discordance between gene and species trees (Degnan & Rosenberg, 2009; Liu *et al.* 2009). Concatenating the sequences prior to analysis infers the assumption that all separate loci have evolved identically along a single evolutionary tree. This method therefore does not account for any recombination events or other loci-independent changes and thus using several different genes could cause contradictions in phylogenies and skewer hypotheses. One suggestion to correct this was to use an increased amount of loci sequences, increasing the accuracy of establishing phylogenetic relationships and evolutionary lineages (Liu *et al.* 2009). This suggestion would however still result in the over-looking of certain lineages as the focus is made on the range of loci, as opposed to single genes.

An alternative process was undertaking analysis through consensus methodology, involving the grouping of taxa in to sets of three, and then calculating the most frequently occurring relationships within each group (the "democratic vote", Degnan & Rosenberg, 2009). This works by averaging out individual taxa lineages compared to one another, along a rooted triple consensus tree.

Overall, the use of consensus and concatenated methods for multilocus analysis were found to result in overconfident support values and often inaccurate species trees, due to the assumption of a "supergene" requiring all loci to have evolved on a single evolutionary tree (analytical methods reviewed in Degnan & Rosenberg, 2009). The inaccuracy and uncertainty found within concatenated and consensus analysis increased the pressure to develop alternative analytical methods; one novel method was multispecies coalescent modelling.

To combat some of the recurring issues from both consensus and concatenated analysis, coalescent theory was used which involves the modelling of genealogies within populations (Hudson, 1983) by calculating probabilities of differences between gene tree and species tree branching patterns: topologies (Degnan & Rosenberg, 2009). This differentiation of gene trees and independent parameters allowed for relaxed molecular clock models to be used, and thus each lineage was assessed with its own individual rates. An example of software using coalescent methods alongside Bayesian analysis for molecular sequences was developed, called *BEAST: Bayesian Evolutionary Analysis by Sampling Trees (Heled & Drummond, 2010). *BEAST uses Markov Chain-Monte Carlo (MCMC) methods and coalescent analysis, thus allowing the most commonly occurring gene topology to be identified and further used as a hypothesis for the species tree.

1.2 The Study Group: Cobras

1.2.1 Systematics of cobras

Cobras are venomous snakes belonging to the Elapidae family, with the name originating from the Portuguese for hooded-snake, *Cobra-de-capello*. The use of binomial nomenclature is vital in research and especially in clinical medical treatment; with the use of efficient antivenom depending on the accurate identification of the specific snake. The ubiquity of venom variation presents complications in the production of effective antivenom (Fry *et al.* 2003). Multiple elapid genera are commonly referred to as cobras, with the following being included in this study: Shield-nose cobras, *Aspidelaps* (2 spp.); Rinkhals, *Hemachatus haemachatus*; True cobras, *Naja* (28 spp.); King cobra, *Ophiophagus hannah*; Tree cobras, *Pseudohaje* (2 spp.). The vague cobra term resulted in the genus *Naja* being labelled as true cobras by Slowinski & Keogh (2000).

Elapids have been subject to various taxonomic changes since the 1940s, with initial phylogenies and identifications being determined from low-resolution morphological methods. Despite there having been 23 species identified by 1943, in a comparison paper sumarising cobras, Bogert (1943) described them as subspecies of only 4 and 2 species from Africa and Asia, respectively; *Naja haje, N. melanoleuca, N. nigricollis, N. nivea,* and *N. naja, N. oxiana*. Klemmer (1963) continued to describe six recognised species, until a series of revisions confirmed the prior identification of 23 species (Broadley, 1968; Wüster, 1996; Broadley & Wüster, 2004; Wüster *et al.* 2007). Several other cobra genera were also historically classified as *Naja*; *Aspidelaps lubricus* (*Naja somersetta*: Smith, 1826), *Aspidelaps scutatus* (*Naja fula-fula*: Bianconi, 1849), *Hemachatus haemachatus* (*Naja haemachatus*: Schlegel, 1837), *Ophiophagus hannah* (multiple inc. *Naja hannah*: Tweedie, 1954), *Pseudohaje goldii* (multiple inc. *Naja goldii*: Mertens, 1941), *Walterinnesia morgani* (*Naja morgani*: Mocquard, 1905).

1.2.2 Gaps in our knowledge

One of the first genetic studies to include cobras was published by Slowinski & Keogh (2000), who sequenced the cytochrome *b* gene for 28 elapid species. The presence of a core cobra group had significant bootstrap support, which included *Aspidelaps, Boulengerina* (synonymized to *Naja*), *Hemachatus, Naja, Paranaja* (synonymized to *Naja*), and *Walterinnesia*. The king cobra, *Ophiophagus hannah,* was however found to not be part of the cobra clade, and instead clustered with *Elapsoidea* (Fig 1. Slowinski & Keogh, 2000). The tree shown in Fig 1. and position of *Ophiophagus hannah* draws uncertainty of the previously assumed monophyly of cobras, and the homology of the characteristic hooding display. The tree also included *Boulengerina* and *Paranaja*, with the exclusion of these two genera rendering the genus *Naja* to be non-monophyletic. Due to the both medical and social importance of the cobras, an arising concern was that retaining these genera could

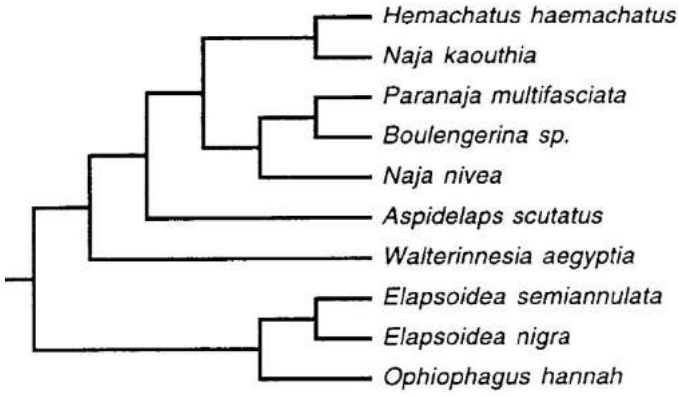


Figure 1. Maximum-likelihood tree of the cytochrome b sequences. Tree Adapted from Slowinksi & Keogh, 2000

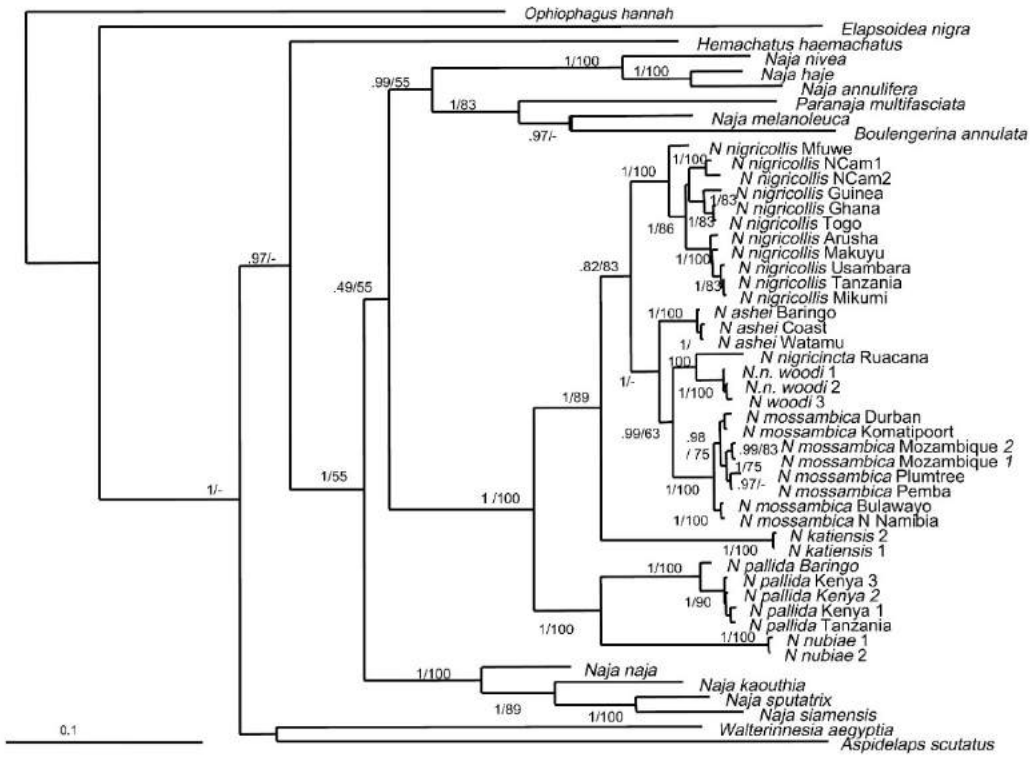


Figure 2. Bayesian tree of 17 *Naja* species and outgroups. Bayesian posterior probabilities and Maximum Parsimony bootstrap values are the support values displayed on the nodes, with a dash indicating <50% support value. Tree from Wüster *et al.* 2007.

result in the destabilization of the nomenclature; and thus it was suggested to synonymize both *Boulengerina* and *Paranaja* to *Naja* (Nagy *et al.*, 2005; Wüster *et al.*, 2007). The act of synonymizing the two aforementioned genera to *Naja* resulted in the expansion of a large genus to one containing 26 species across two continents.

Wüster *et al.* (2007) identified three separate evolutionary lineages within the *Naja* genus: 1) subgen. *Naja*; an Asiatic lineage which includes *Naja kaouthia*, *N. naja, N. siamensis*, and *N. sputatrix*, 2) an African lineage composed of the non-spitting true cobras, which consists of two sister lineages; 2i) subgen. *Uraeus*; *N. annulifera, N. haje,* and *N. nivea*; 2ii) subgen. *Boulengerina*; *N. annulata, N. melanoleuca,* and *N. multifasciata*; 3) subgen. *Afronaja*; the African lineage of spitting cobras including

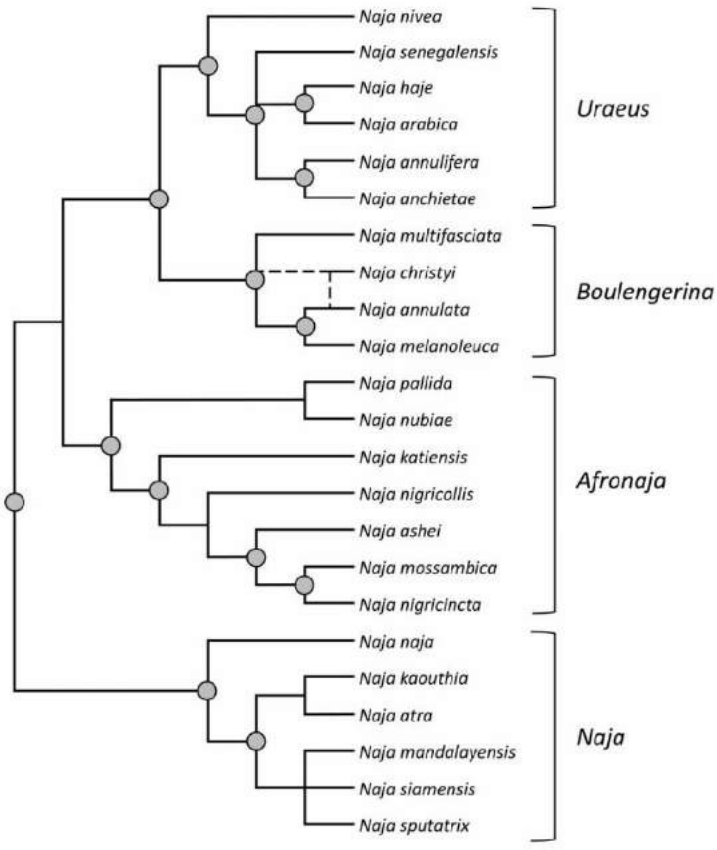


Figure 3. Schematic representation of the *Naja* phylogeny, indicating the recognised subgenera. Grey circles represent nodes with ≥95% Bayesian posterior probability support. Tree adapted from Wallach *et al.* 2009.

N. ashei, N. katiensis, N. mossambica, N. nigricincta, N. nigricollis, N. nubiae, and *N. pallida*. The 17-aforementioned species were included in a phylogenetic study analysing two mitochondrial genes (Fig 2. Wüster *et al.* 2007), with the tree being used for the basis of a schematic representation depicting the position of 23 species (Fig 3: 5 had their positions inferred from previous studies, whilst prior classification (Bogert, 1943) was used to tentatively position *N. christyi* (Wallach *et al.* 2009).

Although cobras have been included in recent phylogenies, they have generally been underrepresented (eg. Pyron *et al.* 2013; Figueroa *et al.* 2016; Lee *et al.* 2016). Both Pyron *et al.* (2013) and Figueroa *et al.* (2016) used sequences predominantly obtained from GenBank to create large multi-locus phylogenetic hypotheses of reptiles, with the latter study focussing on snakes. Lee *et al.* (2016) also predominantly used sequences from GenBank, but their study investigated diversification and body size change in Elapids.

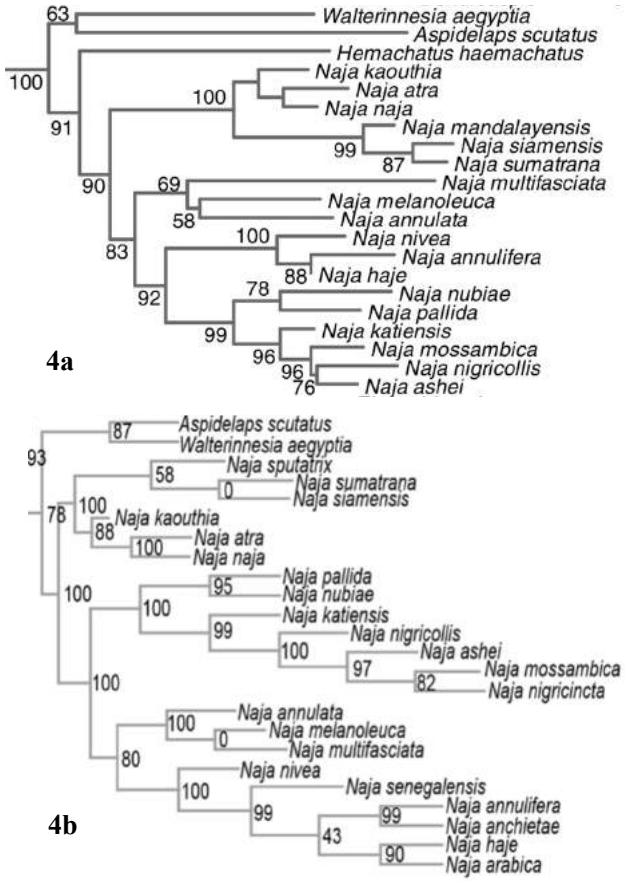


Figure 4. Trees adapted and focussed on the genus *Naja* from Phylogenetic tree of Squamata (**4a.** Pyron *et al.* 2013) and of Serpentes (**4b.** Figueroa *et al.* 2016).

Despite both the Pyron *et al.* (2013) and Figueroa *et al.* (2016) studies using very similar datasets, their resulting phylogenetic hypotheses show inconsistencies, specifically within the genus *Naja* (for trees, see Fig. 4a and 4b, respectively). A problem with using unverified sequences or samples of uncertain localities, is the possibility of mislabelling a species, leading to a false position for that species on the resulting phylogenetic tree. Several studies have used the same mtDNA sequence to represent *N. naja* (GenBank reference DQ343648), for which under further investigation looks to be a mislabelled sequence. The Basic Local Alignment Search Tool (BLAST: Zhang *et al.* 2000) found 99% similarity with two *N. atra* mitochondrial genomes (GenBank accession numbers: EU921898 & EU913475). Further to this, the isolated cyt-b and ND4 segments both grouped within the *N. atra* clades when analysed with the data from this study (Appendix 5). This could be because *N. atra* was previously classified as a subspecies of *N. naja* and the authors did not use the up-to-date taxonomic name. This has led to various studies publishing incorrect phylogenies (e.g. Pyron *et al.* 2013; Figueroa *et al.* 2016; Lee *et al.* 2016) and thus also incorrect assumptions and hypotheses due to the use of those original phylogenetic trees (e.g. Panagides *et al.* 2017).

Despite the advancement of molecular tools and analysis leading to the aforementioned phylogenetic trees, a comprehensive study on the cobra clade elapids has until now not been undertaken. Their findings also lack reliability due to incomplete species sampling and small datasets comprising of few (or individual) loci sequences.

1.3 Genus *Naja* Laurenti, 1768

The altering of an animal's binomial name is not recommended due to the interference with information retrieval, and for a large monophyletic genus containing well-defined lineages, the usefulness of the subgenus rank was highlighted by Smith & Chiszar (2006). To facilitate the grouping and differentiating of distinct lineages within the *Naja* genus, they are partitioned into four subgenera (Wallach *et al.* 2009).

1.3.1 Subgenus *Naja* Laurenti, 1768

The Asian cobras were thought to be only represented by one widespread species, *N. naja* (e.g. Boulenger, 1986; Klemmer, 1963), with a range from the western Caspian Sea, throughout southern and south-eastern Asia including the islands of Indonesia and the Philippines. Initial multivariate analysis of morphological characters suggested the presence of multiple species (Wüster & Thorpe, 1989, 1990, 1991 1992a) with molecular studies (Wüster & Thorpe, 1994) further supporting the presence of various species represented by *N. naja* (sensu lato). With the recent description of a new species of spitting cobra from central Myanmar, *N. mandalayensis* (Slowinski & Wüster, 2000), the number of Asian *Naja* species has risen to 11.
The Borneo population of *N. sumatrana* has historically also been referred to as *N. sumatrana miolepis* Boulenger, 1896, with past research suggesting the need for further investigation (Wüster & Thorpe, 1990; Wüster, 1996) as the population may be a distinct species.

Naja (Naja) atra Cantor, 1842: 482
Naja (Naja) kaouthia Lesson, 1831: 122
Naja (Naja) mandalayensis Slowinski & Wüster, 2000: 260
Naja (Naja) naja (Linnaeus, 1758: 221)
Naja (Naja) oxiana (Eichwald, 1831: 171)
Naja (Naja) philippinensis Taylor, 1922: 265
Naja (Naja) sagittifera Wall, 1913: 247
Naja (Naja) samarensis Peters, 1861: 690
Naja (Naja) siamensis Laurenti, 1768: 91
Naja (Naja) sputatrix Boie, 1827: 557
Naja (Naja) sumatrana Müller, 1890: 277

1. *Naja* cf. *miolepis* Boulenger, 1986

1.3.2 Subgenus *Uraeus* Wagler, 1830

The subgenus *Uraeus* is the sister lineage to *Boulengerina*, within which these two subgenera consist of the African non-spitting species (Wüster *et al.* 2007). The number of species has risen due to recent reviews and new species descriptions (Broadley & Wüster, 2004; Trape *et al.* 2009). The 6 species belonging to the subgenus *Uraeus* inhabit most of Africa and southern Arabia, and are generally found in open formations (Wallach *et al.* 2009).

Naja (Uraeus) anchietae Bocage, 1879: 89
Naja (Uraeus) annulifera Peters, 1854: 624
Naja (Uraeus) arabica Scortecci, 1932: 47
Naja (Uraeus) senegalensis Trape, Chirio & Wüster in Trape et al, 2009: 2236
Naja (Uraeus) haje (Linnaeus, 1758: 225)
Naja (Uraeus) nivea (Linnaeus, 1758: 223)

1.3.3 Subgenus *Boulengerina* Dollo, 1886

The sister lineage of the subgenus *Uraeus*, representing African non-spitters. The subgenus *Boulengerina* is comprised of the remaining 5 non-spitting cobras, which show high morphological and ecological diversity. *Naja melanoleuca* and *N. multifasciata* represent the true size extremes within the cobra clade, with recorded lengths of 2.7m and 0.55m, respectively (Boulenger, 1904; Spawls & Branch, 1995; O'Shea, 2008). Despite all species being restricted to forests or forest edge habitats, their ecology still varies with some being aquatic or semi-fossorial (Wallach *et al.* 2009). They can be found in central Africa, with *N. melanoleuca* extending towards both West and East Africa (Luiselli & Angelici, 2000).
Evidence from previous unpublished work suggests that distinct populations of *N. melanoleuca* may warrant inclusion and be classed as separate proposed species, listed as 1, 2, and 3 (Wüster, *pers. comm.)*.

The continuing taxonomic uncertainty within the genus is further exemplified by the subspecies *N. m. subfulva.* It has been referred to historically as just *N. melanoleuca*, and more recently as a subspecies (Laurent, 1995; Broadley & Baylock, 2013) or even as elevated to species level with little support (Chirio & Ineich, 2006; Wallach et al., 2014; Ceríaco *et al.* 2017)

Naja (Boulengerina) annulata Buchholz & Peters *in* Peters, 1876: 119
Naja (Boulengerina) christyi (Boulenger, 1904: 14)
Naja (Boulengerina) peroescobari (Ceríaco *et al.* 2017: 4325)
Naja (Boulengerina) multifasciata Werner, 1902: 347
Naja (Boulengerina) melanoleuca Hallowell, 1857: 61

1. Western banded cobra
2. West African black form
3. *Naja* cf. *subfulva* Laurent, 1955

1.3.4 Subgenus *Afronaja* Wallach, Wüster, & Broadley, 2009

Prior to 1968, all African spitting cobras were thought to belong to a single species, *Naja* nigricollis, which Broadley (1968) split in to two species, *N. nigricollis* and *N.* mossambica. The subspecies *katiensis, mossambica* and *pallida* were classified under the species *N. mossambica* (Wüster & Broadley, 2003). Recent phylogenetic analysis has supported the inclusion of 7 species to the African spitting clade (Wüster *et al.* 2007), subgenus *Afronaja* (Wallach *et al*. 2009). These species are found in sub-Saharan Africa and alone the Nile Valley, inhabiting open formations and forest edges (Wallach *et al.* 2009).

Naja (Afronaja) ashei Wüster & Broadley, 2007: 58
Naja (Afronaja) katiensis (Angel, 1922: 40)
Naja (Afronaja) mossambica Peters, 1854: 625
Naja (Afronaja) nigricincta Bogert, 1940: 89

1. *Naja* cf. *woodi*, Bauer & Branch 2001

Naja (Afronaja) nigricollis Reinhardt, 1843: 269
Naja (Afronaja) nubiae Wüster & Broadley, 2003: 348
Naja (Afronaja) pallida Boulenger, 1896: 379

1.4 Other Cobras

1.4.1 Genus *Aspidelaps* Fitzinger, 1843

Both *Aspidelaps* species are small venomous snakes which are closely related to the *Naja* genus, however their general biology and ecology is still poorly understood (Broadley & Baldwin, 2006). They are restricted to southern Africa, express fossorial habits aided by their enlarged rostral scale, and are described as nocturnal (Bradley & Baldwin, 2006; Shine *et al.* 2006).

Aspidelaps lubricus (Laurenti, 1768: 80)
Aspidelaps scutatus Smith, 1849: 22

1.4.2 Genus *Hemachatus* Fleming, 1822

The only species within the monotypic genus *Hemachatus* is the ovoviparous Rinkhals, *H. haemachatus*. The ability for this species to spit venom renders it of particular interest due to the increased likelihood of independently evolving this behavioural adaption (Wüster *et al.* 2007). Rinkhals are found in south-eastern Africa, and have been described to be diurnal in the Highveld grasslands (Alexander, 1996), but in the Zimbabwe woodland a more nocturnal diel activity has been noted (Broadley & Cock, 1975).

Hemachatus haemachatus Bonnaterre 1790: 31

1.4.3 Genus *Pseudohaje* Günther, 1858

The genus *Pseudohaje* represents two arboreal elapid snake species found in central and western Africa, commonly called tree cobras. Although similarities exist between the genera *Pseudohaje* and *Naja*, Bogert (1942) found that *Pseudohaje* spp. had proportionally larger eyes, smaller fangs, and a generally smaller bone structure compared to *Naja* spp. The elusiveness of the two species is emphasised by Akani *et al.* (2005) who undertook several years of fieldwork to study the species. The two species have to-date not been included in any published phylogenetic studies, solely within dietary and ecological research (eg. diet: Pauwels & Ohler, 1999; Pauwels *et al.* 1999; ecology: Akani *et al.* 2005).

Pseudohaje goldii (Boulenger, 1895)
Pseudohaje nigra Günther, 1858: 222

1.4.4 Genus *Walterinnesia* Lataste, 1887

Both *Aspidelaps* and *Walterinnesia* form the basal lineages when assessing the phylogenetic relationships within the cobra clade (Fig 2. Wüster *et al.* 2007). The two species of *Walterinnesia* are found in eremial habitat in northeast Africa and the Middle East, ranging from Egypt to Iran (Nilson & Rastegar-Pouyani, 2007), with recent range expansions extending northwards in to Turkey (Uğurtaş *et al.* 2001; Göçmen *et al.* 2009). Little is known about the taxonomy of these two rare nocturnal species, as historically they were often all referred to as *W. aegyptia* (e.g. Uğurtaş *et al.* 2001), or the eastern individuals as the subspecies *W. a. morgani*. The morphological characters alone had been described to warrant the elevation to

species level for *W. a. morgani*, however it was in the study by Nilson & Rastegar-Pouyani (2007) where *W. morgani* was re-established for the eastern populations, justified by the combination of morphological variation and the allopatric distribution.

Walterinnesia aegyptia Lataste, 1887: 411-413
Walterinnesia morgani (Mocquard, 1905)

1.4.5 Genus *Ophiophagus* Günther, 1864

Despite the lack of support from recent phylogenies to include the king cobra, *Ophiophagus hannah* within the cobra clade (Fig. 1 & 2), as a hooded elapid it is of interest and of importance in this study when trying to understand the evolution of the shared defensive adaptations. This iconic snake-eating species is also the longest venomous snake described, and can be found across tropical habitats in south and southeast Asia.

Ophiophagus hannah (Cantor, 1836)

1.5 Defensive adaptations

All cobras share certain characteristic defensive adaptations, most notably the ability to raise the elongated ribs below their heads, stretching the skin and forming a hood. This behaviour is found in multiple elapid genera and also in colubrids putatively mimicking dangerous cobras (Greene, 1997; Young & Kardong, 2010). Exhibited on the dorsal side of the hood on some species, are varying amounts of conspicuous colour markings or patterns, dependent on species or even locality (Wüster, 1998; Lillywhite, 2014). All cobras are highly venomous and have caused fatalities across their range. Due to the presence of different subunits, the multimeric toxin complexes in cobra venom leads to functional types of toxin, listed in Table 1 (Fry *et al*. 2015a)
The severe localized tissue destruction often found in elapid envenomations is caused primarily by the abundance of cytotoxic apotypic three-finger toxins (3FTx, Utkin *et al.* 2015).

Table 1 Functional type of varying multimeric snake toxin complexes within cobras. Adapted from Fry *et al.* 2015a

Functional type	Source (Genus)	Subunit type & uniprot accession	Specific activity
Cardiotoxin	*Ophiophagus*	3FTx (Q69GK0)	ß-blocker
Coagulopathic toxin	*Hemachatus*	3FTx	fVIIa inhibition
	Naja	SVMP (P0DJJ4)	Inhibition of the classical complement pathway
Cytotoxin	*Naja*	3FTx (P60301)	Pore-forming cytotoxin
Neurotoxin	*Naja*	3FTx (α-ntx, P01391)	a7/ß2 antagonist
	Ophiophagus	3FTx (A8N286)	a7 antagonist

Kini & Evans (1989) examined *Naja nigricollis* venom and found cytotoxins characteristically exhibit cytolytic activity due to the presence of both hydrophobic and cationic amino acids on the molecular surface. A major component of the immune system is the complement system, which is heavily involved in adaptive and innate immune responses (Carroll, 2004). The genera *Hemachatus, Naja* and *Ophiophagus* all possess a unique complement-activating protein found in their venom, which is called the cobra venom factor (CVF, Vogel *et al.* 1996; Fry *et al.* 2015b), and has been shown to cause decomplementation lasting for 5 days (Futter *et al.* 1992). The role CVF plays in snake venom is still unknown, as compared to the three-finger toxins present, the action of CVF is not lethal (Fry *et al.* 2015b). Mulligan *et al.* (1996) found that the intravenous injection of CVF caused massive complement activation, with neutrophils being activated and sequestrated to the lungs, damaging lung tissue, however the effects were found to be temporary. It has also been suggested that the inducing of massive complement activation could elicit the release of anaphylatoxins, therefore increasing the blood permeability and thus CVF could be acting as a spreading factor (Fry *et al.* 2015b)

As a further defence mechanism, several species of *Naja* and also *Hemachatus haemachatus* have evolved the behaviourally unique ability to spit their venom towards an aggressor or predator (e.g. Bogert, 1943; Wüster *et al.* 2007). The venom of African spitting cobras is of particular interest as it can cause severe clinical effects on the tissues of the eye, due to being rich in cytotoxic 3FTxs (Boyer *et al.* 2015) and has been recorded to reach as far as two metres (Triep *et al.* 2013). The evolution and divergence of this trait alongside the number of possible origins or losses is still not fully understood, and requires further work (Wüster *et al.* 2007).

A recently published study on cobras investigated their defensive adaptions, predominantly hooding, aposematic marking, venom cytotoxicity and spitting (Panagides *et al.* 2017). Their evolutionary hypotheses were based on the aforementioned incorrect phylogenetic tree from Lee *et al.* (2016). Inconsistencies were also found with their classification of traits, alongside the method for recording said traits. Morphological, specifically pattern and colour descriptions were often vague and were combined with inaccurate assumptions of their role as either cryptic or aposematic traits (Panagides *et al.* 2017). Accompanying these statements are photographs which seem heavily edited (increased saturation levels) or of snakes outside their natural environment; eg. the red *N. pallida* described as aposematic, despite being found in habitats with red sand. Panagides *et al.* (2017) describes how banding correlates with levels of cytotoxicity and hooding, yet uses photos of juveniles of several species that have a much higher contrast in banding, and species for which there is a vast variability in amount of banding across their range, eg. *N. siamensis* which in parts of their range are a drab, brown colour (Wüster & Thorpe, 1994; Wüster et al., 1997), *O. hannah* which as adults can have either faint or no banding at all, and *N. annulifera* which Broadley and Wüster (2004) found to also occur without banding. After stating that banding was aposematic, Panagides *et al*. (2017) described the banded genus *Aspidelaps* as cryptic and non-hooding, alongside *N. annulata,* for which both the *Aspidelaps* genus and *N. annulata* possess and perform hooding as a defensive strategy (Broadley & Baldwin, 2006; O'Shea, 2008).

1.6 Aims and Objectives

As in increasingly more studies, nuclear markers will be used in combination with mitochondrial loci for a multilocus investigation to be undertaken to ensure the most support for a thorough and comprehensive species tree. To circumvent these problems, this study will use the more accurate coalescent analysis (Degnan & Rosenberg, 2009). Advancements in multispecies coalescent models enabled the aforementioned *BEAST software (Heled & Drummond, 2009) to be developed, allowing the simultaneous analysis of multiple loci irrespective of the gene tree incongruences, which previous concatenated methods couldn't do.

Using calibration points of known divergences from fossil records published in previous literature (e.g. *Laticauda,* Scanlon *et al.* 2003; European vipers, Szyndlar & Rage, 1999), Wüster *et al.* (2007) identified a basal divergence for the African spitting cobra group dated at approximately 15 Mya, coinciding with the spread of open grassland formations (Potts & Behrensmeyer, 1992; Jacobs, 2004). Using more recent calibration points (e.g. Sanders *et al.* 2008) *BEAST can further be used to estimate times of cladogenesis within groups, and investigate putative selective pressures having led to the evolution of spitting behaviour.

Due to the gaps in our knowledge of cobra clade elapid systematics, the main objective for this study is to investigate and test previous phylogenetic hypotheses using a greater dataset compiled of more species, samples, and loci, to be analysed using advanced molecular techniques, namely coalescent species trees. The creation of a novel comprehensive multilocus phylogeny should allow the following aims to be addressed:

1. Testing current systematic arrangements, specifically focussing on the monophyly of genera and subgenera, with the addition of some taxa not before included in published phylogenies, especially not comparative studies
2. Investigate the evolution of defensive behaviours, specifically spitting behaviour, hood shape and markings
3. The inclusion of other elapids, specifically Australasian elapids within the phylogeny should facilitate the use of molecular dating techniques to estimate the divergence dates of spitting lineages and other major cladogenic events, including biogeographical scenarios like the migration out of Africa.

2.0 Methods

2.1 Taxon Sampling and Laboratory procedures

For this study, scale clippings, sloughed skin, and blood samples were used to extract DNA. Samples were predominantly provided by Dr Wolfgang Wüster, with sample information available in Appendix 1. Inclusive of possible new or elevated species mentioned in the introduction (i.e. *N. "*miolepis"), a total of 32 *Naja* species were included, *Aspidelaps lubricus* and *A. scutatus*, *Bungarus caeruleus* and *B. fasciatus*, *Dendroaspis angusticeps, Hemibungarus calligaster, Ophiophagus hannah, Pseudohaje goldii,* and *Walterinnesia aegyptia.* Full list of species, samples, and their respective loci analysed can be found in Appendix 1.

Tissues were stored in ethanol, for which small quantities were digested with Proteinase K followed by the Qiagen DNEasy blood and tissue kit being used to extract DNA, with amendments made for blood samples stored in buffer (reduced to 10 mins) and sloughs (longer incubation, up to 36 hours). Blood samples were processed using the tissue protocol if stored in ethanol. Any samples for which there was limited tissue remaining, only 1 final elution was made, using 50µl. DNA was initially quantified using both a Nanodrop spectrophotometer (ND1000), and by using gel electrophoresis (4µl DNA mixed with 1µl loading dye, 1% gel ran for 25 minutes at 80V). Due to inconsistencies and false readings between the DNA quantifying methods, further samples were solely run through the gel electrophoresis to ascertain viability.

The 100bp ladder with known band-concentrations allowed for an estimation of DNA concentrations to be made. Samples were then standardised to a final concentration of 20ng/µl using AE buffer; with samples of lower concentrations having amendments to the final PCR MasterMix.

Samples were prepared for amplification via Polymerase Chain Reaction (PCR) using the reagent combination (PCR MasterMix) in Appendix 2. Two buffers (MasterMix) were used throughout the lab work. Firstly, the ThermoScientific 2X ReddyMix, for which 7.5µl was added to each PCR reaction, yielding 0.625 units ThermoPrime Taq DNA polymerase, 75mM Tris-HCl (pH 8.8 at 25ºC), 20mM $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2mM of the following: dATP, dCTP, dGTP and dTTP, with a red dye to facilitate pipetting for electrophoresis. The second buffer used was ThermoScientific 2X DreamTaq, consisting of DreamTaq Green DNA polymerase, 1X DreamTaq Green buffer, dATP, dCTP, dGTP and dTTP, O.4mM each, and 2mM $Mgcl_2$. The latter buffer increased quality of PCR product and also created one band of the expected amplicon, so was used as standard for all further PCR reactions.

Genes amplified for this study were the mitochondrial Cytochrome B (cytb) and NADH dehydrogenase subunit 4 (ND4), and nuclear Neurotrophin-3 (NT3), prolactin receptor (PRLR), Ubinuclein (UBN1), Oocyte Maturation Factor (c-mos), and Recombination Activating Gene (RAG1); with the latter two nuclear loci being the focus for this study. Appendix 1 lists sequences obtained by this study compared to sequences from previous students. Primers used are listed in Table 2 and corresponding PCR cycle conditions are listed in Appendix 3**.**

The RAG1 primers G396 (R13) and G397 (R18) sourced from literature (Table 2; Groth & Barrowclough, 1999) failed to amplify the DNA for multiple samples of high quality DNA, indicating that the binding region was not compatible for all species. Successfully amplified RAG1 sequences were aligned in MEGA 5.05, and sections with few variable sites at each end (5' and 3') were identified and used to design novel primers (Table 2). Appendix 4 exhibits how new primer sequences were identified, for which a similar process for CMOS was also necessary.

Table 2 The genes used for this study, with their corresponding primers used.

Gene Locus	Direction	Primer Name	Sequence (5' to 3')	Reference
cytB	Forward	Gludg	TGACTTGAARAACCAYCGTTG	Palumbi et al. 1991
	Reverse	ATRCB3	TGAGAAGTTTTCYGGGTCRTT	
ND4	Forward	NADH4	CACCTATGACTACCAAAAGCTCATGT AGAAGC	Arévalo et al. 1994
	Reverse	H12763V	TTCTATCACTTGGATTTGC ACCA	
CMOS	Forward	AV_CMOSF	AAGCACATCAAGGATTCGTCG	This study
	Reverse	AV_CMOSR	TCTGCCTTGGGTGTGATTTTCT	
	Forward	G303	ATTATGCCATCMCCTMTTCC	Hugall et al. 2008
	Reverse	G708	GCTACATCAGCTCTCCARCA	
NT3	Forward	NTF3_F1	ATGTCCAATCTTGTTTTATGTGATATTT	Townsend et al. 2008
	Reverse	NTF3_R1	ACRAGTTTRTTGTTYTCTGAAGTC	
PRLR	Forward	PRLR F1	GACARYGARGACCAGCAACTR ATGCC	Townsend et al. 2008
	Reverse	PRLR R3	GACYTTGTGRACTTCYACRT AATCC AT	
RAG1	Forward	AV_RAG1F	AAATGTGACAGGGTCTCT	This study
	Reverse	AV_RAG1R	GGGCATCTCAAAACCAAATTGT	
	Forward	G396 (R13)	TCTGAATGGAAATTCAAGCTGTT	Groth & Barrowclough 1999
	Reverse	G397 (R18)	GATGCTGCCTCGGTCGGCCACCTTT	
UBN1	Forward	BaUBN_F	CCTCTGGTTACTCAGCAGCA	Barlow, pers. comm.
	Reverse	BaUBN_R	ATTGGCCACTCCTTGTGTTC	

PCR product was quantified and checked for both quality and double-banding using gel electrophoresis. 2µl of each PCR sample was loaded onto a 10% agarose gel, made by microwaving 50ml of TBE buffer mixed with 50mg agarose, with 5µl SafeView added once the mixture had cooled. The gels were run at 80V for 25 minutes and were visualised using the UV transilluminator which allowed for confirmation of amplicon size and quality. All positive PCR products underwent the PCR CleanUp, for which 1µl of CleanUp MasterMix was added to each tube, which was comprised of ultrapure water (0.8µl), Exonuclease I (0.1µl) and Thermosensitive Alkaline Phosphatase (0.1µl). To hydrolyse unwanted dNTP's and remove excess single-stranded PCR product, a CleanUp step was used (Werle *et al.* 1994). The ThermoCycler (Bio-Red T100™ Thermal Cycler) ran the final process of 15 minutes incubation at 37ºC, 15 minutes inactivation temperature of 74ºC, and a final 15 minutes step at 4ºC.

Purified samples were then re-tubed, labelled, and sent to Macrogen Inc. (dna.macrogen.com) for sequencing.

2.2 Sequence Data Preparation

All mitochondrial and nuclear sequences underwent proof-reading in CodonCode Aligner v. 3.7.1 (www.codoncode.com/aligner), as this software allows for the editing and alignment of sequences by displaying the sequencer trace files alongside the nucleobases identified. Nuclear sequences were assembled into contigs as combined forward and reverse amplicons, they were then checked for quality, with low-quality ends being removed. Once aligned, any heterozygous positions were visually identified on the chromatograms as possessing a double peak combined with low quality Phred scores (Ewing *et al.* 1998). These nucleobases were then renamed to their respective IUAPC codes (Cornish-Bowden, 1985).
The nuclear sequences exported in FASTA format were then opened in MEGA 5.05 (Tamura *et al*., 2004; 2011) where they were aligned by MUSCLE (Edgar, 2004). To ensure the smooth running of both PHASE and *BEAST, all sequences were aligned to start and end on the same base pair position, with fewer gaps facilitating analysis.
The program SeqPHASE (www.seqphase.mpg.de/seqphase; Flot, 2010) was used to create the input files required for PHASE (Stephens *et al.* 2001). PHASE is a statistical haplotyping software run through the command-line cmd.exe, in which the input diploid nuclear sequences are read and then individual parental haplotypes are estimated. Due to the assumptions and conditions within PHASE (Stephens *et al.* 2001), gene sequences were split in to the separate *Naja* subgenera prior to analysis. Each analysis was conducted 3 times with different starting seeds, burning in the first 1000 generations. SeqPHASE was then used to convert the PHASE output files in to FASTA files, which were opened in MEGA 5.05. Original sequences with a range of heterozygous positions resulted in various possible parental haplotypes, with the two sequences retained for further analysis being chosen on account for the highest confidence probabilities.

2.3 Multilocus analysis

The software Beauti (Heled and Drummond, 2010) was used to construct the input files for *BEAST (Heled & Drummond, 2010)) analysis. Site models were identified by inputting the genes' NEXUS files in to PAUP* v.4.0 (Swofford, 1998); for which Maximum Likelihood criterion are used to identity the most supported best-fit models, which in this case were SYM+G (Gamma Category Count, 1; Model, GTR, Frequencies, All Equal; Zharkikh, 1994) for all loci except NT3, which was analysed through the best fit model K80+G+I (Gamma Category Count, 1; Proportion Invariant, 0.1; Model, HKY, Frequencies, All Equal). Clock models were adjusted after analysing Tracer files (Rambaut & Drummond, 2013) and attention was paid to any low Effective Sample Size (ESS) figures for the parameters associated with evolutionary rate (i.e. CoefficientOfVariation). Only PRLR yielded low evolutionary rate related ESS readings, and thus had a strict clock model as opposed to the other genes' relaxed clock log normal models.
Several preliminary runs were undertaken using *BEAST, generally running between $5x10^6$ – $10^{8,}$ with the rule of sampling the Markov chain Monte Carlo (MCMC) chain every 500 or 10,000, respectively (chain length divided by 10,000), and the burn in was set at 10%. These preliminary trees were then analysed using Tracer v.1.5 to check ESS values to ensure correct Beauti parameters.
The final *BEAST analysis was set-up to run for 10^9 generations, storing every 10^5 trees to ensure a total of 10,000 trees were created. The pre-burnin was again set for 10%; 10^8.

TreeAnnotator was used to input the 10,000 trees from *BEAST and create a maximum clade credibility tree, with node heights set to median heights and posterior probability limit to 0.5. The single output tree was then viewed and annotated using FigTree.

3.0 Results

3.1 Sequence Data

This multilocus analysis was based upon 1701 sequences totalling 4426 base pairs across 7 loci (cytb, ND4, c-mos, NT3, PRLR, RAG1, UBN1), representing 41 elapid species and all extant *Naja* species excluding *N. christyi*. Further information and characteristics for DNA sequences per gene are listed in Table 2.

Table 2. Characteristics and sequence dataset for loci used in *BEAST analysis. Columns respectively represent number of sequences, length of sequence (number of base-pairs), number of variable sites, and number of parsimony-informative sites.

Gene	No. seq	bp	v.s.	p-i
cytb	246	658	445	160
ND4	266	663	523	184
cmos	116	638	350	83
NT3	256	657	227	98
PRLR	368	551	221	122
RAG1	98	758	146	78
UBN1	351	501	175	80

3.2 Multilocus phylogeny

The *BEAST multilocus species tree in Fig. 5 supports the monophyly of the genus *Naja*, with a Bayesian Posterior Probability (BPP) support value of 1. The monophyly of the core cobra group remains strongly supported, with the addition of *Pseudohaje goldii*. This study found *P. goldii* to form the closest related lineage to the *Naja* genus, with a BPP support value of 0.89, which could be classed as having moderate support for this position. The cobras *Walterinnesia aegyptia, Aspidelaps spp.*, and *H. haemachatus* retain their position as the evolutionarily closest lineages for this group, with the addition of *P. goldii*. The king cobra, *Ophiophagus hannah,* is once again found to not fit amongst the core cobra clade, but its position is poorly resolved in the multilocus phylogeny. *O. hannah* forms a monophyletic clade with the Asian coral snake, *Hemibungarus calligaster*, which is moderate-to-poorly supported with a BPP of 0.83. This clade's position is poorly resolved in regard to its relationship with the sister lineage, the core cobra group, as it yields a BPP support value of 0.61. Within the *Naja* genus, the monophyly of the individual subgenera *Afronaja*, *Boulengerina*, *Naja* and *Uraeus* are strongly supported with BPP support values of 1.

The position of the African spitting lineage, *Afronaja*, has strong support as the sister lineage to the remaining *Naja* species (BPP of 1). Within the African spitting cobras, *N. pallida* and *N. nubiae* form the sister clade to the other *Afronaja* species, which are mostly well resolved except for the position of the *N. nigricincta* & *N. woodi* clade and *N. mossambica* (BPP 0.63).

The other three subgenera nested within the *Naja* clade have relatively poor BPP support values, with the subgenera *Naja* and *Uraeus* forming a monophyletic group (BPP 0.8) with the lineage diverging earlier being *Boulengerina* (BPP 0.74). Interspecific relationships within Boulengerina place *N. multifasciata* as the basal species, with *N. peroescobari, N. melanoleuca,* and the three distinct populations the latter species, "Western banded", "West African black form", and *N.* cf. *subfulva* forming a monophyletic clade, with a BPP support value of 0.73. *Naja* cf. *subfulva* and the Western banded cobra form a strongly supported monophyletic clade (BPP 0.99), as do the sister clade comprising of *N. melanoleuca,* the West African black form and *N. peroescobari*; however, the monophyly of *N. melanoleuca* and West African black form is poorly resolved (BPP 0.48).

The Asian cobras, subgenus *Naja*, form a monophyletic clade with the spectacled cobra *N. naja* positioned at the base of the clade. The remaining species are split in to two clades, with a strong BPP support value of 0.98. *Naja atra, N. oxiana, N. sagittifera, N. kaouthia* form one clade, with the latter two classed as sister species (BPP 1), yet the placement of *N. oxiana* is poorly resolved within the clade (BPP 0.41). The second clade within the subgenus Naja is again strongly supported, with *N. samarensis* and *N. philippinensis* forming a monophyletic clade. Their sister clade is less resolved, with varying support values.

Sister to the subgenus Naja is the African group of non-spitting cobras, Uraeus. The monophyly of this clade is well supported, with *N. nivea* forming the earliest divered lineage. The two

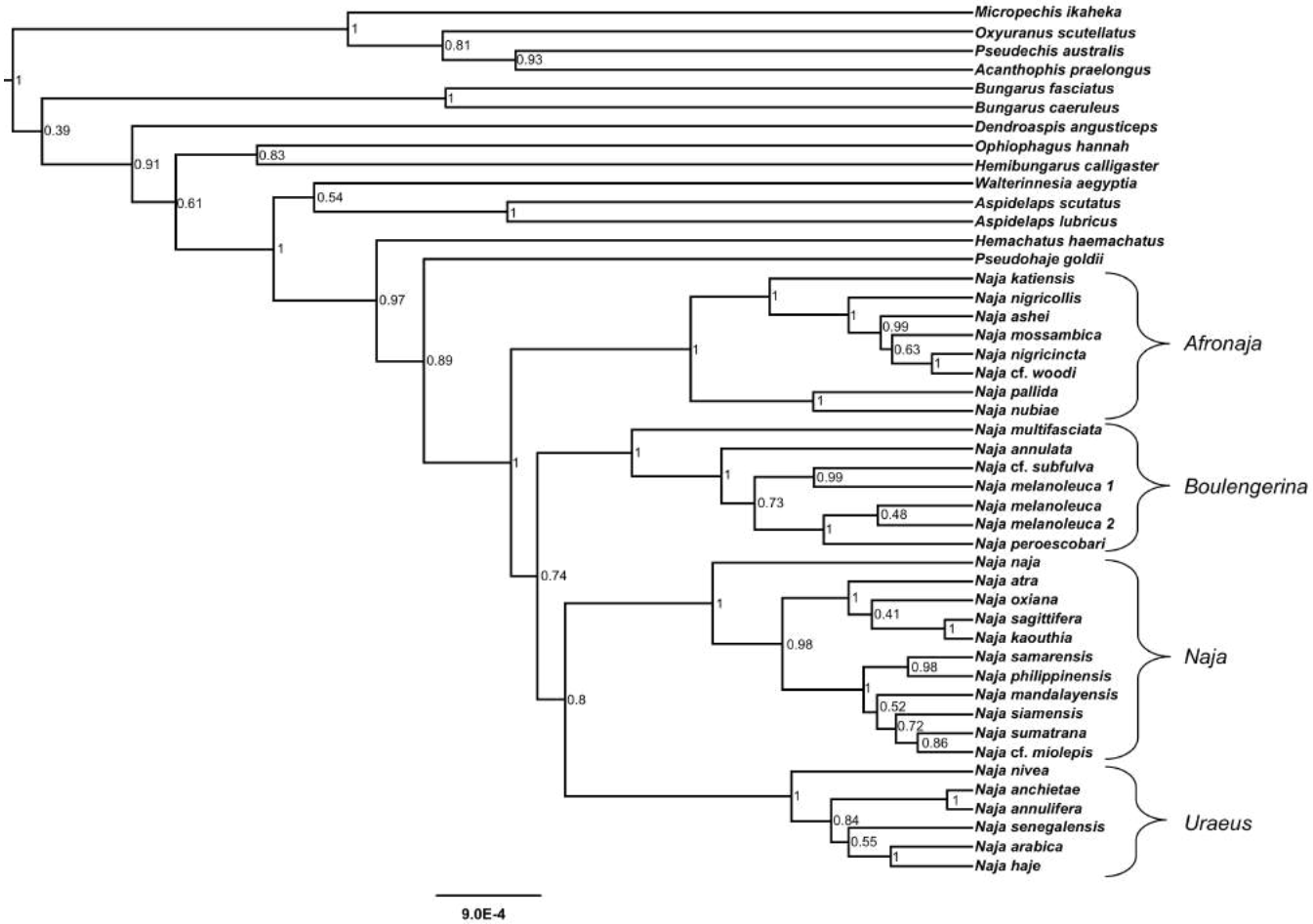


Figure 5 Output *BEAST tree of the cobra clade elapids and outgroups. Branch lengths are representative of divergence time and nucleotide substitution rate. Support values for each node are Bayesian Posterior Probabilities. The clades representing the four *Naja* subgenera are listed on the right.

strongly supported monophyletic clades are comprised of *N. anchietae* & *N. annulifera*, and *N. arabica* & *N. haje*; their positions within the larger clade are however poorly resolved.

3.3 Individual gene trees

The individual gene trees (Appendix 06) predominantly found strong support for the subgenera nesting as individual monophyletic clades. Two exceptions were the nuclear RAG1 and UBN1 trees, for which the former found moderate support (BPP 0.93) in nesting four Boulengerina sequences as a sister clade to Afronaja, rendering the subgenus Boulengerina polyphyletic. The nuclear tree for UBN1 is overall poorly resolved. The monophyly of the subgenus Naja was strongly supported (BPP 1), however the position, monophyly, and inter-relationships between the remaining subgenera has weak-to-no support in BPPs (see Appendix 06).
The paraphyly of the genus *Naja* is weakly supported by the NT3 and PRLR gene trees (Appendix 06d & 06e). The former tree shows strong support for *Pseudohaje goldii* forming a sister lineage to the subgenus Afronaja, and weak support for *Hemachatus haemachatus* forming a basal clade for the remaining three subgenera. The PRLR gene tree shows the inclusion of *P. goldii* as a sister lineage to the African spitting cobras, but this is very poorly supported (BPP 0.13).

4.0 Discussion

The results from this study further support previous phylogenetic hypotheses, whilst also proposing novel and alternative systematic changes to the cobra clade. Although some previous studies have found well supported hypotheses, they have generally either solely used mitochondrial genes (e.g. Wüster *et al.* 2007) and therefore lacked in number of loci used, or more critically, used a small number of cobra species (*Naja* species included: 4, Slowinski & Keogh, 2000; 4, Nagy *et al.* 2005; 5, Kelly *et al.* 17, Wüster *et al.* 2007; 18, Lee *et al.* 2016). The goal for this study was to use a broader range of genetic markers on a greater number of cobra clade elapid species, allowing for the most comprehensive and taxonomically inclusive phylogenetic analysis to be undertaken. All currently accepted species of true cobra, excluding two, were incorporated in this study, with the following having had no prior inclusion in any full genus comparative phylogenies, *N. oxiana, N. sagittifera, N. samarensis, N. philippinensis, N. anchietae, N. senegalensis,* and *N. arabica*. The two extant species excluded from this study are *N. sputatrix* and *N. christyi*, however their phylogenetic positions should not affect the final tree topology. *N. sputatrix* is continued to belong within the Asian subgenus *Naja*; and as in Wallach *et al.* (2009), *N. christyi* is continued to be tentatively placed within the subgenus *Boulengerina*. The results (Fig 5.) offer a new insight in to the evolution of spitting and the relationships between evolutionary lineages within the cobras.

4.1 Phylogeny of the core cobra clade

To-date, the monophyletic core cobra clade (Fig 1. Slowinski & Keogh, 2000) included *Aspidelaps, Hemachatus, Naja,* and *Walterinnesia*; with the two African tree cobra species, *Pseudohaje spp.* not being included in previous phylogenetic hypotheses. They have been commonly referred to as cobras for some time, mostly due to their "cobra-like form" (Cadle, 1987), despite their larger eyes, smaller fangs and reduced bone structure in comparison to true *Naja* cobras (Bogert, 1942). The elusiveness of the two *Pseudohaje* species has resulted in a poor range of literature about this genus (diet: Pauwels & Ohler, 1999; Pauwels et al. 1999; ecology: Akani et al. 2005). Akani *et al*. (2005) noted that over 10 years (c. 100 days field work per year) only 62 individuals were captured, emphasising its rarity and thus the likely attribute to their lack of prior inclusion in phylogenetic studies until now. Samples from *P. goldii* were used for this analysis, however *P. nigra* could not be included. Previous phylogenies have shown *Hemachatus haemachatus* to be the closest taxa to the true cobras (i.e. Wüster *et al.* 2007; Lee *et al.* 2016), however the results from this study support the hypothesis that the arboreal cobra, *P. goldii,* forms a sister lineage to the genus *Naja* (Fig. 5). Confirming previous hypotheses, the genus *Pseudohaje* now has a position on a phylogenetic tree and falls within the aforementioned core cobra group. This novel finding is interesting and further adds to the general knowledge of cobra phylogenetics. Its position as the sister lineage to the genus *Naja* is moderately supported, and due to a small sample size and the exclusion of *P. nigra*, this finding is arguably still unresolved and underrepresented, therefore would benefit from further work.

A continually supported hypothesis is for the exclusion of the king cobra, *Ophiophagus hannah*, within the cobra clade. *O. hannah* has been frequently found to group separately from the remaining cobra-like species, often as a sister lineage to mambas, *Dendroaspis sp.* (Kelly *et al.* 2009; Pyron *et al.* 2013; Lee *et al.* 2016). The results from this study don't however resolve the position of *O. hannah*, with poor support values placing the species as a sister species to the Asian coral snake, *Hemibungarus calligaster*, whereas Figueroa *et al.* (2016) placed *O. Hannah* adjacent to the mambas, *Dendroaspis*. The high BPP value supports the monophyly of the core cobra group to the exclusion of *O. hannah.*

Walterinnesia aegyptia forms a poorly supported sister lineage to the *Aspidelaps spp.*, however the high BPP support value for the monophyly of the remaining core cobra genera would still indicate that both *Walterinnesia* and *Aspidelaps* act as sister lineages to the remaining cobra clade. All extant species within the group reside in Africa, excluding the *Walterinnesia* genus found in north east Africa and the Middle East, and the Asian lineage of *Naja*. This pattern strongly supports an African origin for the entire cobra clade, with an ancestral *Naja* lineage that entered Asia.

4.2 Phylogeny of *Naja*

The Bayesian multispecies coalescent approach used has found that the true cobras, genus *Naja*, have continued strong support in forming a monophyletic clade (Slowinski & Keogh, 2000; Nagy *et al.* 2005; Wüster *et al.* 2007). The four previously identified subgenera, *Afronaja*, *Boulengerina*, *Naja*, and *Uraeus* (Wallach *et al.* 2009) retain strong support in forming individual monophyletic groups, however the interrelationships between them still remains unclear.

Previous phylogenetic hypotheses have found the Asian cobras (subgenus Naja) to form the sister lineage to the other cobra subgenera (Wüster *et al.* 2007; Pyron *et al.* 2013; Lee *et al.* 2016). The combination of nuclear and mitochondrial genes allows for this hypothesis to be rejected, as the results in Fig. 5 support the African spitting group (subgenus *Afronaja*) forming the lineage which diverged at the earliest point in time amongst the *Naja* subgenera. A cause for this conflict and low support values in previous studies may be as a result of the Asian species often being underrepresented (Wüster *et al.* 2007; Pyron *et al.* 2013; Lee *et al.* 2016; 4, 6, and 6 species, respectively), whereas a total of 10 species were included in this study.
The African spitting cobras, currently containing 7 described species, has undergone a vast amount of taxonomic changes and further research within the last 50 years. Historically, all African spitting cobras were described as belonging to one species, *N. nigricollis* Reinhardt, 1843. This was first disputed when *N. mossambica* was formally recognised by Broadley (1968), who continued to propose that the currently-recognised *N. katiensis, N. nigricincta, N. pallida* and *N. n. woodi* were all subspecies of *N. mossambica*. This clade of spitting cobras has received increased previous interest, with most interrelationships having already been investigated, albeit using mitochondrial genes (Wüster *et al.* 2007). Continued support was found for *N. pallida* and *N. nubiae* forming a sister clade to the remaining spitting cobras. Further support was found for the interrelationships between the remaining *Afronaja* species and *N. n. woodi*, hypothesising that *N. n. woodi* remains a subspecies of *N. nigricincta*, and the sister clade to *N. nigricincta* is *N. mossambica*.

The positions of the remaining three subgenera are still poorly resolved, with Bayesian posterior probability support values of 0.74 and 0.8. This emphasises the need for a larger sample size, more sequences specifically for the c-mos and RAG1 loci, and additional loci to be included. The Asian cobra clade has been subject to numerous evolutionary hypothesis, and theories behind a history of multiple-origins; i.e. the proposal that the Asian spitting and non-spitting cobras entered Asia separately (Minton, 1986; Ineich, 1995). Due to the lack of any strict pattern of spitting or non-spitting within the Asian *Naja*, alongside the paraphyly of spitting *Naja* when compared to the subgenus *Afronaja*, the hypothesis from Minton (1986) and Ineich (1995) is rejected.

The next subgenus to diverge after *Afronaja* for the remaining three subgenera is *Boulengerina*, representing some of the African non-spitting cobras, sometimes referred to as Forest cobras (Wallach *et al.* 2009). The small and subterranean *N. multifasciata* sits as the basal lineage for

this morphologically varied subgenus, with its' position supported by a BPP value of 1. The remaining species are *N. annulata, N. melanoleuca,* and the recently described *N. peroescobari.* As previously mentioned due to the morphological, ecological, and spatial differences of populations of *N. melanoleuca*, this species is analysed and assessed as four separate units. Of which, *Naja* cf. *subfulva* and *N. m. "*Western banded" form a monophyletic clade with a very high BPP support value. Their sister clade has a BPP value of 1, and grouped within lie *N. perescobari, N. melanoleuca* sensu stricto, and a population of *N. melanoleuca* described as "West African black form". Similar phylogenetic findings were published by Ceríaco *et al.* (2017) supporting these placements. The phylogenetic position of the uncommon species *N. christyi* is currently still unclear, due to poor inclusion in the analytical literature, and was further unable to be included in this study. Its position however is assumed to be within the subgenus *Boulengerina,* alongside the similar *N. annulata* (Boulenger, 1904; Bogert, 1943).
Previously published phylogenies have included up to 6 Asian species of *Naja* (Lee *et al.* 2016), with this study including 10 described species and a population of *N. sumatrana* from Borneo named here as *N.* cf. *miolepis.* High support values place the spectacled cobra, *N. naja,* as the lineage being genetically isolated for the longest period. The remaining species are split in to two highly supported monophyletic clades (both with a BPP support value of 1), coinciding with their spatial distribution. One group contains *N. atra, N. oxiana, N. sagittifera,* and *N. kaouthia*, with a high BPP for the monophyly of *N. kaouthia* and the island species *N. sagittifera*. Despite the inclusion of *N. oxiana* for the first time, its position within the tree is still poorly resolved, and therefore needs further work to establish its position within this clade. The second clade contains the species endemic to southeast Asia, with high support for the monophyly of *N philippinensis* and *N. samarensis*, from the north and south of the Philippines, respectively. The remaining southeast Asian species have poorly supported positions, with the highest BPP support value supporting the expected position of *N. sumatrana* with its' Bornean population, *Naja* cf. *miolepis.* The one extant Asian cobra not included in this analysis is the spitting *N. sputatrix* found in Indonesia. With all Asian species belonging to the same subgenus *Naja*, and the one previous mitochondrial study supporting that placement (Wüster *et al.* 2007), its position remains the same.
The final subgenus is *Uraeus*, representing the second clade of African non-spitting cobras. Despite the lower support values for the overall positions of the species, a BPP of 1 is presented for the monophyly of *N. anchietae* and *N. annulifera*, with the same high BPP value supporting the monophyly of *N. arabica* and *N. haje*.

4.3 Gene Trees

Individual gene trees were generally found to be poorly resolved, with the exception of high support values for the monophyly of the subgenera. Exceptions to this, however, include for the genes RAG1 and UBN1, with the former gene tree supporting the placement of *N. multifasciata* and *N. annulata* (subgenus *Boulengerina*) within *Afronaja* (0.93 BPP). The latter gene tree showed no true pattern and with a very low support value (0), splitting *Afronaja* half into the subgenus *Naja*.
Despite five gene trees supporting the monophyly of the genus *Naja*, both the NT3 and PRLR trees had high support values hypothesising the inclusion of non-true cobras within the true cobra clade. The NT3 gene tree placed both *P. goldii* and *H. haemachatus* within the true cobra clade (BPP 0.98), with *P. goldii* forming a sister lineage to *Afronaja* (0.94). The PRLR gene tree also highly supports the tree cobra, *P. goldii*, belonging within the true cobra clade (BPP 1), specifically within *Afronaja* and *Uraeus* (BPP 0.91).

4.4 Defensive Adaptions

A notable defensive behaviour for cobras is their ability to form a hood, a trait shared by nearly the cobra species. Within the core cobra group, only *Walterinnesia aegyptia* doesn't utilise hooding as a defence mechanism. Jones (2017) however found that although the other 30 species of cobra included in this study exhibited hooding behaviour, only 22 had extended ribs (see Appendix 07). Despite most species possessing extended ribs to allow for a larger defensive display, Jones (2017) found support for a loss or reduction of rib length in some species. The ecologically varied subgenus *Boulengerina* contains both the semi-fossorial *N. multifasciata* and the large aquatic *N. annulata*, both of which express hooding behaviour yet lack the extended ribs (Jones, 2017; Appendix 07). This is likely caused by their habitat preferences and thus extended ribs may hinder the swimming ability of *N. annulata* or may be less efficient for the different predators encountered by *N. multifasciata*. The king cobra, *Ophiophagus hannah* remains outside the core cobra group, clearly supporting its independent origin for extended ribs and hooding behaviour.

This study further supports the hypothesis of three independent origins of spitting within the cobra clade, as previously proposed by Wüster *et al.* (2007) and Panagides *et al.* (2017). The rinkhals, *Hemachatus haemachatus* is the only non-true cobra to be able to spit and forms an independent origin of spitting. The second origin of spitting lies within the subgenus *Afronaja*, of which all 7 species share this well-adapted trait, with all African cobras either possessing the specialised spitting morphology or not (Berthé, 2011; Wüster & Thorpe, 1992). These prior two origins have also not been questioned, with support found for their independent and uncomplicated spitting status. The Asian subgenus however, *Naja,* proves to be both an interesting yet complicated group.

The monophyletic southeast Asian cobras *N. samarensis*, *N. siamensis, N. sumatrana* and *N.* cf. *miolepis* are all unambiguous spitters, with adapted discharge orifices (Wüster *et al.* 1992). The remaining five Asian cobras show no distinct pattern; *N. atra, N. sagittifera* and *N. kaouthia* are described as occasional or ambiguous spitters (*N. atra* & *N. sagittifera,* Wüster & Thorpe, 1992b; *N. kaouthia,* Santra & Wüster, 2017). *Naja naja* and *N. oxiana* are the only species consistently described as non-spitting, for which Wüster & Thorpe (1992b) found have substantially larger discharge orifices. The uncertainty behind the spitting ability of *N. atra, N. sagittifera* (previous subspecies of *N. kaouthia*), & *N. kaouthia* results in various possible hypotheses. If classed as non-spitters, strong support is found for a single origin within the Asian cobras, at the base of the well-adapted spitters (*N. samarensis*, *N. siamensis, N. sumatrana* and *N.* cf. *miolepis*), further supporting hypotheses from Wüster *et al.* (2007) and Panagides *et al.* (2017). If, however, the aforementioned three ambiguous spitters are included and classed as spitting, a prior origin of this trait at the base of all Asian *Naja* (excluding *N. naja*) is the most parsimonious hypothesis. Although the phylogenetic position of *N. oxiana* is still not highly supported, following this hypothesis would support a single loss in *N. oxiana*, as proposed by Wüster *et al.* (2007). Panagides *et al.* (2017) used the inaccurate phylogeny from Lee *et al.* (2016), and thus grouped *N. naja* as the sister species to *N. atra*, proposing losses of spitting in both *N. naja* and *N. oxiana*.

Spitting as a defensive behaviour is unique to cobras, and is thought to have needed a direct driver for the cobra ancestors to evolve this trait. Barbour (1922) proposed that with the expansion of savannahs and thus broadening the range for ungulates, cobras needed to evolve a more suitable defensive behaviour to warn or deter larger animals. Initial attempts at molecular dating found basal divergences to be around 15mya during the early-mid Miocene (Wüster *et al.* 2007). The molecular dating methodology was then further refined, and estimates were published supporting diverge dates closer to 17mya (Wüster *et al.* 2008; Pook *et al.* 2009), the same estimated time that open grassland formations spread (Potts & Behrensmeyer, 1992;

Jacobs, 2004). The advantage of spitting over biting an aggressor is the additional safe distance for the snake, which proves vital if the aggressors used tools. Primates are known to use tools for foraging and defence (e.g. Chapman, 1986; Boinski, 1988; Osvath & Osvath, 2008), and like the African spitting cobras, primates originated in Africa. This hypothesis could be expanded to include the Asian cobras, as they evolved the ability to spit after the African cobras, possibly coinciding with the expansion of primates or early hominids in to Asia. Molecular timing of primate divergences estimate that the Homo and Pan split occurred between 6-6.6mya (Raaum *et al.* 2004; Steiper & Young, 2006, respectively). Wüster *et al.* (2008) and Pook *et al.* (2009) both found a sudden diversification of species within the subgenus *Afronaja,* since 6mya, thus after the Homo-Pan split. Although the branch lengths in the phylogenetic tree in **Fig. 5** are proportional to divergence times, no thorough molecular dating analysis was undertaken for this study.

4.5 Assessing methods and analytical techniques used

Lab methods used were generally unproblematic and as described in literature. The tissue and blood samples came predominantly from Dr Wolfgang Wüster's tissue/sample archive. The collection dates varied, but many dated back to the 1990s. The age and preservation methods required different DNA extraction techniques and correspondingly resulted in varying success rates. Future sampling should therefore entail scale clipping and preservation in ethanol for the most efficient DNA extraction.

Unlike some previous published phylogenies, this study was undertaken using the more accurate coalescent genetic analysis, as opposed to concatenated analysis. The inconsistencies between gene trees show that the genes possess various evolutionary histories, something that is not compatible with concatenated analysis, a method dependent on the concatenated genes sharing a single evolutionary tree (Degnan & Rosenberg, 2009). Concatenated methods have also been found to falsely increase phylogenetic accuracy (Kubatko & Degnan, 2007). Further sampling by increasing loci used (specifically phylogenetically informative loci) has been found to increase total phylogenetic accuracy (Camargo *et al.* 2012). Camargo *et al.* (2012) found substantial differences in output-accuracy dependent on the number of samples used to represent taxa, even by using two samples. This was a factor considered during this study, and from 335 different samples (46 species and 7 genes), only 21% had a singular representative, of which most were for nuclear genes. Nuclear genes were processed through PHASE, which duplicated sequences and allowing heterozygous positions to be represented by their most-likely parental homozygous haplotypes, thus arguably doubling the effective sample size of nuclear sequences.

4.6 Conclusion

Overall, the phylogenetic positions found were more supportive of previous hypotheses than revolutionary. The species included in each true cobra subgenera stay uncontested with further support from the advanced methodology, combined with an increased number of independent nuclear loci. Support was found to suggest the subgenus *Afronaja* diverged and evolved independently at the earliest time point, thus moving the Asian subgenus *Naja,* but these changes are only moderately supported. The king cobra, *Ophiophagus hannah* remains taxonomically separate to the core cobra group, with the newly supported sister lineage to the genus *Naja* being the forest cobras, *Pseudohaje*. This latter placement cannot be either contested or used to support other hypotheses, as this is the first published phylogeny with its inclusion.

Despite using the currently most accurate multilocus coalescent analysis, there are still several low-supported phylogenetic positions. Increased sampling effort and quantity of loci are needed to represent all species and to obtain higher support values. It would also be beneficial to include both *N. sputatrix* and *N. christyi.* Due to time constraints, it was not possible to undertake molecular dating analysis or estimates using calibration points, but representative branch lengths are shows in Figure 5. Using MCMC algorithms, *BEAST could be used to estimate the dates of divergence events (Heled & Drummond, 2010; Rutschmann, 2006), with the times of cladogenesis within the cobra clade being vital in forming a better understanding of the evolution of some behavioural and morphological characters, including the ability to spit.

References:

Akani, G.C., Angelici, F.M. & Luiselli, L. 2005. Ecological data on the Goldie's tree cobra, *Pseudohaje goldii* (Elapidae) in southern Nigeria. *Amphibia Reptilia*, **26**(3), pp. 382-387.

Alexander, G.J. 1996. *Thermal physiology of Hemachatus haemachatus and its implications to range limitation*. (Doctoral dissertation, Ph. D. Thesis, Faculty of Science, University Witwatersrand, Johannesburg, South Africa).

Avise, J.C. 2004. *Molecular markers, natural history and evolution,* 2nd ed. Sinauer Associates, Inc., Massachusetts, USA

Bauer, A.M., Branch, W.T., 2001. The herpetofauna of the Richtersveld National Park and the adjacent northern Richtersveld, Northern Cape Province, Republic of South Africa. *Herpetological Natural History*. **8**, 111–160.

Beheregaray, L. B. 2008. Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. *Molecular Ecology*. **17,** pp. 3754-3774.

Berthé, R. A. (2011). *Spitting behaviour and fang morphology of spitting cobras*. Doctoral Thesis. Rheinischen Friedrich-Wilhelms-Universität, Bonn, Germany.

Bianconi, J. J. 1849. Specimina zoologica Mossambicana (De piscibus et reptilibus). Alcune nuove specie di rettili del Mozambico. *Nuovi annali delle scienze naturali*, **10**, pp. 106-109 & 202-208.

Bickford, D., Lohman, D.J., Sodhi, N.S., Ng, P.K., Meier, R., Winker, K., Ingram, K.K. & Das, I. 2007. Cryptic species as a window on diversity and conservation. *Trends in ecology & evolution*, **22**(3), pp. 148-155.

Bogert, C.M. 1942. *Pseudohaje* Günther, a valid genus for two West African arboreal cobras. *American Museum of Natural History*, **1174**, 1-9.

Bogert, C.M. 1943. Dentitional phenomena in cobras and other elapids, with notes on adaptive modifications of fangs. *Bulletin of the American Museum of Natural History.* **81**, pp. 285-360.

Boinski, S. 1988. Use of a club by a wild white-faced capuchin (*Cebus capucinus*) to attack a venomous snake (*Bothrops asper*). *American Journal of Prim*atology, **14**(2), pp. 177-179.

Bonnet, X., Naulleau, G. & Shine, R. 1999. The dangers of leaving home: dispersal and mortality in snakes. *Biological conservation*, **89**(1), pp.39-50.

Boulenger, G.A. 1896. *Catalogue of the Snakes in the British Museum (Natural History)*. Vol. 3, British Museum (Natural History), London.

Boulenger, G.A. 1904. Descriptions of two new Elapine snakes from the Congo. *Annals and magazines of natural history*, **7**(14), pp. 15.

Boyer, L., Alagón, A., Fry, B.G., Jackson, T.N.W., Sunagar, K. & Chippaux, J.P. 2015. Signs, symptoms, and treatment of envenomation. In: Fry, B. G. ed. *Venomous Reptiles and Their Toxins: Evolution, Pathophysiology and Biodiscovery*. Oxford University Press, pp. 45.

Broadley, D.G. & Baldwin, A.S. 2006. Taxonomy, natural history, and zoogeography of the southern African shield cobras, genus *Aspidelaps* (Serpentes: Elapidae). *Herpetological natural history*, **9**(2), pp. 163-176.

Broadley, D.G. & Blaylock, R. 2013 *The Snakes of Zimbabwe and Botswana*. Edition Chimaira, Frankfurt am Main, pp. 387.

Broadley, D.G. & Cock, E.V. 1975. *Snakes of Rhodesia*. Salisbury: Longman Rhodesia Ltd.

Broadley, D.G. & Wüster, W. 2004. A review of the southern African 'non-spitting'cobras (Serpentes: Elapidae: *Naja*). *African Journal of Herpetology*, **53**(2), pp. 101-122.

Broadley, D.G. 1968. A review of the African cobras of the genus *Naja* (Serpentes: Elapinae). *Arnoldia Rhodesia*, **3**, pp. 1–14.

Brown, W.M., George, M., & Wilson, A.C. 1979. Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences*, **76**(4), pp. 1967-1971.

Cadle, J.E. 1987. Geographic distribution: problems in phylogeny and zoogeography. *Snakes: ecology and evolutionary biology*, **3**, pp. 77-105.

Camargo, A., Avila, L.J., Morando, M. & Sites Jr, J.W. 2011. Accuracy and precision of species trees: effects of locus, individual, and base pair sampling on inference of species trees in lizards of the *Liolaemus darwinii* group (Squamata, Liolaemidae). *Systematic Biology*, **61**(2), pp. 272-288.

Cantor, T.E. 1836. Sketch of undescribed hooded serpent with fangs and maxillar teeth. *Asiatic Researchers Calcutta*, **19**(8): 87-94

Carroll, M.C. 2004. The complement system in regulation of adaptive immunity. *Nature immunology*, **5**(10), pp. 981-986.

Chapman, C.A. 1986. Boa constrictor predation and group response in white-faced cebus monkeys. *Biotropica*, **18**(2), pp. 171-172.

Conrad, J.L. 2008. Phylogeny and systematics of Squamata (Reptilia) based on morphology. *Bulletin of the American Museum of Natural History*, **310**, pp. 1-182.

Cornish-Bowden, A. 1985. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. *Nucleic acids research*, **13**(9), pp. 3021.

Das, I. 2010. *A field guide to the reptiles of South-East Asia*. London, New Holland Publishing.

Dayrat, B., 2005. Towards integrative taxonomy. *Biological journal of the Linnean society*, **85**(3), pp. 407-415.

Degnan, J.H. & Rosenberg, N.A. 2009. Gene tree discordance, phylogenetic inference and the multispecies coalescent. *Trends in Ecology and Evolution,* **24**, pp. 332–340.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic acids research*, **32**(5), pp.1792-1797.

Eo, S.H. & DeWoody, J.A., (2010). Evolutionary rates of mitochondrial genomes correspond to diversification rates and to contemporary species richness in birds and reptiles. *Proceedings, Biological sciences / The Royal Society*, **277**, 3587–3592

Ewing, B., Hillier, L., Wendl, M.C. & Green, P. 1998. Base-calling of automated sequencer traces usingPhred. I. Accuracy assessment. *Genome research*, **8**(3), pp. 175-185.

Figueroa, A., McKelvy, A.D., Grismer, L.L., Bell, C.D. & Lailvaux, S.P. 2016. A species-level phylogeny of extant snakes with description of a new colubrid subfamily and genus. *PloS one*, **11**(9), p.e.0161070.

Flot, J.F. 2010. SeqPHASE: a web tool for interconverting PHASE input/output files and FASTA sequence alignments. *Molecular Ecology Resources*, **10**(1), pp.162-166.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M. & Gemmell, N.J. 2007. Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS one*, **2**(10), p.e1109.

Fry, B.G., Richards, R., Earl, S., Cousin, X., Jackson, T.N., Weise, C. and Sunagar, K. 2015 **b**. Lesser-known or putative reptile toxins. In: Fry, B. G. ed. *Venomous Reptiles and Their Toxins: Evolution, Pathophysiology and Biodiscovery*. Oxford University Press, pp. 374.

Fry, B.G., Sunagar, K., Casewell, N.R., Kochva, E., Roelants, K., Scheib, H., Wüster, W., Vidal, N., Young, B., Burbrink, F. and Pyron, R.A. 2015**a**. The origin and evolution of the Toxicofera reptile venom system. In: Fry, B. G. ed. *Venomous Reptiles and Their Toxins: Evolution, Pathophysiology and Biodiscovery*. Oxford University Press, pp. 26.

Fry, B.G., Winkel, K.D., Wickramaratna, J.C., Hodgson, W.C. and Wüster, W. 2003. Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. *Journal of Toxicology: Toxin Reviews*, **22**(1), pp. 23-34.

Futter, C.E., Panton, D., Kestin, S. and Flint, D.J. 1992. Mechanism of action of cytotoxic antibodies to adipocytes on adipose tissue, liver and food intake in the rat. *International journal of obesity and related metabolic disorders: journal of the International Association for the Study of Obesity*, **16**(8), pp. 615-622.

Gibbons, J.W. 1987. Activity patterns. *Snakes, ecology and evolutionary biology*, pp. 396-421.

Göçmen, B., Franzen, M., Yildiz, M.Z., Akman, B. & Yalçinkaya, D. 2009. New locality records of eremial snake species in southeastern Turkey (Ophidia: Colubridae, Elapidae, Typhlopidae, Leptotyphlopidae). *Salamandra*, **45**(2), pp. 110-114.

Greene, H.W. 1997. *Evolution of Mystery in Nature*. Berkeley CA, University of California Press.

Gregory, P.T., Macartney, J.M. and Larsen, K.W., 1987. Spatial patterns and movements. *Snakes: ecology and evolutionary biology*, pp. 366-395.

Heled, J. & Drummond, A.J., 2010. Bayesian inference of species trees from multilocus data. *Molecular biology and evolution*, **27**(3), pp. 570-580.

Hillis, D.M. 1987. Molecular versus morphological approaches to systematics. *Annual review of Ecology and Systematics*, **18**, pp. 23-42.

Hudson, R.R. 1983. Testing the constant-rate neutral allele model with protein sequence data. *Evolution,* **37**, pp. 203– 217.

Ineich, I. 1995. Etat actuel de nos connaissances sur la classification des serpents venimeux. *Bulletin de la Société herpétologique de France*, pp.7-24.

Jacobs, B.F. 2004. Palaeobotanical studies from tropical Africa: relevance to the evolution of forest, woodland and savannah biomes. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **359**(1450), pp.1573-1583.

Jones, B. (2017), Masters by Research Thesis, Bangor University, UK.

Kelly, C.M., Barker, N.P., Villet, M.H. & Broadley, D.G. 2009. Phylogeny, biogeography and classification of the snake superfamily Elapoidea: a rapid radiation in the late Eocene. *Cladistics*, **25**(1), pp. 38-63.

Keogh, J.S., Webb, J.K. & Shine, R., 2007. Spatial genetic analysis and long-term mark–recapture data demonstrate male-biased dispersal in a snake. *Biology Letters*, **3**(1), pp. 33-35.

Kini, R.M. and Evans, H.J. 1989. Role of cationic residues in cytolytic activity: modification of lysine residues in the cardiotoxin from *Naja nigricollis* venom and correlation between cytolytic and antiplatelet activity. *Biochemistry*, **28**(23), pp. 9209-9215.

Klemmer, K. 1963. Liste der Rezenten Giftschlangen. In: *Die Giftschlangen der Erde (Behringwerke–Mitteilungen)*. N. G. Elwert, Marburg, pp. 255–464.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X. & Wilson, A.C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences*, **86**(16), pp. 6196-6200.

Kubatko, L.S. & Degnan, J.H. 2007. Inconsistency of phylogenetic estimates from concatenated data under coalescence. *Systematic Biology*, **56**(1), pp. 17-24.

Laurent, R.F., 1955. Diagnoses preliminaires des quelques Serpents venimeux. *Revue de Zoologie et de Botanique Africaines*, **51**, pp.127-139.

Laurenti, J.N. 1768. *Austriaci Viennensis Specimen Medicum, Exhibens Synopsin Reptilium Emendatam Cum Experimentis Circa Venena et Antidota Reptilium Austriacorum*. J.T. De Trattern, Viennae, pp. 214.

Lee, M.S., Sanders, K.L., King, B. and Palci, A. 2016. Diversification rates and phenotypic evolution in venomous snakes (Elapidae). *Royal Society open science*, **3**(150277).

Lillywhite, H.B. 2014. *How snakes work: structure, function and behavior of the world's snakes*. Oxford University Press.

Liu, L., Yu, L., Kubatko, L., Pearl, D.K. & Edwards, S.V. 2009. Coalescent methods for estimating phylogenetic trees. *Molecular Phylogenetics and Evolution*, **53**(1), pp. 320-328.

Losos, J.B., Hillis, D.M. & Greene, H.W. 2012. Who speaks with a forked tongue? *Science*, **338**(6113), pp. 1428-1429.

Luiselli, L. & Angelici, F.M. 2000. Ecological relationships in two Afrotropical cobra species (*Naja melanoleuca* and *Naja nigricollis*). *Canadian Journal of Zoology*, **78**(2), pp. 191-198.

Mertens, R. 1941. Zur Kenntnis der Herpetofauna von Fernando-Poo. *Zoologischer Anzeiger,* **135**, pp. 275-281.

Minton, S.A. 1986. Origins of poisonous snakes: evidence from plasma and venom proteins. *Natural toxins—animal, plant and microbial*. Clarendon Press, Oxford, pp. 3-21.

Mocquard, M.F. 1905. Diagnoses de quelques espèces nouvelles de Reptiles. *Bulletin du Muséum d'histoire naturelle, Paris*, **11**, pp. 76-79.

Moritz, C.T.E.D., Dowling, T.E. & Brown, W M. 1987. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual review of ecology and systematics*, **18**(1), pp. 269-292.

Mulligan, M.S., Schmid, E., Beck-Schimmer, B., Till, G.O., Friedl, H.P., Brauer, R.B., Hugli, T.E., Miyasaka, M., Warner, R.L., Johnson, K.J. and Ward, P.A. 1996. Requirement and role of C5a in acute lung inflammatory injury in rats. *Journal of Clinical Investigation*, **98**(2), pp. 503.

Nagy, Z.T., Vidal, N., Vences, M., Branch, W.R., Pauwels, O.S., Wink, M. & Joger, U. 2005. Molecular systematics of African Colubroidea (Squamata: Serpentes). In *African Biodiversity.* Springer US. pp. 221-228.

Nilson, G. & Rastegar-Pouyani, N. 2007. *Walterinnesia aegyptia* Lataste, 1887 (Ophidia: Elapidae) and the status of *Naja morgani* Mocquard 1905. *Russian Journal of Herpetology,* **14**(1), pp. 7-14.

O'Shea, M. 2008. *Venomous snakes of the world*. New Holland Publishers.

Osvath, M. & Osvath, H. 2008. Chimpanzee (*Pan troglodytes*) and orangutan (*Pongo abelii*) forethought: self-control and pre-experience in the face of future tool use. *Animal cognition*, b(4), pp. 661-674.

Padial, J.M., Miralles, A., De la Riva, I. & Vences, M. 2010. The integrative future of taxonomy. *Frontiers in zoology*, **7**(1), pp. 1-14.

Panagides, N., Jackson, T.N., Ikonomopoulou, M.P., Arbuckle, K., Pretzler, R., Yang, D.C., Ali, S.A., Koludarov, I., Dobson, J., Sanker, B. and Asselin, A. 2017. How the cobra got its flesh-eating venom: Cytotoxicity as a defensive innovation and its co-evolution with hooding, aposematic marking, and spitting. Toxins, **9**(3), pp. 103-125.

Pauwels, O.S.G. & Ohler, A. 1999: *Pseudohaje nigra* (Günther,1858): black tree cobra. Diet. *African Herpetological News,* **30**: 33-34.

Pauwels, O.S.G., David, P. & Lenglet, G. 1999. *Pseudohaje goldii* (Boulenger, 1895); Goldie's Tree Cobra. Diet. *African Herpetological News,* **30**, pp.32-33.

Pook, C. E., Joger, U., Stümpel, N. & Wüster, W. 2009. When continents collide: phylogeny, historical biogeography and systematics of the medically important viper genus *Echis* (Squamata: Serpentes: Viperidae). *Molecular Phylogenetics and Evolution*, **53**(3), 792-807.

Potts, R. & A. K. Behrensmeyer. 1992. *Terrestrial Paleoecology in the Cenozoic*. In: Behrensmeyer, A. K., J. D. Damuth, W. A. DiMichele, R. Potts, H.-D. Sues, & S. L. Wing, *Terrestrial Ecosystems Through Time*, Chicago: University of Chicago Press. pp. 419-541.

Presgraves, D.C. 2010. The molecular evolutionary basis of species formation. *Nature Reviews Genetics*, **11**(3), pp. 175-180.

Pyron, R.A., Burbrink, F.T. & Wiens, J.J. 2013. A phylogeny and revised classification of Squamata, including 4161 species of lizards and snakes. *BMC evolutionary biology*, **13**(1), pp. 1-53.

Raaum, R. L., Sterner, K. N., Noviello, C. M., Stewart, C. B. & Disotell, T. R. 2005. Catarrhine primate divergence dates estimated from complete mitochondrial genomes: concordance with fossil and nuclear DNA evidence. *Journal of Human Evolution*, **48**(3), 237-257.

Rambaut, A. & Drummond, A.J. 2013. Tracer v1.5. Available from http://beast. bio. ed. ac. uk/Tracer.

Reece, J.B., Urry, L.A., Cain, M.L., Wasserman, S.A., Minorsky, P.V. & Jackson, R.B. 2011. *Campbell biology*. Boston: Pearson.

Rutschmann, F. 2006. Molecular dating of phylogenetic trees: a brief review of current methods that estimate divergence times. *Diversity and Distributions*, **12**(1), 35-48.

Sanders, K.L., Lee, M.S.Y., Leys, R., Foster, R. & Scott Keogh, J. 2008. Molecular phylogeny and divergence dates for Australasian elapids and sea snakes (Hydrophiinae):

evidence from seven genes for rapid evolutionary radiations. *Journal of evolutionary biology*, **21**(3), pp. 682-695.

Scanlon, J.D., Lee, M.S. & Archer, M. 2003. Mid-Tertiary elapid snakes (Squamata, Colubroidea) from Riversleigh, northern Australia: early steps in a continent-wide adaptive radiation. *Geobios*, **36**(5), pp. 573-601.

Schlegel, H. 1837. Abbildungen neuer oder unvollständig bekannter Amphibien, nach der Natur oder dem Leben entworfen, herausgegeben und mit einem erläuternden Texte begleit. *Dusseldorf: Arnz und Comp*.

Slowinski, J.B. & Keogh, J.S. 2000. Phylogenetic relationships of elapid snakes based on cytochrome b mtDNA sequences. *Molecular phylogenetics and evolution*, **15**(1), pp. 157-164.

Slowinski, J.B. & Wüster, W. 2000. A new cobra (Elapidae: *Naja*) from Myanmar (Burma). *Herpetologica*, **56**(2), pp. 257-270.

Smith, A. 1826. On the snakes of Southern Africa. *Edinburgh new philosophical journal*, **1**, pp. 248-254.

Smith, H.M. & Chiszar, D. 2006. Dilemma of name-recognition: why and when to use new combinations of scientific names. *Herpetological Conservation and Biology*, **1**(1), pp. 6-8.

Spawls, S. & Branch, W.R. 1995. *The dangerous snakes of Africa: natural history, species directory, venoms, and snakebite*. Ralph Curtis Pub.

Steiper, M. E. & Young, N. M. 2006. Primate molecular divergence dates. *Molecular Phylogenetics and Evolution*, **41**(2), 384-394.

Stephens, M., Smith, N.J. & Donnelly, P. 2001. A new statistical method for haplotype reconstruction from population data. *The American Journal of Human Genetics*, **68**(4), pp. 978-989.

Swofford, D.L. 1998. *Paup 4.0 beta version for windows: phylogenetic analysis using parsimony*. Sinauer Associates.

Szyndlar, Z. & Rage, J.C. 1999. Oldest fossil vipers (Serpentes: Viperidae) from the old World. *Kaupia*, **8**, pp. 9-20.

Tamura K., Nei M., & Kumar S. 2004. Prospects for inferring very large phylogenies by using the neighbour-joining method. *Proceedings of the National Academy of Sciences* (USA), **101**(30), pp. 11030-11035.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular biology and evolution,* **28**(10), pp. 2731–9.

Townsend, T.M., Larson, A., Louis, E. & Macey, J.R. 2004. Molecular phylogenetics of Squamata: the position of snakes, amphisbaenians, and dibamids, and the root of the squamate tree. *Systematic biology*, **53**(5), pp. 735-757.

Trape, J.F., Chirio, L., Broadley, D.G. & Wüster, W. 2009. Phylogeography and systematic revision of the Egyptian cobra (Serpentes: Elapidae: *Naja haje*) species complex, with the description of a new species from West Africa. *Zootaxa*, **2236**, pp. 1-25.

Triep, M., Hess, D., Chaves, H., Brücker, C., Balmert, A., Westhoff, G. & Bleckmann, H. 2013. 3D flow in the venom channel of a spitting cobra: do the ridges in the fangs act as fluid guide vanes? *PloS one*, **8**(5), p.e61548.

Tweedie, M.W.F. 1954. Notes on Malayan reptiles, No.3. *Bulletin of the Raffles Museum*, **25**, pp. 107-117.

Uğurtaş, I.H., Papenfuss, T.J. & Orlov, N.L. 2013. New record of *Walterinnesia aegyptia* Lataste, 1887 (Ophidia: Elapidae: Bungarinae) in Turkey. *Russian Journal of Herpetology*, **8**(3), pp. 239-245.

Utkin, Y., Sunagar, K., Jackson, T., Reeks, T. And Fry, B. 2015. Three-finger Toxins (3FTxs). In: Fry, B. G. ed. *Venomous Reptiles and Their Toxins: Evolution, Pathophysiology and Biodiscovery*. Oxford University Press, pp. 222.

Valdecasas, A.G., Williams, D. & Wheeler, Q.D. 2008. 'Integrative taxonomy' then and now: a response to Dayrat (2005). *Biological Journal of the Linnean Society*, **93**(1), pp. 211-216.

Vogel, C.W., Bredehorst, R., Fritzinger, D.C., Grunwald, T., Ziegelmüller, P. and Kock, M.A. 1996. Structure and function of cobra venom factor, the complement-activating protein in cobra venom. In: *Natural Toxins 2.* Springer US. pp. 97-114.

Wagler, J.G. 1830. *Natürliches System der Amphibien: mit vorangehender Classification der Säugethiere und Vögel: ein Beitrag zur vergleichenden Zoologie*. JG Cotta, München, pp. 354.

Wallach, V., Wüster, W. & Broadley, D.G. 2009. In praise of subgenera: taxonomic status of cobras of the genus *Naja* Laurenti (Serpentes: Elapidae). *Zootaxa*, **2236**, pp. 26-36.

Werle, E., Schneider, C., Renner, M., Völker, M. & Fiehn, W. 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. *Nucleic Acids Research*, **22**(20), pp. 4354.

Wheeler, Q. D., Platnick, N. I. 2000. The phylogenetic species concept (sensu Wheeler & Platnick). In: Wheeler, Q. D.; Meier, R., editors. *Species concepts and phylogenetic theory: a debate*. New York: Columbia University Press. pp. 55–69.

Wiens, J.J., Hutter, C.R., Mulcahy, D.G., Noonan, B.P., Townsend, T.M., Sites, J.W. & Reeder, T.W. 2012. Resolving the phylogeny of lizards and snakes (Squamata) with extensive sampling of genes and species. *Biology letters*, **8**(6), pp. 1043-1046.

Wüster, W. 1990. *Population Evolution of the Asiatic Cobra (Naja naja) Species Complex*. Doctoral Thesis. University of Aberdeen, UK.

Wüster, W. & Broadley, D.G. 2003. A new species of spitting cobra (*Naja*) from north-eastern Africa (Serpentes: Elapidae). *Journal of Zoology*, **259**(04), pp. 345-359.

Wüster, W. & Thorpe, R.S. 1989. Population affinities of the asiatic cobra (*Naja naja*) species complex in south-east Asia: reliability and random resampling. *Biological Journal of the Linnean Society*, **36**(4), pp. 391-409.

Wüster, W. & Thorpe, R.S. 1990. Systematics and biogeography of the Asiatic cobra (Naja naja) species complex in the Philippine Islands. *Peters, G. and Hutterer, R. Vertebrates in the Tropics.* Museum Alexander Koenig, Bonn, pp. 333-344.

Wüster, W. & Thorpe, R.S. 1991. Asiatic cobras: systematics and snakebite. *Experientia*, **47**(2), pp. 205-209.

Wüster, W. & Thorpe, R.S. 1992a. Asiatic cobras: population systematics of the *Naja naja* species complex (Serpentes: Elapidae) in India and Central Asia. *Herpetologica*, **48**(1), pp.69-85.

Wüster, W. & Thorpe, R.S. 1992b. Dentitional phenomena in cobras revisited: spitting and fang structure in the Asiatic species of Naja (Serpentes: Elapidae). *Herpetologica*, pp.424-434.

Wüster, W. & Thorpe, R.S. 1994. *Naja siamensis*, a cryptic species of venomous snake revealed by mtDNA sequencing. *Experientia*, **50**(1), pp. 75-79.

Wüster, W. 1996. Taxonomic changes and toxinology: systematic revisions of the Asiatic cobras (*Naja naja* species complex). *Toxicon*, **34**(4), pp. 399-406.

Wüster, W., Crookes, S., Ineich, I., Mané, Y., Pook, C.E., Trape, J.F. & Broadley, D.G. 2007. The phylogeny of cobras inferred from mitochondrial DNA sequences: evolution of venom spitting and the phylogeography of the African spitting cobras (Serpentes: Elapidae: *Naja nigricollis* complex). *Molecular phylogenetics and evolution*, **45**(2), pp. 437-453.

Wüster, W., Peppin, L., Pook, C. E. & Walker, D. E. 2008. A nesting of vipers: phylogeny and historical biogeography of the Viperidae (Squamata: Serpentes). *Molecular Phylogenetics and Evolution*, **49**(2), 445-459.

Young, B.A. & Kardong, K.V. 2010. The functional morphology of hooding in cobras. *Journal of Experimental Biology*, **213**(9), pp. 1521-1528.

Zhang, Z., Schwartz, S., Wagner, L. & Miller, W. 2000. A greedy algorithm for aligning DNA sequences. *Journal of Computational biolo*gy, **7**(1-2), pp. 203-214.

Zharkikh, A. 1994. Estimation of evolutionary distances between nucleotide sequences. Journal of molecular evolution, **39**(3), pp.315-329.

Appendix

01 Appendix. Sample list

Details of all the samples included in this study are listed below. Most sample numbers refer to Dr Wolfgang Wüster's sample archive, with the others being the accession numbers for GenBank. Genus, subgenus, species and country are also listed, where possible. Sequences labelled 1* were obtained throughout this study by the author.

Sample no.	Genus	Subgenus	Species	Country	CMOS	cytb	ND4	NT3	PRLR	RAG1	UBN
3360	*Acanthophis*		*praelongus*	Australia		1	1	1	1		1
3363	*Acanthophis*		*praelongus*	Australia		1		1	1		1
EU546887	*Acanthophis*		*praelongus*							1	
EU546926	*Acanthophis*		*praelongus*		1						
1469	*Aspidelaps*		*lubricus*	South Africa				1	1		
2599	*Aspidelaps*		*lubricus*	Namibia		1	1		1	1*	1
2600	*Aspidelaps*		*lubricus*	Namibia		1	1	1	1		1
2601	*Aspidelaps*		*lubricus*	Namibia				1	1		1
4346	*Aspidelaps*		*lubricus*	South Africa					1		1
4348	*Aspidelaps*		*lubricus*	South Africa		1	1	1	1		
4364	*Aspidelaps*		*lubricus*	South Africa				1	1		1
4365	*Aspidelaps*		*lubricus*	South Africa		1	1	1			1
4366	*Aspidelaps*		*lubricus*	South Africa		1	1				
4367	*Aspidelaps*		*lubricus*	South Africa	1*	1	1	1		1*	1
4368	*Aspidelaps*		*lubricus*	South Africa		1	1				
4370	*Aspidelaps*		*lubricus*	South Africa		1	1				
288	*Aspidelaps*		*scutatus*	Namibia			1	1	1		1
1324	*Aspidelaps*		*scutatus*	South Africa			1				
2269	*Aspidelaps*		*scutatus*	Mozambique			1	1	1	1*	1
2291	*Aspidelaps*		*scutatus*	Mozambique	1*			1	1		1
2309	*Aspidelaps*		*scutatus*	South Africa	1*						
4339	*Aspidelaps*		*scutatus*	South Africa			1				
4341	*Aspidelaps*		*scutatus*	South Africa			1				
4357	*Aspidelaps*		*scutatus*	South Africa			1				
4358	*Aspidelaps*		*scutatus*	South Africa			1				
4363	*Aspidelaps*		*scutatus*	South Africa			1				
AY058969	*Aspidelaps*		*scutatus*				1				
AY188007	*Aspidelaps*		*scutatus*			1					
U96790	*Aspidelaps*		*scutatus*			1					
14-33	*Bungarus*		*caeruleus*		1*					1*	
14-33	*Bungarus*		*caeruleus*			1					
14-33	*Bungarus*		*caeruleus*					1	1		
14-34	*Bungarus*		*caeruleus*		1*						
14-34	*Bungarus*		*caeruleus*				1	1	1		1
14-36	*Bungarus*		*fasciatus*			1		1	1		
14-36	*Bungarus*		*fasciatus*								1

14-37	*Bungarus*		*fasciatus*		1*		1	1	1		
14-37	*Bungarus*		*fasciatus*							1*	
192	*Dendroaspis*		*angusticeps*	Mozambique		1	1	1		1*	
1322	*Dendroaspis*		*angusticeps*	South Africa		1	1	1			1
1327	*Dendroaspis*		*angusticeps*	South Africa		1		1	1		1
1335	*Dendroaspis*		*angusticeps*	South Africa			1				
1408	*Dendroaspis*		*angusticeps*	Tanzania			1		1		
1413	*Dendroaspis*		*angusticeps*	Tanzania		1	1	1			1
1414	*Dendroaspis*		*angusticeps*	Tanzania		1			1		
1423	*Dendroaspis*		*angusticeps*	Kenya		1	1	1			
1424	*Dendroaspis*		*angusticeps*	Kenya			1	1	1		1
1425	*Dendroaspis*		*angusticeps*	Kenya	1*		1	1			
2423	*Dendroaspis*		*angusticeps*						1		1
2455	*Dendroaspis*		*angusticeps*								1
3158	*Dendroaspis*		*angusticeps*						1		1
4059	*Dendroaspis*		*angusticeps*	Zimbabwe			1	1			1
4060	*Dendroaspis*		*angusticeps*	Zimbabwe			1	1			1
4061	*Dendroaspis*		*angusticeps*	Zimbabwe			1	1			1
4062	*Dendroaspis*		*angusticeps*	Zimbabwe			1	1			1
2263	*Hemachatus*		*haemachatus*	Swaziland		1	1	1	1		1
2264	*Hemachatus*		*haemachatus*	Swaziland	1*	1	1		1	1*	1
2266	*Hemachatus*		*haemachatus*	South Africa		1	1		1		1
2319	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
2320	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		
2337	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
2338	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
2361	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
2412	*Hemachatus*		*haemachatus*	South Africa	1*	1	1	1	1		1
2413	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
2415	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
2431	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
2432	*Hemachatus*		*haemachatus*	South Africa		1	1	1	1		1
1823	*Hemibungarus*		*calligaster*	Philippines	1		1	1	1	1*	1
EF137411	*Hemibungarus*		*calligaster*			1					
1704	*Micropechis*		*ikaheka*	Papua New Guinea		1	1	1	1		1
1775	*Micropechis*		*ikaheka*	Indonesia		1	1	1	1		
EU366435	*Micropechis*		*ikaheka*							1	
EU366449	*Micropechis*		*ikaheka*		1						
FJ587160	*Micropechis*		*ikaheka*		1						
1267	*Naja*	*Afronaja*	*ashei*	Kenya	1*	1	1			1*	
1268	*Naja*	*Afronaja*	*ashei*	Kenya		1	1				
1270	*Naja*	*Afronaja*	*ashei*	Kenya		1	1				
1394	*Naja*	*Afronaja*	*ashei*	Kenya		1	1	1	1		1
1430	*Naja*	*Afronaja*	*ashei*	Kenya	1*	1	1	1	1	1*	1*
1540	*Naja*	*Afronaja*	*katiensis*	Mali	1*	1	1	1	1	1*	1

1541	*Naja*	*Afronaja*	*katiensis*	Mali		1	1				
1543	*Naja*	*Afronaja*	*katiensis*	Mali		1	1				
2022	*Naja*	*Afronaja*	*katiensis*	Senegal	1*	1	1	1	1		1
190	*Naja*	*Afronaja*	*mossambica*	Mozambique		1	1	1	1		1
191	*Naja*	*Afronaja*	*mossambica*	Mozambique		1	1				
590	*Naja*	*Afronaja*	*mossambica*	South Africa		1	1				
882	*Naja*	*Afronaja*	*mossambica*	Zimbabwe		1	1	1	1		1
1289	*Naja*	*Afronaja*	*mossambica*	Zimbabwe	1*	1	1			1*	
1300	*Naja*	*Afronaja*	*mossambica*	South Africa		1	1				
1391	*Naja*	*Afronaja*	*mossambica*	Tanzania		1	1				
1392	*Naja*	*Afronaja*	*mossambica*	Tanzania		1	1	1	1		1
1416	*Naja*	*Afronaja*	*mossambica*	Tanzania		1	1				
2110	*Naja*	*Afronaja*	*mossambica*	South Africa	1*					1*	
877	*Naja*	*Afronaja*	*nigricincta*	Namibia	1*	1	1			1*	
879	*Naja*	*Afronaja*	*nigricincta*	Namibia				1			1
2631	*Naja*	*Afronaja*	*nigricincta*	Namibia		1	1	1	1		1
296	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1		1		1
297	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1				
842	*Naja*	*Afronaja*	*nigricollis*	Ghana		1	1				
1062	*Naja*	*Afronaja*	*nigricollis*	Togo		1	1				
1074	*Naja*	*Afronaja*	*nigricollis*	Cameroon		1	1	1	1		1
1075	*Naja*	*Afronaja*	*nigricollis*	Cameroon		1	1	1	1		1
1076	*Naja*	*Afronaja*	*nigricollis*	Cameroon		1	1		1		
1271	*Naja*	*Afronaja*	*nigricollis*	Kenya		1	1	1	1		1
1272	*Naja*	*Afronaja*	*nigricollis*	Kenya		1	1	1	1		1
1288	*Naja*	*Afronaja*	*nigricollis*	Zambia		1	1				
1393	*Naja*	*Afronaja*	*nigricollis*	Zambia		1	1		1		1
1403	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1				
1404	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1	1	1		1
1405	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1	1	1		1
1406	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1				
1407	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1				
1415	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1				
1538	*Naja*	*Afronaja*	*nigricollis*	Guinea		1	1				
1614	*Naja*	*Afronaja*	*nigricollis*	Tanzania		1	1				
3103	*Naja*	*Afronaja*	*nigricollis*	Kenya		1	1	1	1		
3160	*Naja*	*Afronaja*	*nigricollis*	Angola		1	1	1	1		1
3162	*Naja*	*Afronaja*	*nigricollis*	Angola		1	1		1		1
3760	*Naja*	*Afronaja*	*nigricollis*	Guinee		1	1		1		1
3777	*Naja*	*Afronaja*	*nigricollis*	Congo	1*				1	1*	
4010	*Naja*	*Afronaja*	*nigricollis*	Kenya		1	1	1	1		
4559	*Naja*	*Afronaja*	*nigricollis*	Tanzania				1	1		1
4560	*Naja*	*Afronaja*	*nigricollis*	Tanzania							1
4566	*Naja*	*Afronaja*	*nigricollis*	Nigeria		1	1	1	1		
EBG2006	*Naja*	*Afronaja*	*nigricollis*						1		
836	*Naja*	*Afronaja*	*nubiae*		1*	1	1	1	1	1*	1

837	*Naja*	*Afronaja*	*nubiae*			1	1	1	1		1
4565	*Naja*	*Afronaja*	*nubiae*		1*	1	1		1		1
834	*Naja*	*Afronaja*	*pallida*			1	1				
835	*Naja*	*Afronaja*	*pallida*			1	1				
1080	*Naja*	*Afronaja*	*pallida*	Tanzania		1	1	1	1		1
1081	*Naja*	*Afronaja*	*pallida*	Kenya		1	1				
1082	*Naja*	*Afronaja*	*pallida*	Kenya		1	1				
1083	*Naja*	*Afronaja*	*pallida*	Kenya		1	1				
1273	*Naja*	*Afronaja*	*pallida*	Kenya	1*	1	1			1*	
1431	*Naja*	*Afronaja*	*pallida*	Kenya		1	1	1	1		1
2305	*Naja*	*Afronaja*	*pallida*	Tanzania	1*					1*	
1395	*Naja*	*Afronaja*	*woodi*	South Africa	1*	1	1	1	1	1*	1
1396	*Naja*	*Afronaja*	*woodi*	South Africa		1	1				
1563	*Naja*	*Afronaja*	*woodi*	South Africa		1	1				
1564	*Naja*	*Afronaja*	*woodi*	South Africa					1		1
1588	*Naja*	*Afronaja*	*woodi*	South Africa		1	1				
2818	*Naja*	*Afronaja*	*woodi*	South Africa	1*			1*			
1303	*Naja*	*Boulengerina*	*annulata*	Tanzania		1	1		1		
1304	*Naja*	*Boulengerina*	*annulata*	Democratic Republic of Congo		1	1	1	1		1
1305	*Naja*	*Boulengerina*	*annulata*	Democratic Republic of Congo		1	1		1		
2463	*Naja*	*Boulengerina*	*annulata*						1		
2716	*Naja*	*Boulengerina*	*annulata*	Congo					1		
2717	*Naja*	*Boulengerina*	*annulata*	Congo		1	1	1	1		1
2718	*Naja*	*Boulengerina*	*annulata*	Congo	1*			1	1	1*	1
1084	*Naja*	*Boulengerina*	*"West African black form"*	Ghana	1*	1	1	1		1*	1
1658	*Naja*	*Boulengerina*	*"West African black form"*	Ghana		1	1		1		
2456	*Naja*	*Boulengerina*	*"West African black form"*	Togo		1	1		1		1
2491	*Naja*	*Boulengerina*	*"West African black form"*	Togo		1	1	1	1		
2492	*Naja*	*Boulengerina*	*"West African black form"*	Togo		1	1		1		1
2493	*Naja*	*Boulengerina*	*"West African black form"*	Togo		1	1		1		1
3749	*Naja*	*Boulengerina*	*"West African black form"*	Guinee		1	1		1		1
3751	*Naja*	*Boulengerina*	*"West African black form"*	Guinee		1	1		1		
3752	*Naja*	*Boulengerina*	*"West African black form"*	Liberia		1	1		1		1
3753	*Naja*	*Boulengerina*	*"West African black form"*	Guinee		1	1		1		1
3754	*Naja*	*Boulengerina*	*"West African black form"*	Guinee					1		1
3757	*Naja*	*Boulengerina*	*"West African black form"*	Liberia		1	1		1		1

3758	*Naja*	*Boulengerina*	*"West African black form"*	Liberia		1	1		1		1
3759	*Naja*	*Boulengerina*	*"West African black form"*	Guinee		1	1		1		
182	*Naja*	*Boulengerina*	*melanoleuca*	Cameroon		1	1	1	1		1
1877	*Naja*	*Boulengerina*	*melanoleuca*	Cameroon		1	1		1		1
1878	*Naja*	*Boulengerina*	*melanoleuca*	Cameroon	1*	1	1				1
1879	*Naja*	*Boulengerina*	*melanoleuca*	Cameroon		1	1		1		1
2719	*Naja*	*Boulengerina*	*melanoleuca*	Congo		1	1				
2720	*Naja*	*Boulengerina*	*melanoleuca*	Congo	1*	1	1	1	1	1*	
2721	*Naja*	*Boulengerina*	*melanoleuca*	Congo		1	1		1		1
2722	*Naja*	*Boulengerina*	*melanoleuca*	Congo		1	1	1	1		1
2873	*Naja*	*Boulengerina*	*melanoleuca*	Gabon					1		1
2996	*Naja*	*Boulengerina*	*melanoleuca*	Congo		1	1		1		1
3163	*Naja*	*Boulengerina*	*melanoleuca*	Angola		1	1		1		1
3779	*Naja*	*Boulengerina*	*melanoleuca*	Congo				1	1		1
4673	*Naja*	*Boulengerina*	*melanoleuca*	Democratic Republic of Congo				1	1		1
4674	*Naja*	*Boulengerina*	*melanoleuca*	Democratic Republic of Congo					1		1
4675	*Naja*	*Boulengerina*	*melanoleuca*	Democratic Republic of Congo				1	1		1
CRT3797	*Naja*	*Boulengerina*	*melanoleuca*			1	1		1		
CRT3913	*Naja*	*Boulengerina*	*melanoleuca*			1	1		1		
CRT4044	*Naja*	*Boulengerina*	*melanoleuca*			1	1		1		
4313	*Naja*	*Boulengerina*	*multifasciata*	Democratic Republic of Congo	1*	1	1	1	1	1*	1
AF217837	*Naja*	*Boulengerina*	*multifasciata*			1					
AY058985	*Naja*	*Boulengerina*	*multifasciata*				1				
1085	*Naja*	*Boulengerina*	*"western banded"*	Ghana	1*	1	1	1	1	1*	1
1539	*Naja*	*Boulengerina*	*"western banded"*	Guinea		1	1		1		1
2046	*Naja*	*Boulengerina*	*"western banded"*	Benin		1	1				
2047	*Naja*	*Boulengerina*	*"western banded"*	Benin		1	1				
2495	*Naja*	*Boulengerina*	*"western banded"*	Senegal	1*	1	1	1	1	1*	1
3755	*Naja*	*Boulengerina*	*"western banded"*	Guinee		1	1		1		1
189	*Naja*	*Boulengerina*	*subfulva*	Mozambique		1	1	1	1		1
1086	*Naja*	*Boulengerina*	*subfulva*	Cameroon		1	1		1		1
1087	*Naja*	*Boulengerina*	*subfulva*	Cameroon		1	1		1		1
1088	*Naja*	*Boulengerina*	*subfulva*	Cameroon		1	1	1	1		1
1089	*Naja*	*Boulengerina*	*subfulva*	Central African Republic		1	1		1		
1090	*Naja*	*Boulengerina*	*subfulva*	Burundi	1*	1	1	1	1	1*	

1264	*Naja*	*Boulengerina*	*subfulva*	Kenya		1	1	1	1		1
1265	*Naja*	*Boulengerina*	*subfulva*	Kenya		1	1		1		1
1266	*Naja*	*Boulengerina*	*subfulva*	Kenya		1	1	1	1		1
1292	*Naja*	*Boulengerina*	*subfulva*	South Africa		1	1		1		1
1326	*Naja*	*Boulengerina*	*subfulva*	South Africa		1	1	1	1		1
1587	*Naja*	*Boulengerina*	*subfulva*	Central African Republic		1	1	1	1		1
1649	*Naja*	*Boulengerina*	*subfulva*	Cameroon		1	1		1		
1912	*Naja*	*Boulengerina*	*subfulva*	Central African Republic		1	1		1		1
2654	*Naja*	*Boulengerina*	*subfulva*	South Africa		1	1		1		1
2723	*Naja*	*Boulengerina*	*subfulva*	Congo		1	1		1		
2876	*Naja*	*Boulengerina*	*subfulva*	Uganda		1	1	1			1
2997	*Naja*	*Boulengerina*	*subfulva*	Congo		1	1		1		1
3776	*Naja*	*Boulengerina*	*subfulva*	Congo				1	1		1
3778	*Naja*	*Boulengerina*	*subfulva*	Congo					1		1
3780	*Naja*	*Boulengerina*	*subfulva*	Congo				1	1		1
3781	*Naja*	*Boulengerina*	*subfulva*	Congo					1		
3782	*Naja*	*Boulengerina*	*subfulva*	Congo					1		1
4006	*Naja*	*Boulengerina*	*subfulva*	Kenya		1	1	1	1		1
4007	*Naja*	*Boulengerina*	*subfulva*	Kenya		1	1	1	1		1
4008	*Naja*	*Boulengerina*	*subfulva*	Kenya		1	1	1	1		1
4009	*Naja*	*Boulengerina*	*subfulva*	Kenya		1	1	1	1		1
CRT3585	*Naja*	*Boulengerina*	*subfulva*			1	1		1		1
CRT3690	*Naja*	*Boulengerina*	*subfulva*			1	1		1		1
CRT4004	*Naja*	*Boulengerina*	*subfulva*			1	1		1		1
CRT4056	*Naja*	*Boulengerina*	*subfulva*			1	1		1		1
1196	*Naja*	*Boulengerina*	*peroescobari*	São Tomé	1*						
1197	*Naja*	*Boulengerina*	*peroescobari*	São Tomé	1*	1	1	1	1	1*	1
582	*Naja*	*Naja*	*atra*	China		1	1	1	1	1*	1
793	*Naja*	*Naja*	*atra*	Taiwan	1*	1	1	1	1	1*	1
585	*Naja*	*Naja*	*kaouthia*	Thailand		1	1	1	1		1
812	*Naja*	*Naja*	*kaouthia*	Vietnam	1*	1	1			1*	
813	*Naja*	*Naja*	*kaouthia*	Vietnam		1	1				
818	*Naja*	*Naja*	*kaouthia*	Thailand		1	1				
839	*Naja*	*Naja*	*kaouthia*	Myanmar		1	1	1	1		1
3375	*Naja*	*Naja*	*kaouthia*	India					1		
592	*Naja*	*Naja*	*mandalayensis*	Burma	1*	1	1	1	1	1*	1
593	*Naja*	*Naja*	*mandalayensis*	Burma		1	1	1	1		1
186	*Naja*	*Naja*	*miolepis*	Malaysia		1	1	1	1		1
188	*Naja*	*Naja*	*miolepis*	Malaysia		1	1	1	1	1*	1
1827	*Naja*	*Naja*	*miolepis*	Philippines	1*	1	1	1*		1*	
579	*Naja*	*Naja*	*naja*	Nepal		1	1				
580	*Naja*	*Naja*	*naja*	Sri Lanka		1	1	1	1		1
581	*Naja*	*Naja*	*naja*	Sri Lanka		1	1				

595	*Naja*	*Naja*	*naja*	Nepal		1	1	1	1		1
831	*Naja*	*Naja*	*naja*	Sri Lanka	1*					1*	
832	*Naja*	*Naja*	*oxiana*			1	1		1		
838	*Naja*	*Naja*	*oxiana*			1	1	1	1		1
NO2	*Naja*	*Naja*	*oxiana*		1*					1*	
1800	*Naja*	*Naja*	*philippinensis*	Philippines	1*	1	1				
1801	*Naja*	*Naja*	*philippinensis*	Philippines		1	1	1	1		1
1822	*Naja*	*Naja*	*philippinensis*	Philippines	1*					1*	
1824	*Naja*	*Naja*	*philippinensis*	Philippines		1	1	1	1		1
1825	*Naja*	*Naja*	*philippinensis*	Philippines		1	1				
1828	*Naja*	*Naja*	*philippinensis*	Philippines		1	1				
400	*Naja*	*Naja*	*sagittifera*	Brazil	1*	1	1				
1815	*Naja*	*Naja*	*sagittifera*	India	1*	1	1	1	1	1*	1
841	*Naja*	*Naja*	*samarensis*	Philippines		1	1	1	1	1*	1
1803	*Naja*	*Naja*	*samarensis*	Philippines		1	1				
1806	*Naja*	*Naja*	*samarensis*	Philippines		1	1	1	1		1
1807	*Naja*	*Naja*	*samarensis*	Philippines	1*						
26	*Naja*	*Naja*	*siamensis*	Brazil		1	1				
40	*Naja*	*Naja*	*siamensis*	Brazil		1	1				
810	*Naja*	*Naja*	*siamensis*	Vietnam		1	1	1	1		1
811	*Naja*	*Naja*	*siamensis*	Vietnam	1*	1	1	1	1	1*	1
4562	*Naja*	*Naja*	*siamensis*	unknown	1*			1			1
294	*Naja*	*Naja*	*sumatrana*	Sumatra	1*	1	1	1	1	1*	1
295	*Naja*	*Naja*	*sumatrana*	Sumatra	1*	1	1			1*	
586	*Naja*	*Naja*	*sumatrana*	Malaysia		1	1	1	1		1
587	*Naja*	*Naja*	*sumatrana*	Malaysia	1*	1	1			1*	
589	*Naja*	*Naja*	*sumatrana*	Indonesia		1	1				
289	*Naja*	*Uraeus*	*anchietae*	Namibia		1	1				
591	*Naja*	*Uraeus*	*anchietae*	Namibia		1	1	1	1		1
1892	*Naja*	*Uraeus*	*anchietae*	Botswana	1*	1	1	1	1	1*	1
1893	*Naja*	*Uraeus*	*anchietae*	Botswana		1	1				
193	*Naja*	*Uraeus*	*annulifera*	South Africa		1	1	1	1		1
881	*Naja*	*Uraeus*	*annulifera*	Zimbabwe		1	1	1	1		1
2109	*Naja*	*Uraeus*	*annulifera*	South Africa	1*					1*	
4564	*Naja*	*Uraeus*	*annulifera*	unknown					1		1
1677	*Naja*	*Uraeus*	*arabica*	Saudi Arabia		1	1				
1678	*Naja*	*Uraeus*	*arabica*	Saudi Arabia		1	1				
1679	*Naja*	*Uraeus*	*arabica*	Saudi Arabia		1	1				
1681	*Naja*	*Uraeus*	*arabica*	Saudi Arabia	1*	1	1	1*	1	1*	1
1682	*Naja*	*Uraeus*	*arabica*	Saudi Arabia		1	1				
2035	*Naja*	*Uraeus*	*arabica*	Yemen	1*	1	1	1	1	1*	1
893	*Naja*	*Uraeus*	*haje*	Egypt		1	1		1		1
1057	*Naja*	*Uraeus*	*haje*	Morocco		1	1	1			
1077	*Naja*	*Uraeus*	*haje*	Egypt	1*	1	1	1	1	1*	1
1078	*Naja*	*Uraeus*	*haje*	Egypt		1	1				
1262	*Naja*	*Uraeus*	*haje*	Kenya		1	1		1		1

1263	*Naja*	*Uraeus*	*haje*	Kenya		1	1	1	1		1
1651	*Naja*	*Uraeus*	*haje*	Niger		1	1				
1652	*Naja*	*Uraeus*	*haje*	Niger		1	1				
1653	*Naja*	*Uraeus*	*haje*	Niger		1	1				
1659	*Naja*	*Uraeus*	*haje*	Nigeria	1*	1	1			1*	
1660	*Naja*	*Uraeus*	*haje*	Nigeria		1	1				
1661	*Naja*	*Uraeus*	*haje*	Nigeria		1	1				
4554	*Naja*	*Uraeus*	*haje*	Uganda				1	1		1
4563	*Naja*	*Uraeus*	*haje*	Morocco					1		1
1295	*Naja*	*Uraeus*	*nivea*	South Africa		1	1	1	1	1*	1
1482	*Naja*	*Uraeus*	*nivea*	South Africa	1*	1	1	1	1	1*	1
AY058983	*Naja*	*Uraeus*	*nivea*				1				
FR693729	*Naja*	*Uraeus*	*nivea*			1					
1079	*Naja*	*Uraeus*	*senegalensis*	Mali		1	1	1	1	1*	1
1542	*Naja*	*Uraeus*	*senegalensis*	Mali		1	1				
2018	*Naja*	*Uraeus*	*senegalensis*	Mali		1	1				
2039	*Naja*	*Uraeus*	*senegalensis*	Benin		1	1				
2203	*Naja*	*Uraeus*	*senegalensis*	Senegal	1*	1	1	1	1		1
187	*Ophiophagus*		*hannah*	Malaysia		1	1				
500	*Ophiophagus*		*hannah*	India			1				
501	*Ophiophagus*		*hannah*	India			1				
502	*Ophiophagus*		*hannah*	India			1				
574	*Ophiophagus*		*hannah*	Indonesia			1				
577	*Ophiophagus*		*hannah*	Indonesia			1				
1060	*Ophiophagus*		*hannah*	Indonesia		1	1				
1802	*Ophiophagus*		*hannah*	Philippines	1*	1	1	1*		1*	1*
1816	*Ophiophagus*		*hannah*	India			1	1			
1820	*Ophiophagus*		*hannah*	Philippines	1*	1	1	1	1	1*	1
2922	*Ophiophagus*		*hannah*	Indonesia			1	1	1		1
2923	*Ophiophagus*		*hannah*	Malaysia			1		1		1
2924	*Ophiophagus*		*hannah*	Malaysia			1		1		1
2925	*Ophiophagus*		*hannah*	Indonesia			1		1		1
U96803	*Ophiophagus*		*hannah*			1					
274	*Oxyuranus*		*scutellatus*	Indonesia		1	1	1*	1	1*	1
1199	*Oxyuranus*		*scutellatus*	Australia		1	1				
1256	*Oxyuranus*		*scutellatus*	Papua New Guinea		1					
1274	*Oxyuranus*		*scutellatus*	Australia			1	1	1		1
EU546916	*Oxyuranus*		*scutellatus*		1						
3207	*Pseudechis*		*australis*	Australia		1	1	1	1		1
4051	*Pseudechis*		*australis*	Australia		1	1	1	1		1
EU546873	*Pseudechis*		*australis*							1	
EU546912	*Pseudechis*		*australis*		1						
1336	*Pseudohaje*		*goldii*	Uganda	1*	1	1	1	1	1*	1
2967	*Pseudohaje*		*goldii*	Angola		1	1				
4337	*Walterinnesia*		*aegyptia*		1*	1	1	1	1	1*	1

4338	*Walterinnesia*	*aegyptia*	1*	1	1	1	1	1*	1
AY058988	*Walterinnesia*	*aegyptia*			1				
U96807	*Walterinnesia*	*aegyptia*		1					

02 Appendix. PCR Mastermix composition

PCR Mastermix composition

Reagants	Initial Concentration	Final Concentration	1 sample (µl)
H2O			5.6
DreamTaq PCR Buffer	2 X	1 X	7.5
F-Primer	10 µM	0.27 µM	0.45
R-Primer	10 µM	0.27 µM	0.45
DNA	20 ng/µl	20 ng/µl	1
Total Volume			**15**

03 Appendix. Cycling conditions

Cycling conditions for the 8 different primer sets used.

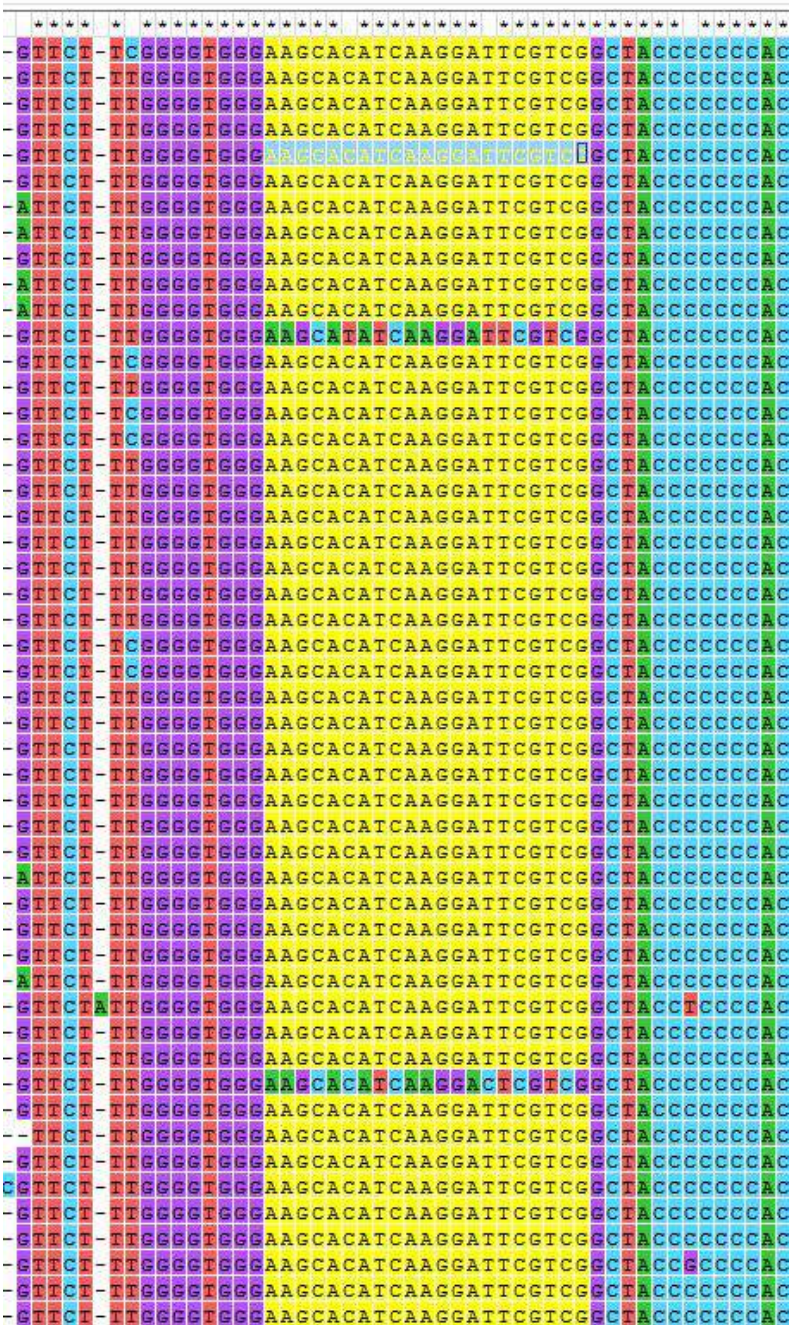
	ND4	**NT3**	**PRLR**	**UBN**	**CMOS**	**CMOS**	**RAG1**
Forward	NADH4	NTF3_F1	PRLR F1	BaUBN_F	G303	AV_CMOSF	AV_RAG1F
Reverse	H12763V	NTF3_R1	PRLR R3	BaUBN_R	G708	AV_CMOSR	AV_RAG1R
1 - Initial denature	94°C 2:00	94°C 2:00	94°C 2:00	94°C 2:00	94°C 2:00	94°C 2:00	94°C 2:00
2 - Denature	94°C 0:30	94°C 0:30	94°C 0:30	94°C 0:30	94°C 0:45	94°C 0:30	94°C 0:30
3 - Anneal	57°C 0:30	42°C 0:30	48°C 0:30	40°C 0:30	58°C 0:45	60.5°C 0:30	59°C 0:30
4 - Extension	72°C 1:00	72°C 0:45	72°C 0:45	72°C 0:45	72°C 1:00	72°C 1:00	72°C 1:00
Repeat steps 2-4:	**x39**	**x39**	**x39**	**x39**	**x35**	**x44**	**x44**
5 - Final Extension	72°C 5:00	72°C 5:00	72°C 5:00	72°C 5:00	72°C 6:00	72°C 5:00	72°C 5:00
6 - Cooling	4°C 15:00	4°C 15:00	4°C 15:00	4°C 15:00	4°C 15:00	4°C 15:00	4°C 15:00

03 Appendix continued

	RAG1
Fwd	G396 (R13)
Rev	G397 (R18)
1 - Initial denature	94°C 2:00
2 - Denature	95°C 0:20
3 - Anneal	61°C 0:20
4 - Extension	72°C 1:00
Repeat steps 2-4:	**x5**
5 - Denature	95°C 0:20
6 - Anneal	59°C 0:20
7 - Extension	72°C 1:00
Repeat steps 5-7:	**x5**
8 - Denature	95°C 0:20
9 - Anneal	57°C 0:20
10 - Extension	72°C 1:00
Repeat steps 8-10:	**x5**
11 - Denature	95°C 0:20
12 - Anneal	55°C 0:20
13 - Extension	72°C 1:00
Repeat steps 11-13:	**x25**
14 - Final Extension	72°C 5:00
15 - Cooling	4°C 15:00

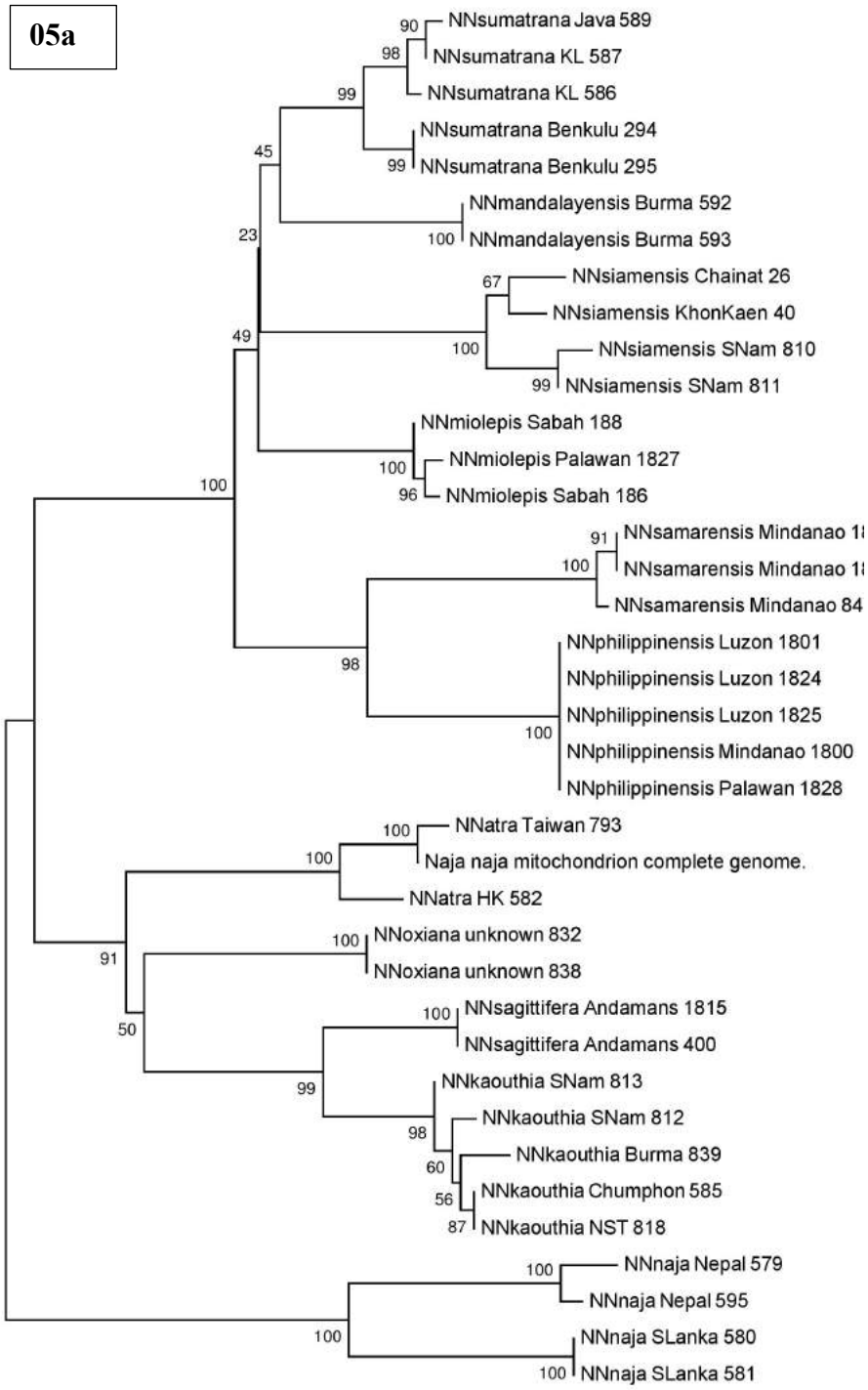
04 Appendix. Primer identification

Screenshot of MEGA 5.05 (Tamura et al., 2004; 2011). Due to the primers for CMOS and RAG1 not binding to, or amplifying many samples, those which were successfully sequenced were analysed and new possible binding regions were identified. Below is an example of where the CMOS forward primer was identified. Same methods were used for RAG1.

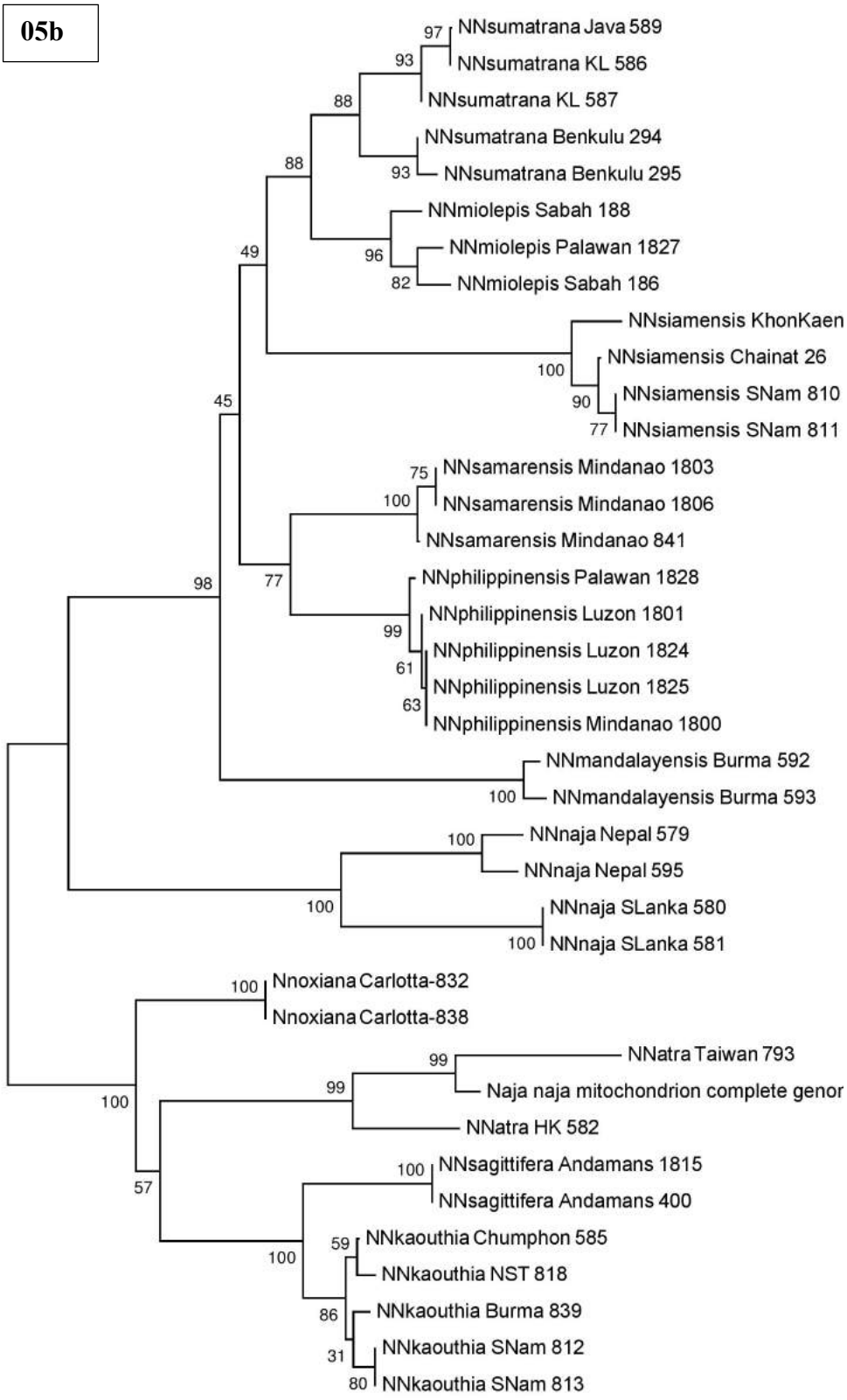


05 Appendix. *Naja naja* discrepancy

05a and 05b show simple bootstrap neighbour joining trees for the cyt-b and ND4 gene, respectively. Used for this analysis were the sequences from this study, compared alongside the "*Naja naja"* mitochondrial sequences from GenBank (EU921898 & EU913475) in MEGA 5.05 (Tamura et al., 2004; 2011). Both incorrectly labelled sequences group alongside *Naja atra* sequences from this study.

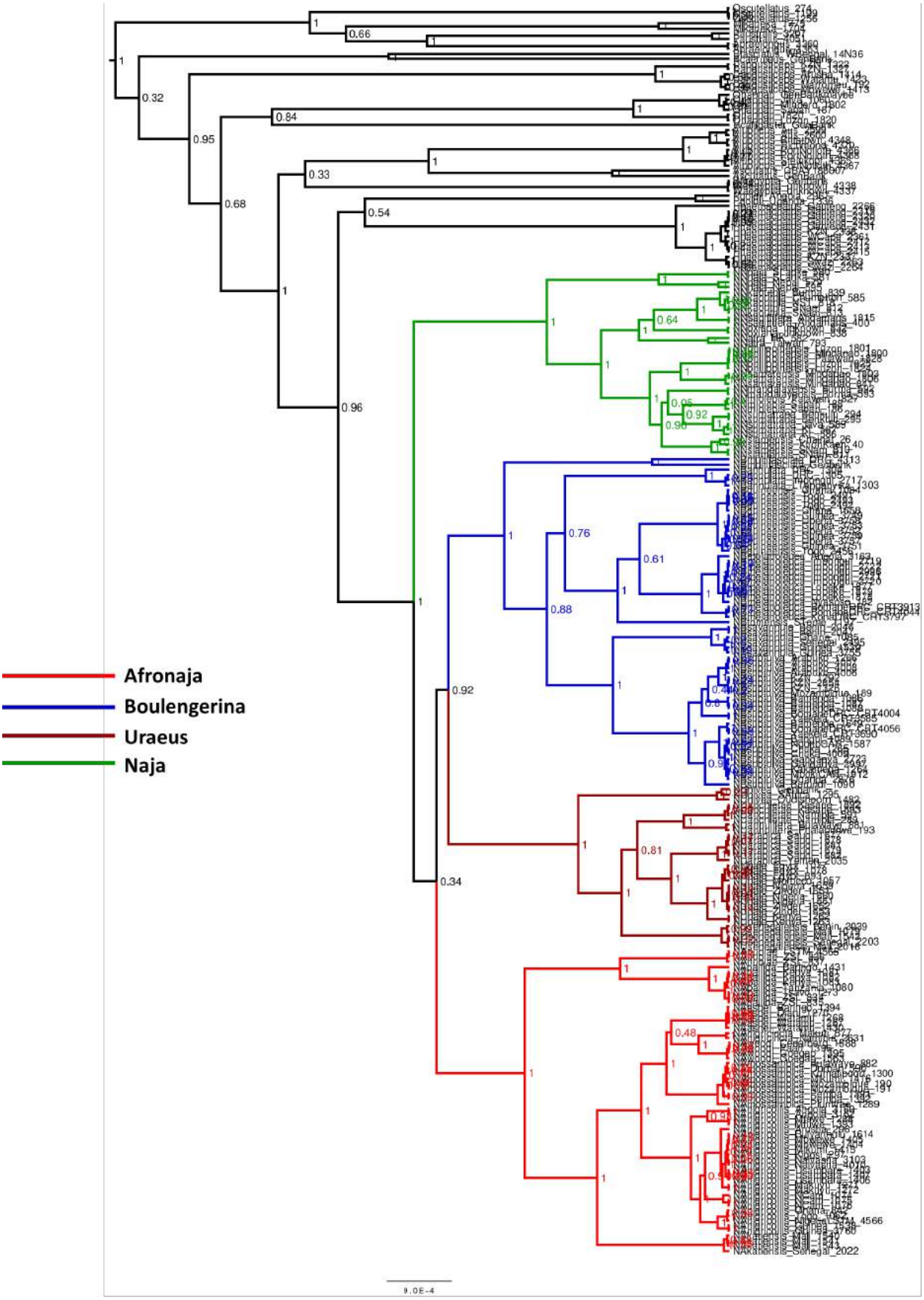


05b

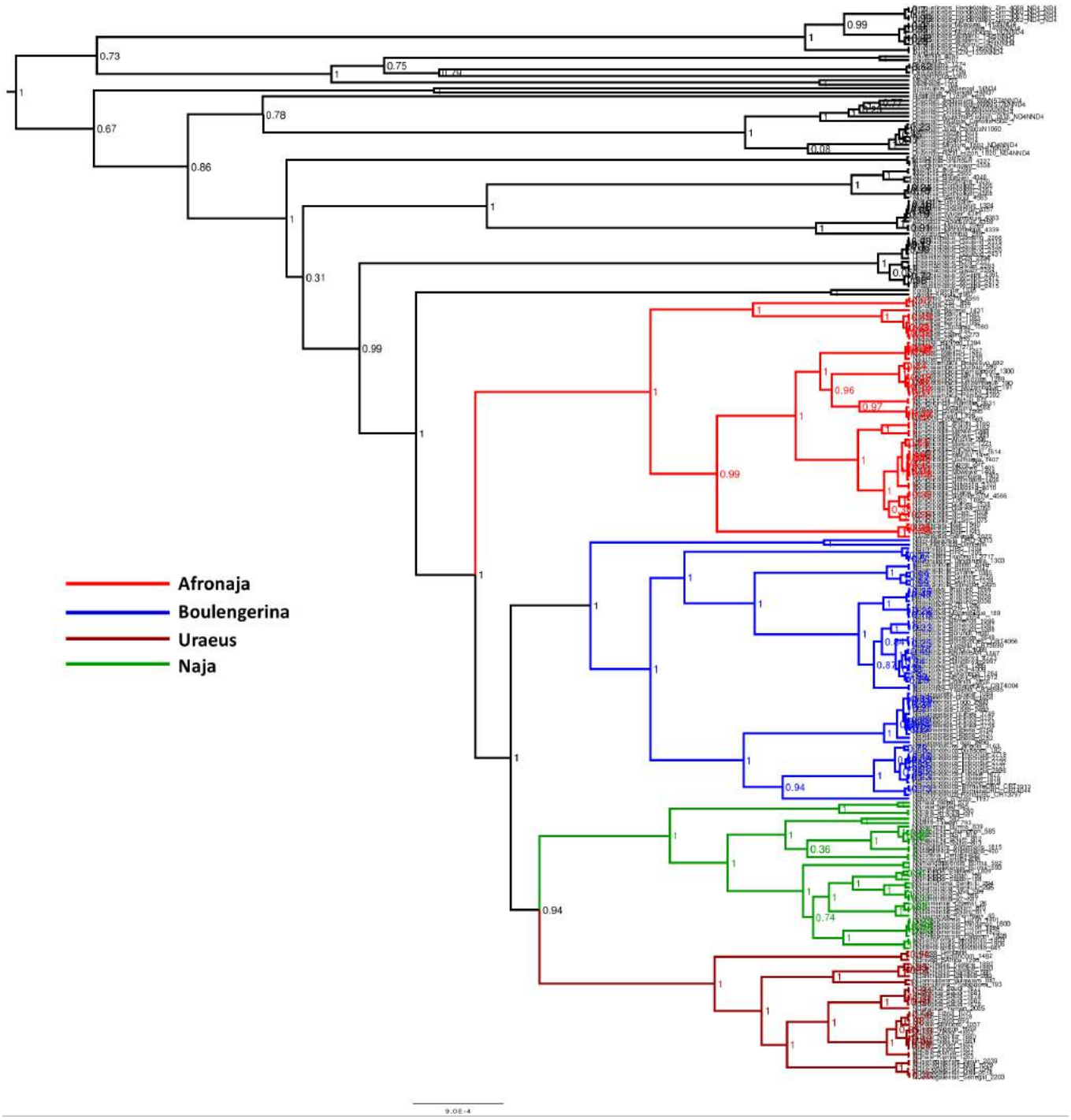

NNsumatrana Java 589
NNsumatrana KL 586
NNsumatrana KL 587
NNsumatrana Benkulu 294
NNsumatrana Benkulu 295
NNmiolepis Sabah 188
NNmiolepis Palawan 1827
NNmiolepis Sabah 186
NNsiamensis KhonKaen
NNsiamensis Chainat 26
NNsiamensis SNam 810
NNsiamensis SNam 811
NNsamarensis Mindanao 1803
NNsamarensis Mindanao 1806
NNsamarensis Mindanao 841
NNphilippinensis Palawan 1828
NNphilippinensis Luzon 1801
NNphilippinensis Luzon 1824
NNphilippinensis Luzon 1825
NNphilippinensis Mindanao 1800
NNmandalayensis Burma 592
NNmandalayensis Burma 593
NNnaja Nepal 579
NNnaja Nepal 595
NNnaja SLanka 580
NNnaja SLanka 581
Nnoxiana Carlotta-832
Nnoxiana Carlotta-838
NNatra Taiwan 793
Naja naja mitochondrion complete genor
NNatra HK 582
NNsagittifera Andamans 1815
NNsagittifera Andamans 400
NNkaouthia Chumphon 585
NNkaouthia NST 818
NNkaouthia Burma 839
NNkaouthia SNam 812
NNkaouthia SNam 813

06 Appendix. Supplementary Gene Trees

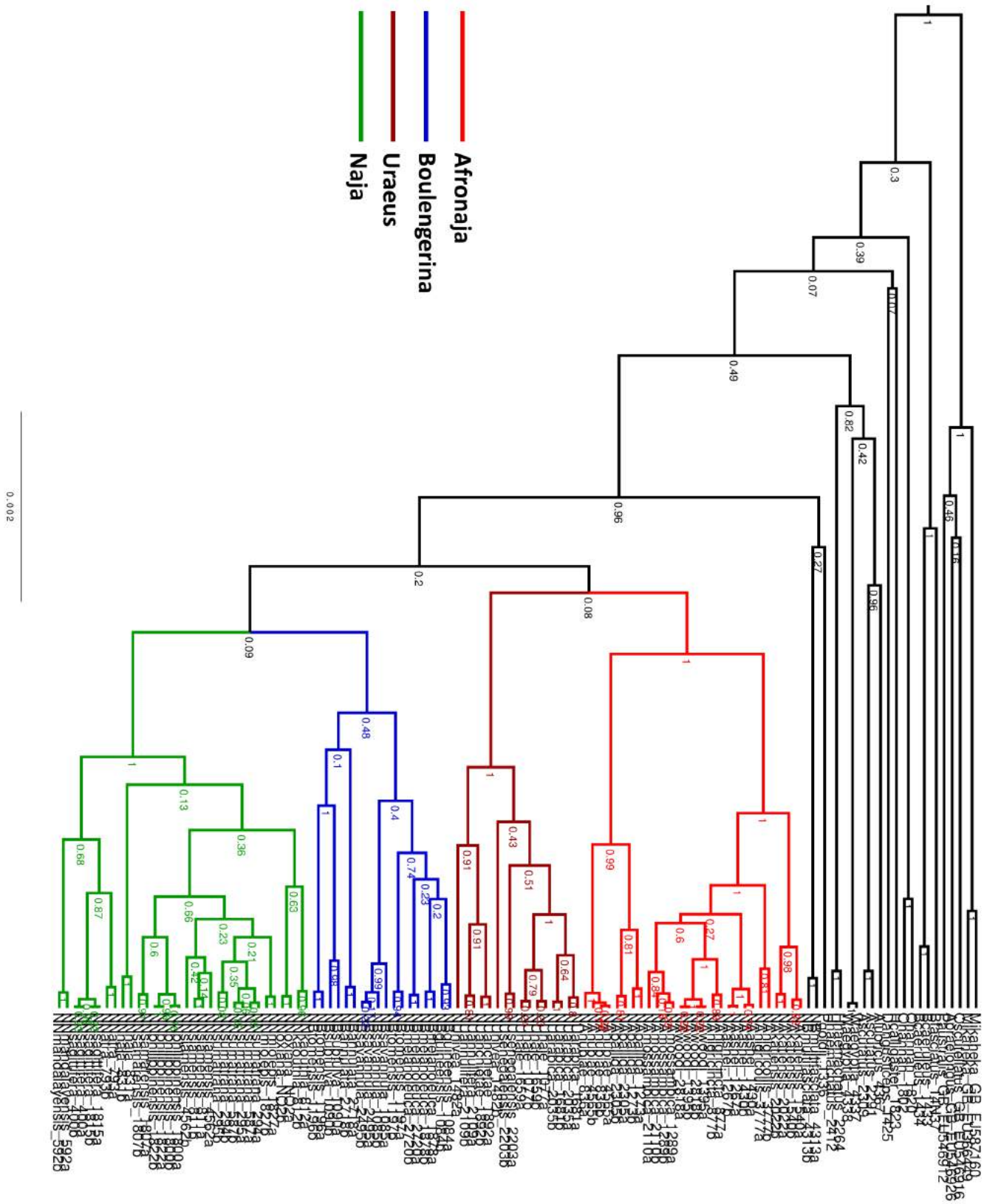
06a. CytB Gene Tree. Exported from *BEAST (Heled & Drummond, 2010)



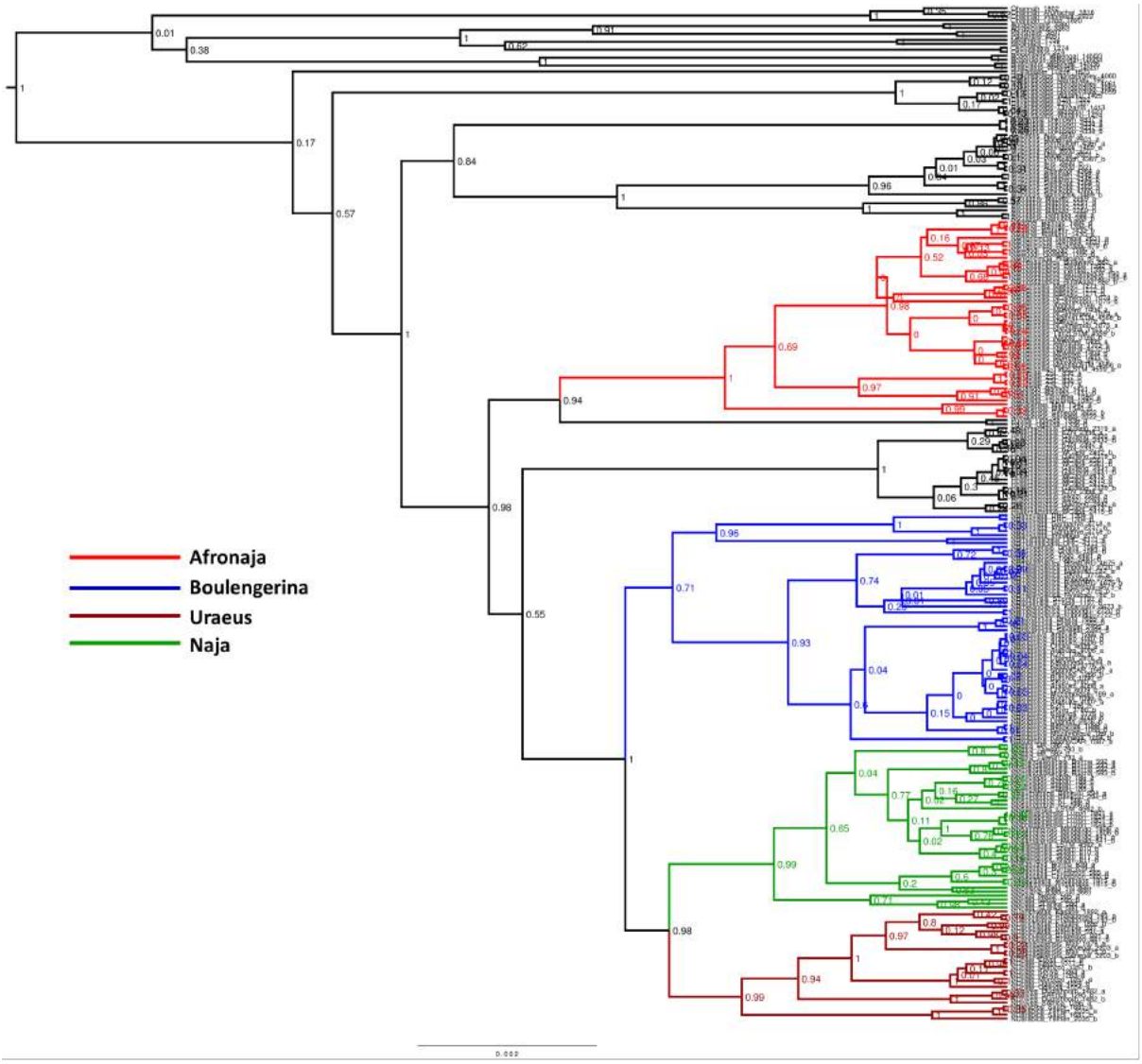
06b. ND4 Gene Tree. Exported from *BEAST (Heled & Drummond, 2010)



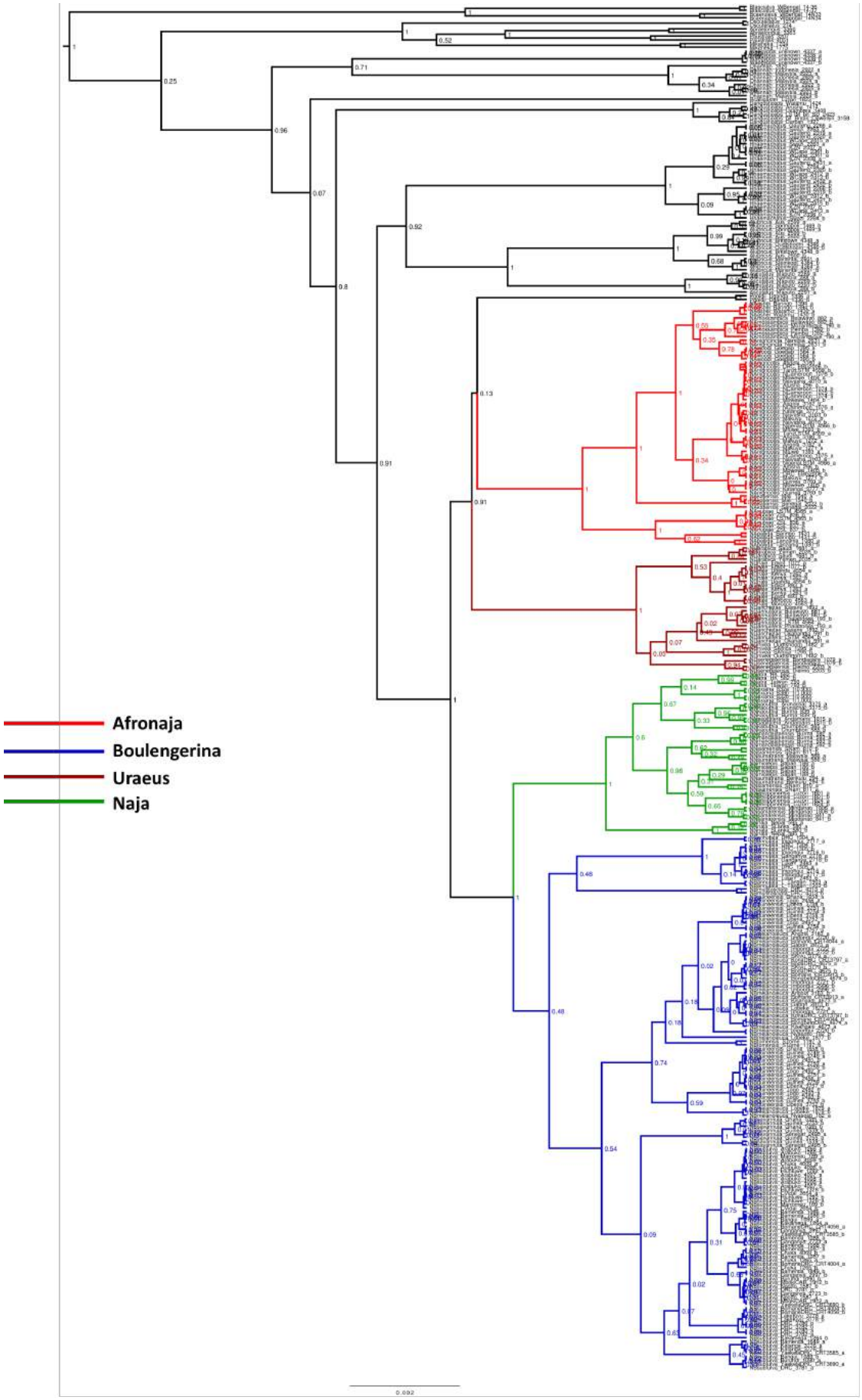
06c. CMOS Gene Tree. Exported from *BEAST (Heled & Drummond, 2010)



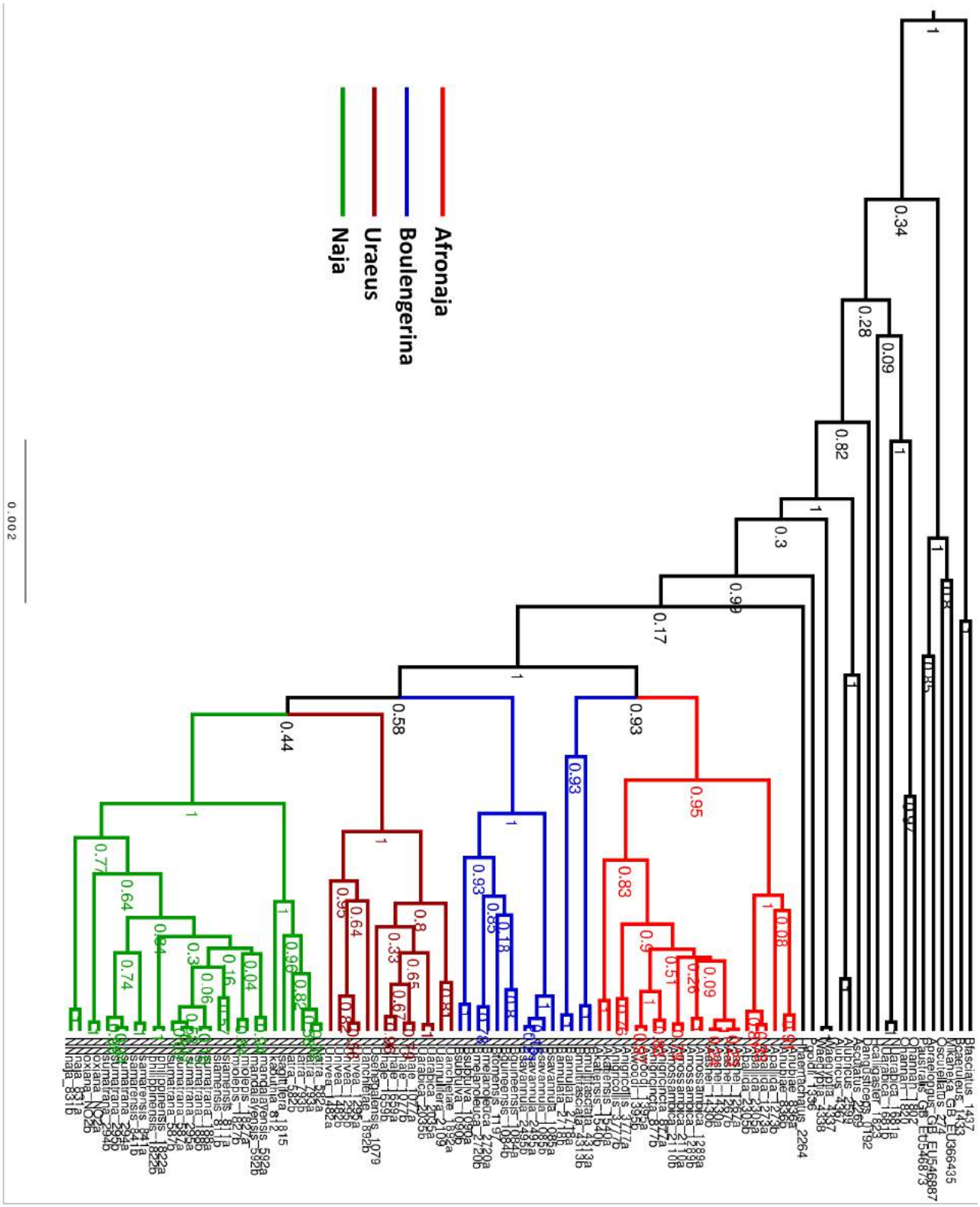
06d. NT3 Gene Tree. Exported from *BEAST (Heled & Drummond, 2010)



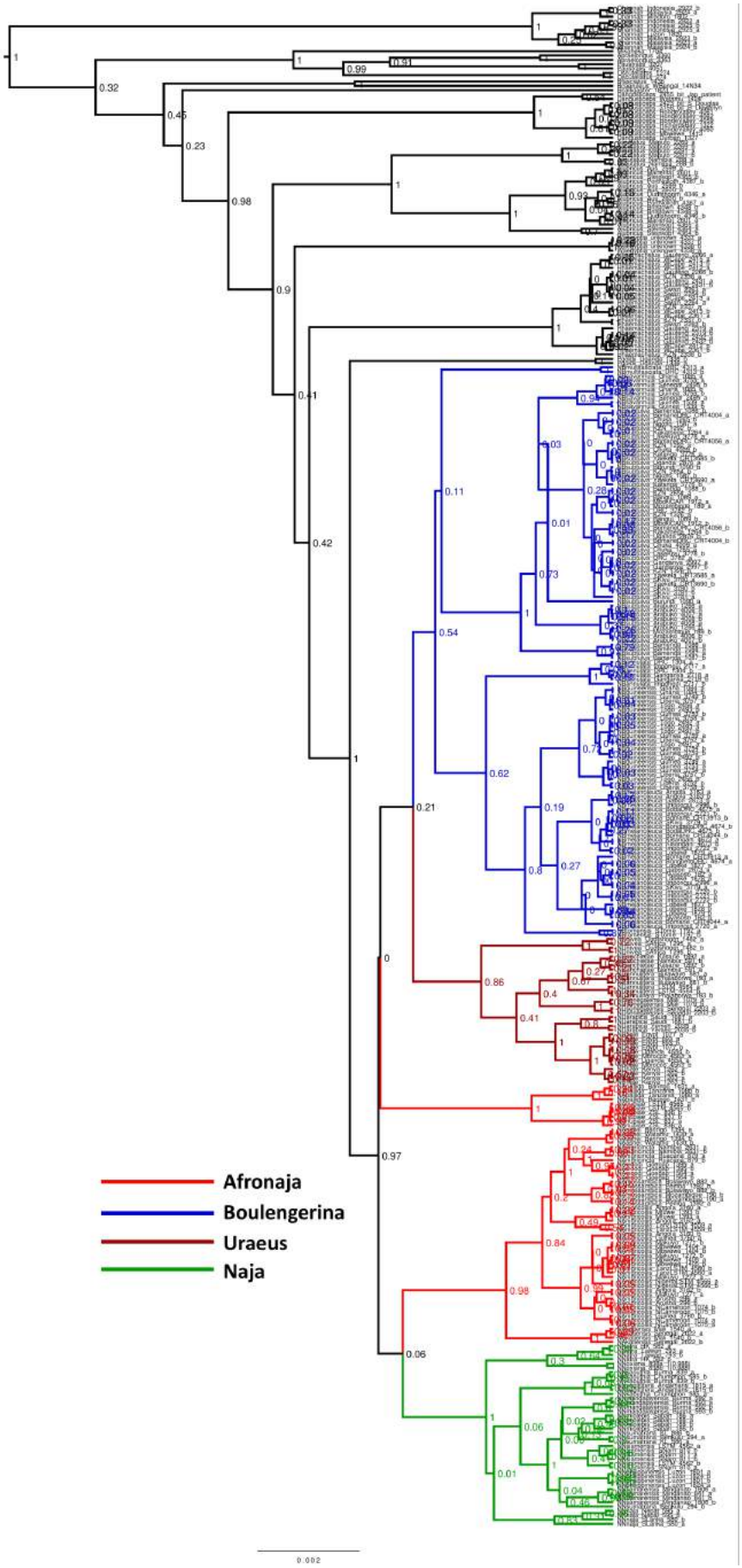
06e. PRLR Gene Tree. Exported from *BEAST (Heled & Drummond, 2010)

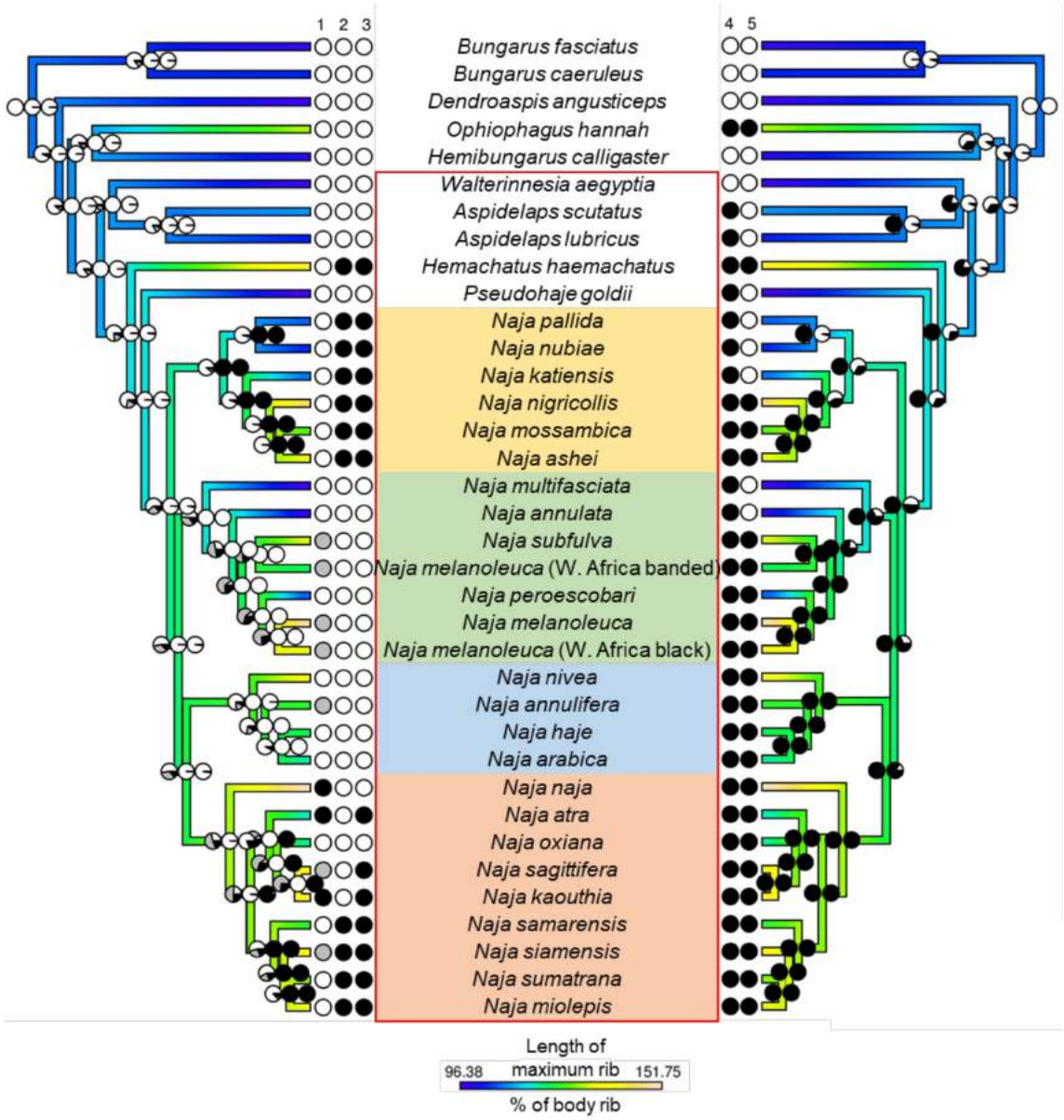


06f. RAG1 Gene Tree. Exported from *BEAST (Heled & Drummond, 2010)



06f. UBN1 Gene Tree. Exported from *BEAST (Heled & Drummond, 2010)



07 Appendix. Ancestral State Reconstruction

Ancestral reconstruction of maximum rib length and discrete characters, taken from Jones (2017). ContMap was used for ancestral reconstruction of maximum rib length with make.simmap reconstructions of discrete characters: 1- Hood pattern, 2- Spitting (ambiguous non-spitting), 3- Spitting (ambiguous spitting), 4- Hooding behaviour and 5- Extended ribs. Pies at nodes represent posterior probabilities. Black- present, white- absent, grey- possible or indistinct. Colours behind species name represent Genus *Naja* subgenera: yellow- *Afronaja*, green- *Boulengerina*, blue- *Uraeus*, red- *Naja*. Red box- core cobra group.