

Molecular Phylogeny of Genus *Horaglanis* (Indian Blind Catfishes) Within the Family Clariidae

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Abstract

Background: India is one of the mega biodiverse countries with a large number of endemic freshwater fishes. Recently, species of genus *Horaglanis* (family: Clariidae) have been reported from the southern part of India. Due to their unique morphological adaptations, these enigmatic species have been subjected to phylogenetic studies to understand the evolution of adaptive traits. Further, the taxonomic status of these species has not been verified using molecular data. **Methods:** In the present study, secondary data i.e. reported sequences of mitochondrial cytochrome *c* oxidase subunit I gene was used to estimate the genetic divergence values for 14 species of the family Clariidae. Phylogenetic trees were reconstructed using maximum parsimony, maximum likelihood and Bayesian Inference methods. **Results:** The average genetic divergence value among genera *Clarias-Clariallabes-Platyallabes-Dolichallabes-Gymnallabes-Tanganikallabes-Channallabes* was 0.082 ± 0.01 . However, these genera showed an average divergence value of 0.296 ± 0.02 with genus *Horaglanis*. In all tree topologies, species of the genus *Horaglanis* formed a basal group to all other clariids. **Discussion:** The higher genetic divergence value between genus *Horaglanis* and other genera of Clariidae family suggested that genus *Horaglanis* may belong to a separate sub family. Based on phylogenetic trees, it is evident that species of *Horaglanis* might have originated early in the evolution of Clariids than other species.

Keywords: Troglomorphic fish, Molecular phylogeny, Cytochrome *c* oxidase subunit I, Clariidae, *Horaglanis*

1 Introduction

2 India is one of the mega biodiverse countries with a large number of endemic freshwater
3 fishes. A total of 946 freshwater fish species (endemic species: 195 nos) have been reported from
4 India (FishBase, 2017). Among different ecosystems, caves and other subterranean habitats are
5 one of the most challenging environments for fishes. Nevertheless, bony fishes are the only
6 vertebrate group that has been successfully colonized these habitats (Soares & Niemiller, 2013).
7 Due to their unique morphological adaptations such as lack of eyes and pigments, these cave
8 fishes / hypogean fishes have been used as a model organism to study the evolution of adaptive
9 traits (Romeo & Paulson, 2001).

10
11 In India, eight hypogean fishes representing three orders viz., Cypriniformes: *Nemacheilus*
12 *evezardi* Day, 1872, *Schisturasi juensis* Menon, 1987; Synbranchiformes: *Monopterus eapeni* Talwar,
13 1991, *M. roseni* Bailey and Gans, 1998 and *M. digressus* Gopi 2002; Siluriformes: *Horaglanis krishnai*
14 Menon, 1950, *Horaglanis alikunhii* Babu & Nayar, 2004 and *Horaglanis abdulkalami* Babu, 2012
15 have been reported from different subterranean waters / caves (Vincent, 2012).

16
17 Among these, species of *Horaglanis* (order: Siluriformes) are endemic to India and
18 commonly known as Indian blind catfish. Earlier, Menon (1951) described blind catfishes from
19 India and placed them in family Clariidae under genus *Horaglanis*. Later, Jayaram (2006; 2010)
20 categorized genus *Horaglanis* under a new subfamily Horaglanidinae. However, the evolutionary
21 relationship/phylogeny of genus *Horaglanis* has not been investigated using either morphological
22 or molecular data. Further, the Phylogenetic analysis will provide new insights into the
23 taxonomical position and character evolution of this species. Among the molecular markers,
24 phylogenetically informative gene mitochondrial cytochrome *c* oxidase subunit I (COI “barcode
25 gene”) has been successfully used for metazoan identification and species delimitation (Hebert et
26 al., 2004, Hajibabaei, 2012). It resulted in a global initiative to develop a comprehensive DNA
27 barcode database (Barcode of Life Database: BOLD) including species specific markers (COI
28 gene) along with their taxonomic details. At present, this database contains taxonomic
29 information for 11256 fish species (Ratnasingam and Hebert, 2007). The COI sequences of this
30 database could be used to infer phylogenetic relationship between species at lower taxonomic
31 level (i.e. within the family). Previously several researchers have used the DNA sequences
32 available from databases and resolved the phylogenetic relationship between different taxon
33 through *in silico* approach (Dangre et al., 2009; Vélez-Zuazo and Agnarsson, 2011).

34 With this background, the present study was aimed to investigate the phylogenetic
35 position of genus *Horaglanis* within Clariidae family using mitochondrial COI gene sequences
36 available from public databases.

37 **Materials and Methods**

38

39 **Sequence data mining and analysis**

40 The public data portal of BOLD and core nucleotide database of GenBank were
41 searched for COI sequences of Clariidae fishes. A total of 39 COI sequences representing 8
42 genera, 14 species were downloaded from databases (Table 1). In BOLD / GenBank database,
43 the species name of genus *Horaglanis* was not defined and reported as *Horaglanis* species.
44 *Heteropneustes fossilis* (Heteropneustidae family) was taken as an outgroup for phylogenetic
45 studies. COI sequences with a minimum length of 600 bp, with no missing nucleotides or gaps,
46 were included for analyses. Open reading frame was predicted for all the COI sequences using
47 NCBI ORF finder to confirm the lack of NUMTs (nuclear DNA originating from mitochondrial
48 DNA sequences, Zhang & Hewitt, 1996).

49 **Model of evolution and phylogeny reconstruction methods**

50 The sequences were aligned using ClustalW and genetic divergence values were
51 calculated using Kimura two parameter (K2P) distance model implemented in MEGA V.5.2
52 (Tamura et al., 2011). The size of the alignment, number of variable sites, number of parsimony
53 informative sites among all taxa were calculated using MEGA V.5.2 software. Chi-square (χ^2) test
54 was used to test the base compositional evenness and stationarity among taxa, implemented in
55 PAUP*v.4.0b10 (Swofford, 2003) software. J MODELTEST v.0.1.1 (Posada, 2008) was used to
56 estimate the most likely model of sequence evolution for Maximum likelihood (ML) and
57 Bayesian Inference (BI) methods. Based on likelihood values and the Akaike information
58 Criterion (AIC), most likely sequence evolutionary models were selected.

59 Distance based (K2P models), Maximum Parsimony and Maximum likelihood analyses
60 were performed by PAUP* v.4.0b10 (Swofford, 2003). For Maximum Parsimony analysis, all
61 characters were treated as unordered and un-weighted. A heuristic search for the most
62 parsimonious trees was conducted with random addition sequence (100 replicates) and tree
63 bisection–reconnection (TBR) branch-swapping. A majority-rule consensus tree was constructed
64 with 1000 bootstrap resampling (Felsenstein, 1985) to assess support of relationships. MR
65 BAYES v3.1.2 (Huelsenbeck & Ronquist, 2003) was used for Bayesian inference. For all

66 analyses, the prior probability of a flat Dirichlet distribution for the substitution rates and
67 stationary nucleotide frequencies was used under the assumption of no prior knowledge. The
68 Bayesian analysis was run using the Metropolis coupled Markov Chain Monte Carlo (MCMC)
69 algorithm from randomly generated starting trees for 5 million generations with sampling every
70 1000 generations. Two runs were performed simultaneously in which there were three heated
71 chains and one cold chain, each with a temperature parameter of 0.2. The first 25% of sampled
72 trees were discarded as burn-in.

73 **Results and Discussion**

74 Mitochondrial COI genes with 602 nucleotide characters were aligned to the
75 homologous position. The number of conserved, variable and parsimony informative
76 nucleotides is 374, 228 and 218, respectively. These values show that the COI gene has sufficient
77 phylogenetic signal to infer the evolutionary relationship. The average nucleotide frequencies for
78 COI gene among the species were A = 26.9, T=29, G=17.1 and C= 26.9% with a GC content
79 of 44%. The GC content of COI gene region is slightly lesser than previous reports for teleosts
80 (Ward et al., 2005). The average GC content values at codon 1st, 2nd, and 3rd base positions are
81 55.4, 42.5 and 34.2%, respectively. The average transitional pairs ($S_i=50$) were more than the
82 transversional pairs ($S_v=29$) with an average ratio of 1.75. It showed that the sequences were not
83 saturated and useful for phylogenetic analysis. Chi-square (χ^2) test applied for detecting
84 homogeneity of base composition across the taxa indicated that there was no significant variation
85 ($P = 0.99$) in AT/GC content among species.

86 The species name for the COI sequences of *Horaglanis* genus were not described in
87 NCBI GenBank /BOLD databases. To confirm whether these COI sequences belongs to the
88 same species or not, genetic divergence values were estimated and the results revealed a genetic
89 distance value of “0” suggesting that the four specimens indeed belong to the same species of
90 genus *Horaglanis*.

91 The average genetic divergence value among genera *Clarias*, *Clariallabes*, *Platyallabes*,
92 *Dolichallabes*, *Gymnallabes*, *Tanganikallabes* and *Channallabes* was 0.082 ± 0.01 . However, the average
93 genetic divergence value between these genera and genus *Horaglanis* was 0.296 ± 0.12 (Table 2).
94 In the present study, degree of increase in genetic distance value (3X) among genera is more than
95 the previous reports (Ward et al., 2005; Lakra et al., 2011). Based on the high genetic divergence
96 value between genus *Horaglanis* and other genera of family Clariidae, genus *Horaglanis* could be
97 grouped under separate subfamily “Horaglanidinae”. Nucleotide diagnostic characters of COI

98 sequences exclusive to genus *Horaglanis* were identified by comparing with other Clariidae
99 species. Simple nucleotide diagnostic characters were identified in 56 informative sites. Among
100 these, 26 characters correspond to transitions and remaining 30 sites are transversional changes
101 (Supplementary Figure 1). These diagnostic characters could help in species of the genus
102 *Horaglanis* identification. Generally, nucleotide diagnostic characters are useful for discriminating
103 closely related species (Wong et al., 2009). However, in this study we used the only one reported
104 *Horaglanis* species, but we believe that in view of discovering more number of different species of
105 *Horaglanis* (Babu, 2012), this information will help in identifying/discriminating the closely
106 related *Horaglanis* species.

107 The phylogenetic relationship among Clariidae species was estimated by Neighbor-
108 joining, Maximum Parsimony, Maximum likelihood (TIM1+I+G model) and Bayesian Inference
109 (GTR+I+G model) methods. Maximum parsimony method yielded most parsimonious tree with
110 a length of 583. The consistency, homoplasy and retention indexes of the tree are 0.58, 0.41 and
111 0.79, respectively.

112 All methods yielded similar topologies with a slight difference in Bootstrap values
113 (Supplementary figure 2-4). Some of the tree topologies displayed soft polytomies at internal
114 nodes and this could be due to the usage of a single gene. However, in all tree topologies, species
115 of the genus *Horaglanis* formed the basal group to all other Clariids (distinct lineages) and the
116 branch length was relatively high (Fig. 1). It showed that the species might have originated early
117 in the evolution of Clariids than other species. Previous studies have compared the cranial
118 osteology of species of *Horaglanis* with other genera of Clariidae fishes and reported Genus
119 *Horaglanis* as a primitive Silurid fishes (Bhimachar, 1933, Mercy & Pillai, 2001, Mercy et al., 2001).
120 Bhimachar (1933) reported that species of *Horaglanis* would be a connecting link between family
121 Clariids and Bagrids. Based on the COI divergence and phylogeny analyses, the present study
122 also supports that the genus *Horaglanis* could be grouped into a separate subfamily. However, it
123 needs further studies with more number of molecular markers and species to understand the
124 evolutionary relationship of these enigmatic species. The phylogenetic tree also revealed the
125 paraphyletic nature of species of *Clarias* as reported by previous workers (Devaere et al., 2005,
126 2007; Jansen et al., 2006; Mwita & Nkwengulila, 2008).

127 In conclusion, this study provides preliminary information on the phylogenetic position
128 of the genus *Horaglanis* within the clariidae family based on the available molecular marker

129 information. Further, this study identified nucleotide diagnostic characters exclusive to the genus

130 *Horaglanis*.

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215 Table 1: List of species along with GenBank / BOLD BIN numbers

| S.No | Species (number of specimen) | Characteristics | GenBank accession / Bold ID |
|------|---|--|---|
| 1. | <i>Channallabes apus</i> (Günther, 1873) (4) | Endemic to Africa, anguilliform shape, reduced suprabranchial organ (fishbase) | AMNHI001-09 AMNHI008-09 AMNHI059-09 AMNHI321-10 |
| 2. | <i>Gymnallabes nops</i> Roberts & Stewart, 1976 (1) | Endemic to Africa, anguilliform shape, reduced surabranhial organ (fishbase) | AMNHI021-09 |
| 3. | <i>Clariallabes teugelsi</i> Ferraris, 2007 (3) | Endemic to Africa, elongate body shape, suprabranchial organ present (fishbase) | AMNHI025-09 AMNHI026-09 AMNHI498-12 |
| 4. | <i>Dolichallabes microphthalmus</i> Poll, 1942 (3) | Endemic to Africa, anguilliform shape, reduced suprabranchial organ (fishbase) | AMNHI054-09 AMNHI056-09 AMNHI096-09 |
| 5. | <i>Platyallabes tiboni</i> (Poll, 1944) (2) | Endemic to Africa, intermediate shape between anguilliform and fusiform, reduced suprabranchial organ (Devaere <i>et al.</i> , 2005) | AMNHI071-09 AMNHI073-09 |
| 6. | <i>Tanganikallabes mortiauxi</i> Poll, 1943 (2) | Endemic to deeper waters in Lake Tanganyika (East Africa), Fusiform shape, reduced suprabranchial organ (Write and Bailey 2012) | AMNHI090-09 AMNHI091-09 |
| 7. | <i>Clarias gabonensis</i> Günther, 1867 (5) | Endemic to Africa, anguilliform shape, developed suprabranchial organ (Fishbase) | AMNHI322-10 AMNHI324-10 AMNHI494-12 AMNHI495-12 BAFEN135-10 |
| 8. | <i>Clarias angolensis</i> Steindachner, 1866 (1) | Africa; Lower and middle Congo river, fusiform, developed suprabranchial organ | HM880232 |
| 9. | <i>Clarias teijsmanni</i> Bleeker, 1857 (1) | Endemic to Asia, anguilliform shape (Fishbase) | JN646093 |
| 10. | <i>Clarias gariepinus</i> (Burchell, 1822) (5) | Native of Africa, fusiform Suprabranchial organ (Fishbase) | JQ699199-203 |
| 11. | <i>Clarias dussumieri</i> Valenciennes, 1840 (5) | Native to Asia, Fusiform, slightly reduced suprabranchial organ (Fishbase) | JQ699209-213 |
| 12. | <i>Clarias camerunensis</i> Lönnberg, 1895 (1) | Endemic to Africa, anguilliform, developed suprabranchial organ (fishbase) | HM882808 |
| 13. | <i>Clarias jaensis</i> Boulenger, 1909 (2) | Africa, anguilliform, developed suprabranchial organ (Fishbase) | BAFEN138-10 BAFEN139-10 |
| 14. | <i>Horaglanis</i> species (4) | Asia, anguilliform shape, reduced suprabranchial organ (Fishbase) | HE819391-94 |
| 15. | <i>Heteropneustes fossilis</i> (2) | | GQ466396-97 |

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217 Table 2. COI Average genetic divergence values (K2P method) among Clariidae species

| Species | <i>C. apus</i> | <i>G. nops</i> | <i>C. teugelsi</i> | <i>D. microphthalmus</i> | <i>P. tiboni</i> | <i>T. mortiauxi</i> | <i>C. gabonensis</i> | <i>C. teijsmanni</i> | <i>C. gariepinus</i> | <i>C. dussumieri</i> | <i>C. camerunensis</i> | <i>C. jaensis</i> | <i>Horaglanis</i> |
|-------------------------------------|----------------|----------------|--------------------|--------------------------|------------------|---------------------|----------------------|----------------------|----------------------|----------------------|------------------------|-------------------|-------------------|
| <i>Channallabes apus</i> | | 0.013 | 0.013 | 0.013 | 0.012 | 0.008 | 0.011 | 0.015 | 0.013 | 0.013 | 0.014 | 0.011 | 0.026 |
| <i>Gymnallabes nops</i> | 0.095 | | 0.014 | 0.011 | 0.014 | 0.014 | 0.014 | 0.016 | 0.016 | 0.015 | 0.014 | 0.013 | 0.027 |
| <i>Clariallabes teugelsi</i> | 0.102 | 0.108 | | 0.014 | 0.016 | 0.012 | 0.014 | 0.017 | 0.016 | 0.016 | 0.015 | 0.015 | 0.027 |
| <i>Dolichallabes microphthalmus</i> | 0.089 | 0.059 | 0.105 | | 0.014 | 0.013 | 0.013 | 0.016 | 0.017 | 0.015 | 0.015 | 0.014 | 0.026 |
| <i>Platyallabes tiboni</i> | 0.077 | 0.110 | 0.127 | 0.113 | | 0.012 | 0.012 | 0.016 | 0.015 | 0.013 | 0.015 | 0.012 | 0.026 |
| <i>Tanganikallabes mortiauxi</i> | 0.038 | 0.103 | 0.086 | 0.091 | 0.077 | | 0.010 | 0.015 | 0.014 | 0.013 | 0.014 | 0.011 | 0.026 |
| <i>Clarias gabonensis</i> | 0.064 | 0.101 | 0.111 | 0.091 | 0.079 | 0.058 | | 0.015 | 0.015 | 0.013 | 0.014 | 0.013 | 0.027 |
| <i>Clarias teijsmanni</i> | 0.132 | 0.130 | 0.153 | 0.136 | 0.132 | 0.132 | 0.121 | | 0.016 | 0.015 | 0.016 | 0.016 | 0.024 |
| <i>Clarias gariepinus</i> | 0.098 | 0.133 | 0.131 | 0.147 | 0.118 | 0.114 | 0.117 | 0.132 | | 0.015 | 0.013 | 0.015 | 0.028 |
| <i>Clarias dussumieri</i> | 0.096 | 0.126 | 0.141 | 0.116 | 0.104 | 0.104 | 0.104 | 0.125 | 0.123 | | 0.015 | 0.014 | 0.027 |
| <i>Clarias camerunensis</i> | 0.106 | 0.112 | 0.112 | 0.118 | 0.120 | 0.104 | 0.108 | 0.134 | 0.095 | 0.113 | | 0.013 | 0.028 |
| <i>Clarias jaensis</i> | 0.068 | 0.095 | 0.112 | 0.099 | 0.085 | 0.071 | 0.091 | 0.130 | 0.119 | 0.108 | 0.098 | | 0.026 |
| <i>Horaglanis</i> species | 0.298 | 0.301 | 0.317 | 0.308 | 0.303 | 0.301 | 0.314 | 0.280 | 0.316 | 0.308 | 0.321 | 0.306 | |

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219 *Below diagonal distance values; above diagonal standard error values for corresponding distance value

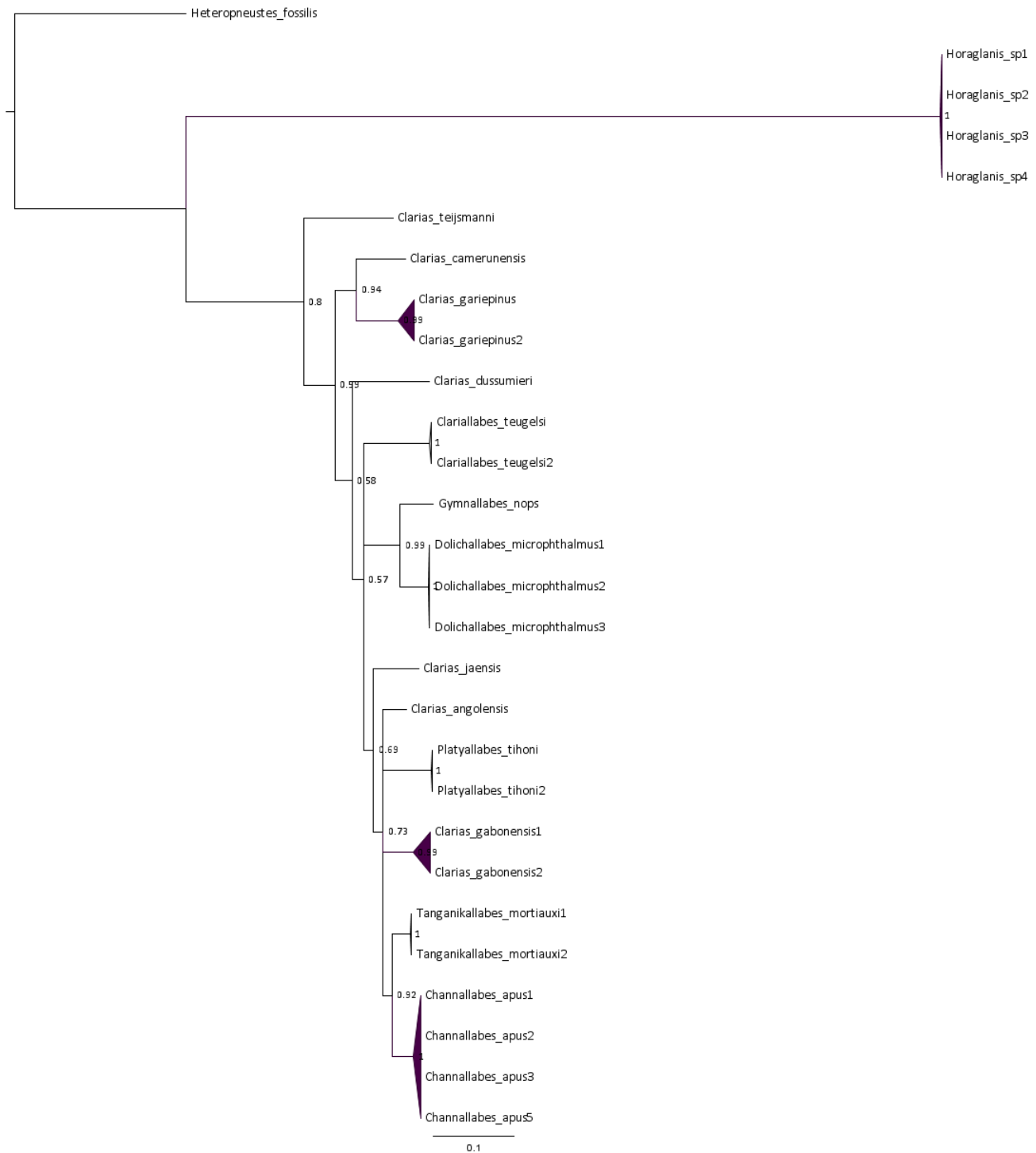
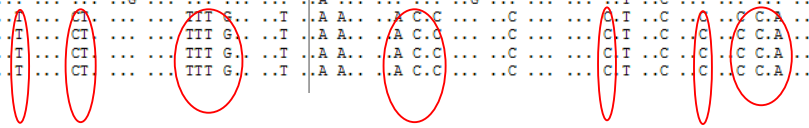


Fig.1. Bayesian inference based phylogeny depicting relationship among clariidae species

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!Domain=Data property=Coding CodonStart=1;
#Channallabes_apus1      CTT AGC TTA TTA ATC CGG GCA GAA CTA GCA CAA CCC GGG GCC CIT TTA GGA GAT GAC CAG ATC TAT AAT GIT ATT GTT
#Channallabes_apus2      ...
#Channallabes_apus3      ...
#Channallabes_apus5      ...
#Gymnallabes_nops        ...
#Clariallabes_teugelsi    ..C ..
#Clariallabes_teugelsi2   ..C ..
#Dolichallabes_microphthalmus1 ..
#Dolichallabes_microphthalmus2 ..
#Dolichallabes_microphthalmus3 ..
#Platyallabes_tihoni      ..G ..
#Platyallabes_tihoni2     ..G ..
#Tanganikallabes_mortiauxi1 ..
#Tanganikallabes_mortiauxi2 ..
#Clarias_gabonensis1     ..
#Clarias_gabonensis2     ..A ..
#Clarias_angolensis      ..C ..
#Clarias_teijsmanni      ..C ..
#Clarias_gariepinus      T.. ..G ..G ..
#Clarias_gariepinus2     ..G ..
#Clarias_dussumieri      ..C ..
#Clarias_camerunensis    ..G ..G ..
#Clarias_jaensis         ..C ..
#Horaglanis_sp1          ..C ..
#Horaglanis_sp_MV-2012   ..C ..
#Horaglanis_sp2          ..C ..
#Horaglanis_sp3          ..C ..

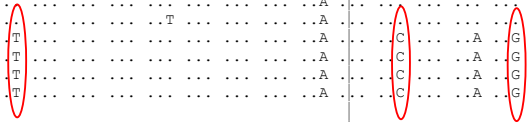
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#Channallabes_apus1      ACT GCT CAC GCC TTC GTA ATA ATC TTC TTT ATA GTG ATA CCA ATT ATG ATT GGC GGT TTC GGG AAC TGA CTT GTC CCA
#Channallabes_apus2      ...
#Channallabes_apus3      ...
#Channallabes_apus5      ...
#Gymnallabes_nops        ..C ..T ..T ..T ..T ..A ..
#Clariallabes_teugelsi    ..C ..T ..G ..
#Clariallabes_teugelsi2   ..C ..T ..G ..
#Dolichallabes_microphthalmus1 ..C ..T ..T ..T ..T ..A ..
#Dolichallabes_microphthalmus2 ..C ..T ..T ..T ..T ..A ..
#Dolichallabes_microphthalmus3 ..C ..T ..T ..T ..T ..A ..
#Platyallabes_tihoni      ..C ..T ..T ..T ..T ..A ..
#Platyallabes_tihoni2     ..C ..T ..T ..T ..T ..A ..
#Tanganikallabes_mortiauxi1 ..T ..
#Tanganikallabes_mortiauxi2 ..T ..
#Clarias_gabonensis1     ..C ..T ..
#Clarias_gabonensis2     ..C ..T ..
#Clarias_angolensis      ..C ..T ..
#Clarias_teijsmanni      ..C ..C ..T ..
#Clarias_gariepinus      ..C ..A ..
#Clarias_gariepinus2     ..A ..
#Clarias_dussumieri      ..C ..
#Clarias_camerunensis    ..A ..
#Clarias_jaensis         ..C ..C ..T ..
#Horaglanis_sp1          ..C ..C ..T ..
#Horaglanis_sp_MV-2012   ..C ..C ..T ..
#Horaglanis_sp2          ..C ..C ..T ..
#Horaglanis_sp3          ..C ..C ..T ..

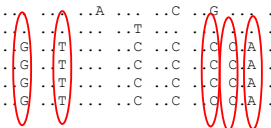
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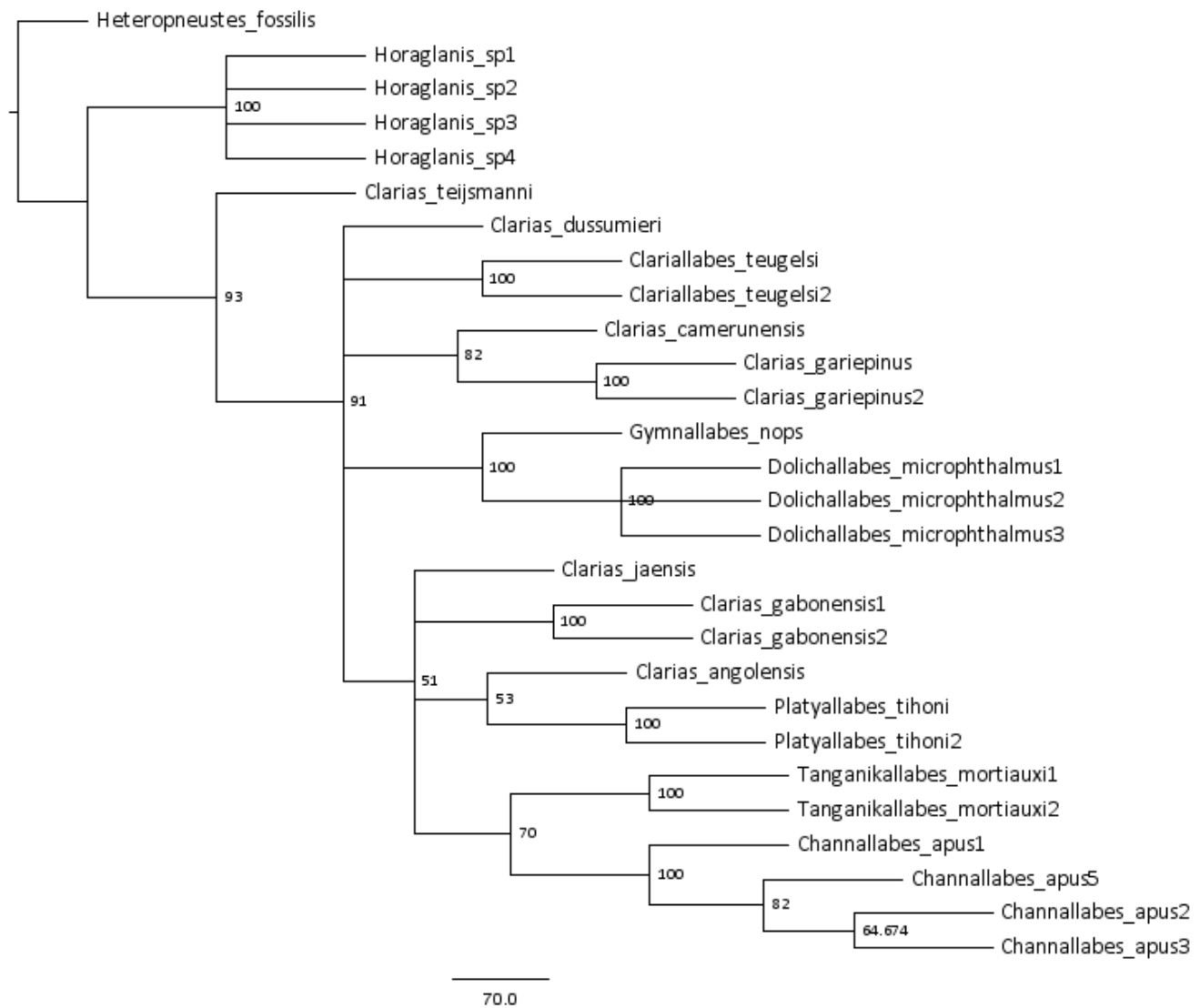


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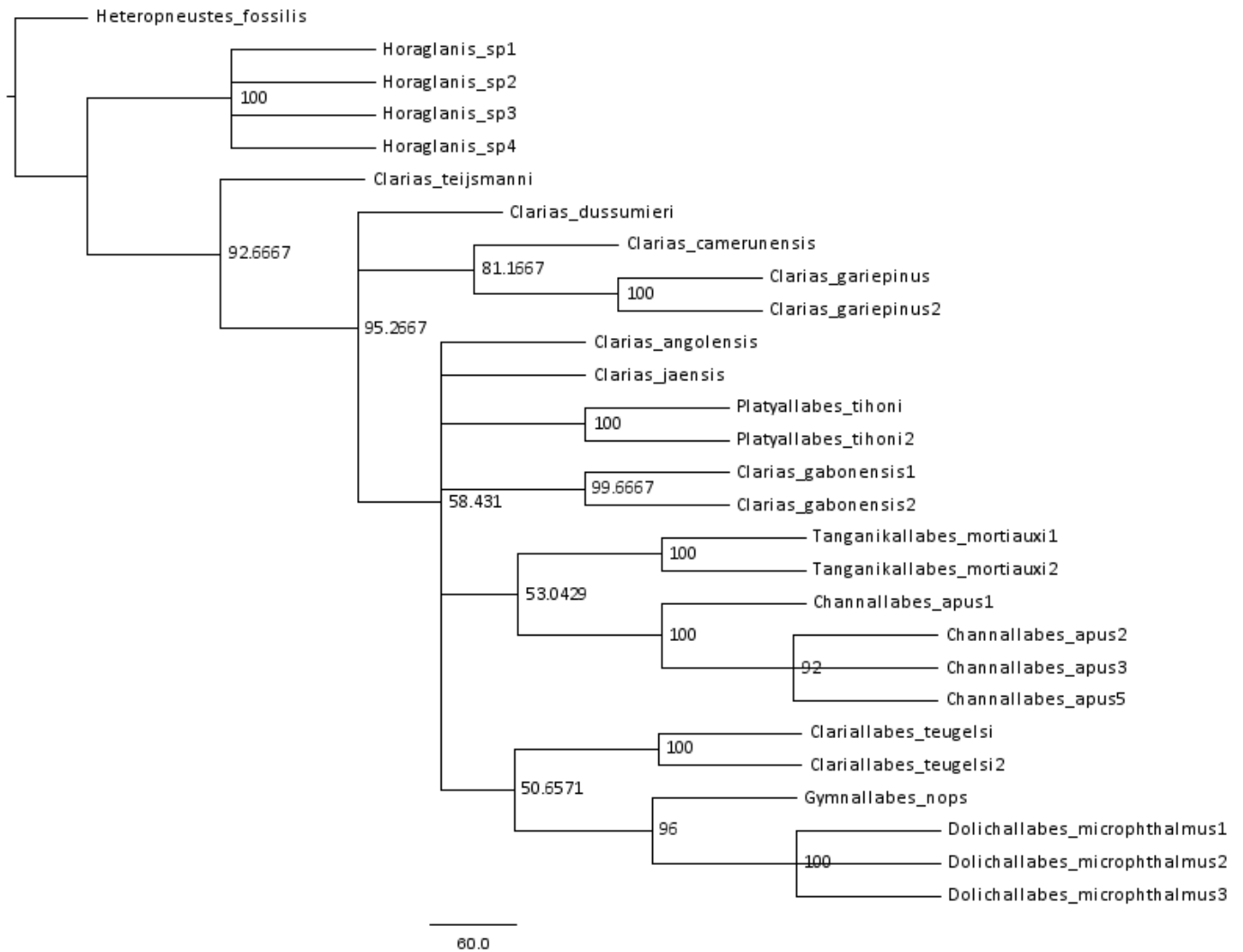
#Channallabes_apus1      CTA ATA CTA GGA GCC CCT GAT ATA GCA TTC CCA CGA ATG AAT AAC ATA AGC TTT TGA CTA CTC CCA CCA TCA TTC CTA
#Channallabes_apus2      ...
#Channallabes_apus3      ...
#Channallabes_apus5      ...
#Gymnallabes_nops        T.. ..G ..A ..C ..
#Clariallabes_teugelsi    ..T ..G ..C ..G ..G ..A ..
#Clariallabes_teugelsi2   ..T ..G ..C ..G ..G ..A ..
#Dolichallabes_microphthalmus1 ..
#Dolichallabes_microphthalmus2 ..
#Dolichallabes_microphthalmus3 ..
#Platyallabes_tihoni      ..G T.. ..T ..
#Platyallabes_tihoni2     ..G T.. ..T ..
#Tanganikallabes_mortiauxi1 ..
#Tanganikallabes_mortiauxi2 ..
#Clarias_gabonensis1     ..G ..
#Clarias_gabonensis2     ..C ..
#Clarias_angolensis      ..G ..T ..
#Clarias_teijsmanni      T.. ..A.T ..A ..C ..
#Clarias_gariepinus      ..G ..G ..T ..
#Clarias_gariepinus2     ..G ..G ..
#Clarias_dussumieri      ..G ..C ..
#Clarias_camerunensis    ..G ..
#Clarias_jaensis         ..G ..T ..G ..A ..C ..
#Horaglanis_sp1          ..T ..A.T ..G ..A ..C ..
#Horaglanis_sp_MV-2012   ..T ..A.T ..G ..A ..C ..
#Horaglanis_sp2          ..T ..A.T ..G ..A ..C ..
#Horaglanis_sp3          ..T ..A.T ..G ..A ..C ..

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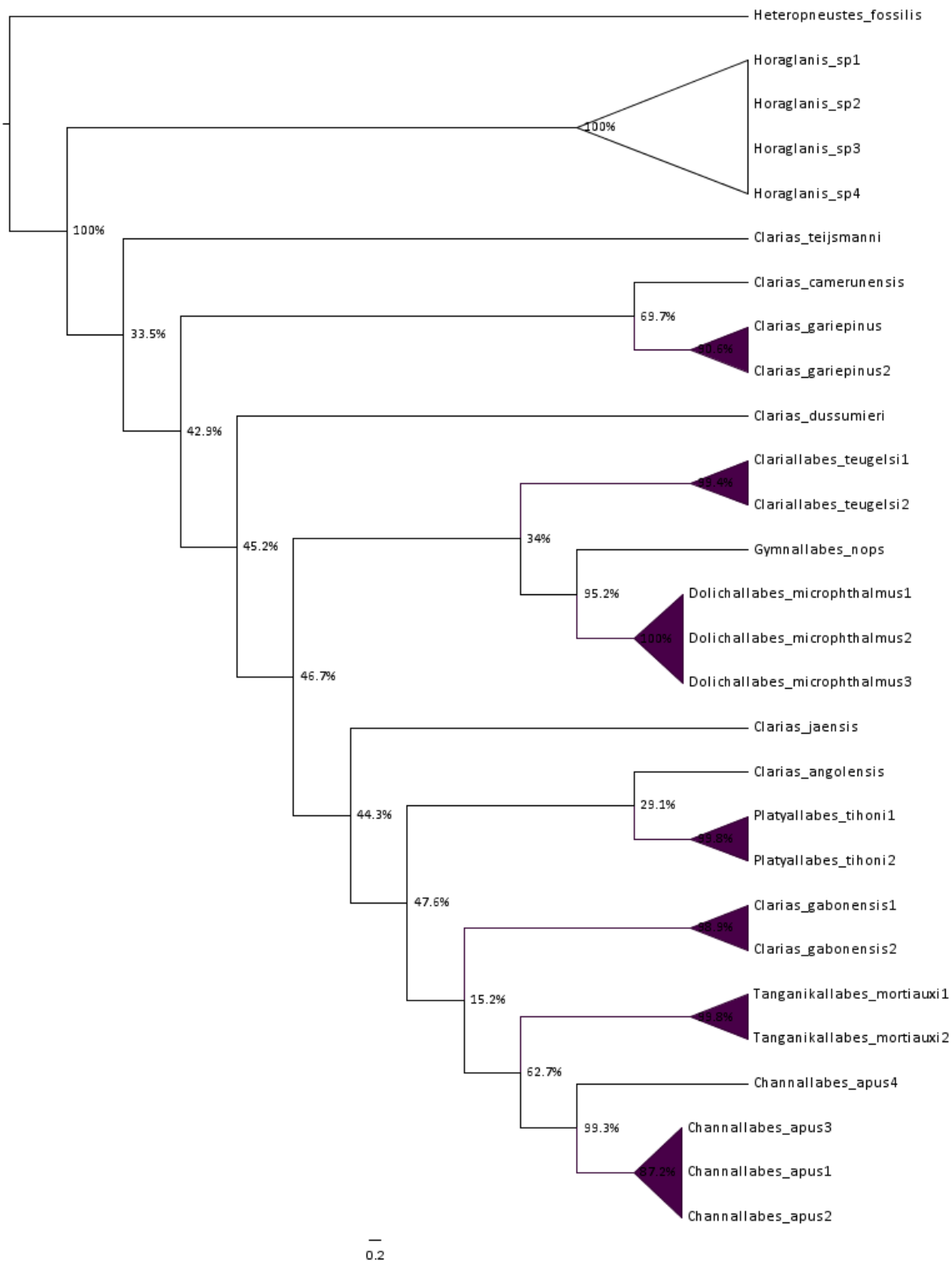




Sup. Fig. 2. Neighbor- Joining tree of Clariidae species constructed by COI gene



Sup. Fig.3. Clariidae species phylogeny tree constructed by COI gene using Maximum Parsimony method



Sup. Fig. 4. Clariidae Species phylogeny tree constructed by Maximum Likelihood method.