Unveiling the molecular identity of the diminutive cyprinid, Horadandia brittani (Teleostei: Cyprinidae), a species endemic to Southern India

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Short Report

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Abstract

Horadandia brittani is a small cyprinid fish species found in the coastal floodplains of southern India. For almost 50 years, the Horadandia genus was monotypic with only one species (*Horadandia atukorali*) confined to Sri Lanka until the discovery of *H. brittani* in south-western India in 1992. Despite being described as a separate species, *H. brittani*was later considered a synonym of *H. atukorali* until 2013 when researchers recognized it as a distinct species based on some morphological differences. However, no one had yet generated DNA sequences to validate the identity of *H. brittani* and its evolutionary relationship with its closely related species. To address this gap, this study was conducted to generate DNA sequences and validate the identity *of H. brittani* using molecular data. The results showed that *H. brittani* is genetically distinct from *H. atukorali* with mitochondrial cytochrome C oxidase (COI) gene distance values ranging from 3.21-3.63%. The study also established the phylogenetic relationships between these two species, confirming *H. brittani* as a valid species based on COI gene sequences. These sequences can be used to identify *H. brittani* quickly and accurately in the future.

INTRODUCTION

Horadandia is a genus closely related to two other genera, Trigonostigma and Rasboroides. This genus can be distinguished from Trigonostigma by the absence of a conspicuous black stripe that runs from below the origin of the dorsal fin to the middle of the caudal fin base. Additionally, the shape of the Horadandia is usually broadened anteriorly and has a triangular or hatchet shape, which sets it apart from Trigonostigma. In contrast, Horadandia differs from Rasboroides in the absence of a lateral line, while Rasboroides has an incomplete one. Horadandia is widely distributed in the coastal floodplains of southern India and southern and western Sri Lanka. Currently, Horadandia has two valid species, namely *Horadandia atukorali* Deraniyagala, 1943 which is restricted to Sri Lanka, and *Horadandia brittani* Rema Devi & Menon, 1992 which is restricted to India. In India, *H. brittani* is mostly distributed in the southern states of Kerala, Tamil Nadu, and perhaps Karnataka (Kottelat & Witte, 1999; Batuwita *et al*., in 2013).

H. atukorali (Deraniyagala, 1943) from Sri Lanka was considered a monotypic genus for almost half a century until the description of *H. brittani* from India (Rema Devi & Menon, 1992). However, some authors subsequently considered *H. brittani* to be a synonym of *H. atukorali* (Rema Devi, 1996; Menon, 1999), until when it was resurrected by Batuwita *et al*. (2013) from synonymy based on few diagnostic characters. The main difference among the two species is that dorsal profile approximately flat, eye diameter 27-37 % of head length, pelvic fin just reaching the anal fin origin, and dorsal fin origin located closer to the hypural notch than to the snout tip in *H. brittani* (vs. dorsal profile distinctly arched, eye diameter 37-41 of % head length, pelvic fin reaching beyond anal-fin origin, and dorsal-fin origin located half way between snout tip and hypural notch in *H. atukorali*). While DNA sequences for *H. atukorali* have already been generated and deposited in the NCBI GenBank under accession numbers MH780760–MH780764 and FJ753505, there is a lack of genetic information available for *H. brittani*. To address this gap, we collected *H. brittani* specimens from southern India and generated mitochondrial cytochrome c oxidase subunit I (COI) gene sequences for this species for the first time. Our main objective was to

generate DNA sequences for *H. brittani* and to investigate its genetic distance from its closely related congeners to confirm the taxonomic validity of this species, as well as to perform a phylogenetic analysis to explore its evolutionary relationships with the other closely related species.

MATERIALS AND METHODS

Sample Collection and Preparation

The specimens were collected from the streams of pechiparai reservoir, Kanyakumari, South India [(8°09'08.7"N 77°29'48.9"E); (8°09'09.9"N 77°28'30.1"E); (8°08'42.8"N 77°27'22.4"E); (8°08'28.3"N 77°26'53.3"E)] using hand net of mesh size 2mm on December 18-20, 2022. The collected specimens were brought live in a bucket to the laboratory for photography (See Fig. 1) and further analysis. Species identity was confirmed using standard literatures (Rema Devi & Menon, 1992; Rema Devi, 1996; Batuwita *et al*., 2013).

Genomic DNA Extraction, PCR Amplification and DNA Sequencing

Tissue samples were taken from the right pectoral fin of the species for genetic analysis. DNA was extracted using the standard phenol-Chloroform method as described by Sambrook *et al*. (2001). For the present study, the mitochondrial cytochrome c oxidase subunit (COI) gene (650bp size) was amplified and sequenced. Primers (26bp length) designed by Ward *et al*. (2005) were used to amplify the mitochondrial COI gene. The primers had the following sequences: Fish F1–5'-TCAACCAACCACAAAGACATTGGCAC-3' and Fish R1–5'-TAGACTTCTGGGTGGCCAAA GAATCA-3'. PCR amplification was carried out using a thermocycler programmed with the recommended conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 30 sec at 94°C, 30 sec at 54°C and 60 sec at 72°C, and a final extension of 10 min at 72°C. The PCR amplified products were purified using a Gel extraction kit (Fermantas, Pittsburgh, PA) as per the manufacturer's protocol and were then sequenced in both forward and reverse direction. The homology of the generated sequences was checked using BLAST to find the closest sequences available in GenBank (https://www.ncbi.nlm.nih.gov). The sequences generated in this study were deposited in NCBI GenBank under accession numbers OQ361850 – OQ361853. Gene sequences were aligned using MUSCLE. The genetic distances among the sequences for different species were determined by Kimura's two-parameter (K2P) model in MEGA X (Molecular Evolutionary Genetics Analysis. For comparison, available sequences for other closely related species were retrieved from the NCBI database (https://www.ncbi.nlm.nih.gov/nucleotide/).

RESULTS

The partial segment of mitochondrial COI gene sequences generated in this study were first checked with Finchtv software to ensure the quality of the sequences. Bases with a Phred score of >30 alone retained for further analysis, and any low-quality sequences were end trimmed and discarded. The NCBI Open Reading Frames (ORFs) Finder tool was used to predict the Open Reading Frame (ORF) of the COI

sequences. There were no insertions or deletions (indels) observed, indicating the absence of NUMTS (nuclear mitochondrial DNA) in the studied samples.

In this study, the average intra-specific genetic distance values showed low variation within *H. brittani* and within *H. atukorali*, with average distances of 0.39% and 0.23%, respectively. However, the average inter-specific genetic distance value between *H. brittani* and *H. atukorali* was higher, at 3.38% (see Table 1). Notably, the average genetic distance values for *H. brittani* increased further when compared to other closely related species of a different genus: 14.55% with *Rasboroides pallidus* and 15.05% with *Rasboroides vaterifloris*.

The study identified a clear separation between the maximum intra-specific distance values (ranging from 0.39% to 0.79%) and the minimum inter-specific distance value (3.21%) for *H. brittani* and *H. atukorali*. These results clearly indicate the genetic distinction between the two species which shows the existence of a barcoding gap between them, as illustrated in Fig 2.

The average nucleotide frequency was estimated for *H. brittani*, *H. atukorali, R. pallidus*, and *R. vaterifloris* in this study. The results showed that the frequency of adenine (A) ranged from 25.4% to 28.1%, thymine (T) ranged from 33.9% to 35.1%, cytosine (C) ranged from 22.2% to 23.1%, and guanine (G) ranged from 14.6% to 17.1%. The mean GC content was found to be between 36.8% and 39.7%. Furthermore, the study also estimated the GC% values at the first, second, and third base positions of the codons for all the four species. The GC% values for the first, second, and third positions were found to be 50.3% to 53.1%, 42.5% to 42.7%, and 17.4% to 23.1%, respectively which are illustrated in Fig. 3.

In this study, we used MEGA XI software to identify specific nucleotide sequences in the mitochondrial COI gene that differentiate between two closely related species: *H. brittani* and *H. atukorali*. The analysis revealed that *H. brittani* can be distinguished from *H. atukorali* based on differences in the following base positions: 6 (C vs. T), 135 (G vs. A), 174 (T vs. C), 192 (T vs. C), 207 (G vs. A), 234 (A vs. G), 243 (A vs. G), 264 (G vs. A), 277 (C vs. T), 357 (A vs. G), 366 (T vs. A), 369 (C vs. T), 408 (A vs. G), 414 (G vs. A), 424 (C vs. T), and 450 (A vs. G).

In this study, we used the partial mitochondrial COI gene sequences of *H. brittani* generated by us and combined them with sequences from other species including *H. atukorali*, *R. pallidus* and *R. vaterifloris* (retrieved from the NCBI GenBank database) to construct a phylogenetic tree (Fig. 4) with the objective to investigate the evolutionary relationships between these species. The maximum likelihood method with 1000 bootstrap replications was used to reconstruct the phylogenetic tree, which clearly established the relationship between the four different species. The topology of the phylogenetic tree showed that sequences from the same species were grouped under the same nodes, while those from different species were grouped under separate nodes. Moreover, significant bootstrap values supported the major nodes in the phylogenetic tree.

DISCUSSION

Western Ghats region of India is a mountain range running parallel to the western coast and covering six states, including Tamil Nadu, Kerala, Karnataka, Goa, Maharashtra, and Gujarat. On account of the high degree of fish endemism, Western Ghats are considered as the key hot spot for freshwater fish biodiversity. In addition, descriptions of new species are regular in these regions, which call for attention to research on the validation of taxonomic identities (Raghavan *et al*., 2023). According to data published in 2014, the region is home to 379 fish species from 48 families and 143 genera, of which 151 (39.84%) are endemic, 220 (58.05%) are non-endemic, and eight (2.11%) are exotic (Dayal *et al*., 2014). Among them, Cyprinidae alone contributes to 45.12% and remain the major group dominate in terms of overall fish composition in the region (Dayal *et al*., 2014). However, for most species of freshwater fish that have been described, the only information that exist are those on their morphology and type locality. One of such poorly studied fish is *H. brittani* described by Rema Devi & Menon (1992). Despite this species described from south-western India in 1992, later it was treated as a synonym of *H. atukorali* until 2013. Although the synonym of *H. brittani* was resurrected from *H. atukorali*, it was only based on few morphological differences (Batuwita *et al*., 2013), and so far no studies have focused on generating DNA sequences for *H. brittani* or exploring its genetic relationship with *H. atukorali*. Species misidentification can significantly affect biodiversity metrics and lead to inaccurate estimates of aquatic biodiversity. As a result, experts recommend using DNA barcoding techniques to validate the identity of the species (Carreiro *et al*., 2023).

In recent years, molecular taxonomy is gaining considerable attention among researchers, particularly those who work on biodiversity. They believe that molecular taxonomy can accelerate species identification and new species discovery. One of the main molecular markers used to delimit fish species is the mitochondrial COI gene. Researchers generally agree that a threshold genetic divergence value of less than 2-3% within a species and greater than 3% among different species in the mitochondrial COI gene is appropriate for fish species delimitation. In this study, the COI gene was used to determine the genetic divergence for four different species of fish. The results showed that the average genetic divergence values were 0.39% and 0.20 - 0.79% within the species of *H. brittani* and *H. atukorali* (intraspecific genetic distance values), respectively. Additionally, the inter-specific genetic distance values between the species of *H. brittani* and *H. atukorali* were 3.21 - 3.63% (See Table 1). The genetic distance values between Horadandia and Rasboroides were even higher (14.55% to 15.05%), indicating that genetic distance value increases with the taxonomic rank. Similar results were obtained by earlier researchers who also noticed that genetic distance increases from lower taxa towards the higher taxonomic rank in fish, i.e., species > genera > family > order, and they used a threshold genetic distance value of >2-3% to delimit fish species (Kundu *et al*., 2019; Pandey *et al*., 2020; Laskar *et al*., 2022).

Hebert *et al*. (2004) proposed that distinguishing between species based on genetic variation can be achieved by identifying a significant gap between intra and interspecific genetic variation. Subsequently, researchers identified that the effectiveness of using a gene sequence for species identification depends on the degree of separation between the variation within a species and the divergence between different species in the selected marker. This concept is now known as the "DNA barcoding gap" (Meier *et al*., 2008). Several previous studies in fish have demonstrated the existence of barcoding gaps between

species (Bamaniya *et al*., 2016; Bingpeng *et al*., 2018; Tsoupas *et al*., 2022). In this study, we also observed the absence of any overlap between the maximum intraspecific distance values and the minimum interspecific distance values for the two species, *H. brittani* and *H. atukorali* (Fig. 2). This clearly indicates the existence of a barcoding gap between these two species and confirms the effectiveness of the COI gene in delimiting these two species.

A growing body of literature shows that DNA base composition, particularly GC content, can differ between organisms due to variations in selection, mutational bias, and biased recombination-associated DNA repair. In our study, we examined the average nucleotide frequencies of the COI gene in four different species and found that A, T, C, and G content were 26.6%, 34.7%, 22.5%, and 16%, respectively, resulting in an average GC content of 38.6%. These findings are consistent with previous studies that have reported variations in average GC-content in different fish species (Lakra *et al*., 2011; Pavan-Kumar *et al*., 2015; Bamaniya *et al*., 2016). These observations suggest that the variation in base composition can serve as a distinguishing characteristic to differentiate between different fish species.

Research has indicated that the nucleotide composition of mitochondrial genes, particularly the GC content, can impact the codon usage bias (Jenkins & Holmes, 2003). It has also been found that the GC content is non-uniform across the first, second, and third bases of codons, indicating the influence of mutational pressure on these sites (Clare *et al*., 2008). Due to the conservation of the amino acid sequence, substitutions at the third position of codons occur more frequently than at the first and second positions, because substitutions at the first and second positions can alter the amino acid sequence, whereas those at the third position typically do not (Wakeley, 1994). Our study confirms these patterns of nucleotide changes, as we observed that most nucleotide (GC content) changes occurred at the third codon position (See Fig. 3), consistent with previous findings in fish (Lakra *et al*., 2011; Bamaniya *et al*., 2016), as well as sharks and rays (Pavan-Kumar *et al*., 2015). Taken together, our results provide strong evidence that the COI gene can be effectively used to differentiate between species of fish based on these patterns of nucleotide changes.

Researchers often use nucleotide diagnostic methods to support the effectiveness of DNA barcoding in distinguishing fish species. This method involves identifying a specific nucleotide sequence that is unique to a particular species, much like traditional taxonomy based on morphological characteristics. Previous studies have successfully employed nucleotide diagnostic approaches to differentiate closely related sharks (Wong *et al*., 2009), and fishes (Chakraborty *et al*., 2017) using the COI gene. In the present study, we generated nucleotide diagnostic characters specific to *H. brittani* and *H. atukorali* using the COI gene. The unique nucleotide characters generated in this study can be combined to identify these two species more quickly and accurately in future.

According to taxonomists, species delimitation requires more than one line of evidence beyond traditional taxonomic validation. Therefore, taxonomists use phylogenetic validation using genetic data to support the classical taxonomy for species validation. Since the genomic data carries extensive information about the degree of genetic isolation among species and about ancient and recent introgression (transfer

of genetic information from one species to another), researchers use genomic data as a complementary tool to traditional taxonomy for delimitating species. In this study, a phylogenetic tree was constructed using COI gene sequences from *H. brittani*, *H. atukorali*, *R. pallidus*, and *R. vaterifloris* to determine the evolutionary relationship between these four species. The maximum likelihood method was used to reconstruct the phylogenetic tree, which clearly established the relationships among these species. The COI gene sequences of *H. brittani* were clustered under the same node, while those of *H. atukorali* were clustered under separate node. Similarly, *R. pallidus* and *R. vaterifloris* were clustered under different nodes with significant bootstrap values, indicating that these four species are phylogenetically distinct (See Fig. 4). Previously, Batuwita *et al*. (2013) confirmed the taxonomic validity of *H. brittani* and *H. atukorali* based on morphological differences. The results of our study lend support to previous research and confirmed the taxonomic validity of *H. brittani* and *H. atukorali* based on the phylogenetic relationship using the COI gene sequences generated in this study.

CONCLUSION

In conclusion, this study affirms the utility of DNA barcoding as a dependable approach for identifying the fish genus Horadandia and emphasizes the genetic uniqueness of the species *Horadandia brittani*. The study further suggests that the mitochondrial COI gene sequences obtained for *H. brittani* in this study can be used to identify this fish rapidly and accurately in future.

Declarations

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AUTHOR CONTRIBUTION

All authors contributed to the study conception and design. Material preparation and data collection and analysis was performed by N. Daniel. The first draft of the manuscript was written by N. Daniel, and later version of the manuscript was improved by Hemam Nanaobi, J. Praveenraj, V. Balaji, and J. Stephen Sampath Kumar**.** All authors read and approved the final version of the manuscript.

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DATA AVAILABILITY

The DNA sequences generated in this study are available at NCBI GenBank under accession numbers OQ361850 – OQ361853.

COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with this publication.

ETHICAL APPROVAL

This study did not involve human participants. Fish specimens used in this study are not classified as threatened by IUCN Red List or protected under Indian Wildlife Protection Act, 1972. The procedure described does not seek ethical approval because experimental animals were not subjected to any kind of noxious stimulus such as pain or distress or genetically modified as per the guidelines given by Government of India, Ministry of Fisheries, Animal Husbandry and Dairying Department of Animal Husbandry and Dairying, Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA/Annexure 9/2021), and the possible pain and distress were mitigated by the investigators while handling of fish. The procedure to handle live fish were also permitted by Institutional Animal Ethics Committee, Committee for purpose of Control/Supervision of Experiments on Animals, and Animal Dissection Monitoring Committee of Tamil Nadu Dr. J. Jayalalithaa Fisheries University, Tamil Nadu, India (KKPCeSA/TNJFU/12/2022).

INFORMED CONSENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

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table

Table 1. Genetic distances within & between species of *H. brittani* and *H. atukorali*

Comparisons	Minimum Distance	Maximum Distance	Mean Distance
Within *H. brittani*	0.39	0.39	0.39±0.0000
Within *H. atukorali*	0.20	0.79	0.23±0.0008
Between *H. brittani* and *H. atukorali*	3.21	3.63	3.38±0.0012

Figures

Figure 1

Live specimen of H. brittani collected from the natural habitat

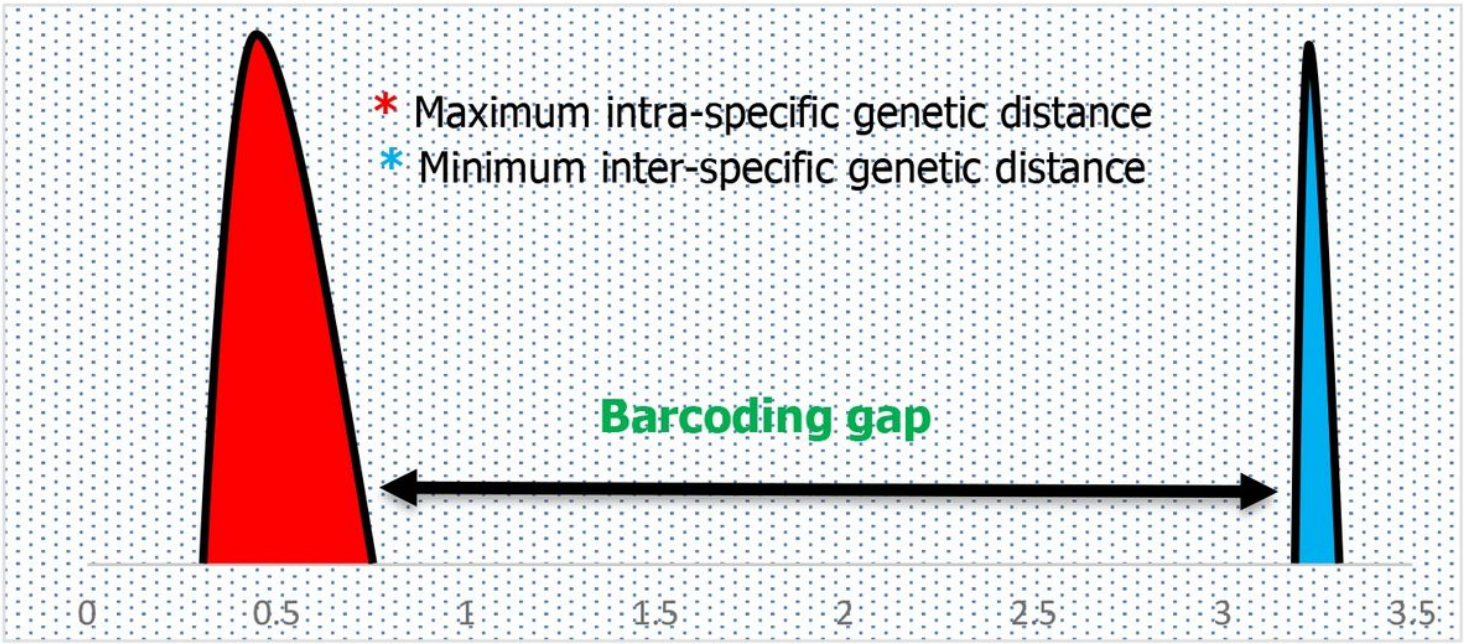

Figure 2

Barcode gap between minimum intra-specific & maximum inter-specific genetic distances for *H. brittani* and *H. atukorali*

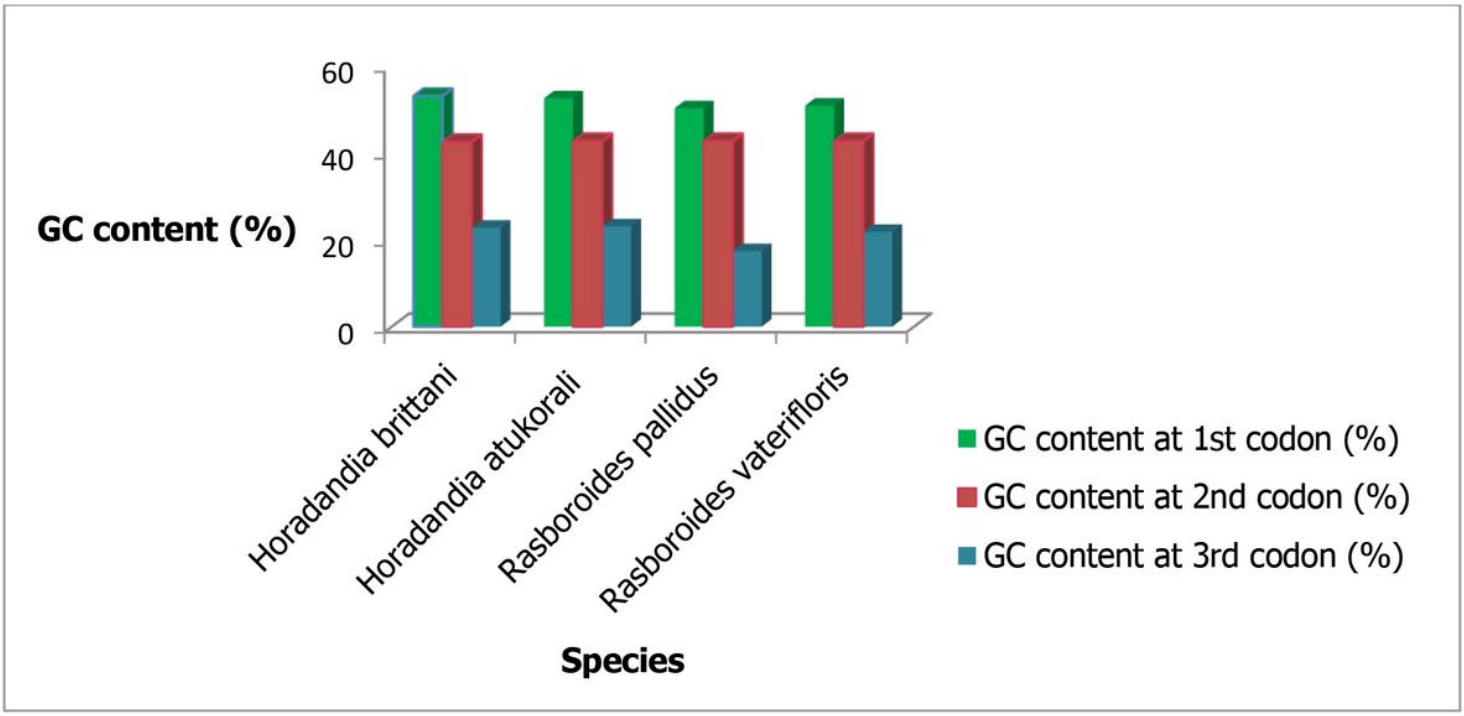

Figure 3

Percentage of GC content at codon 1st, 2nd and 3rd base positions among the species of Horadandia

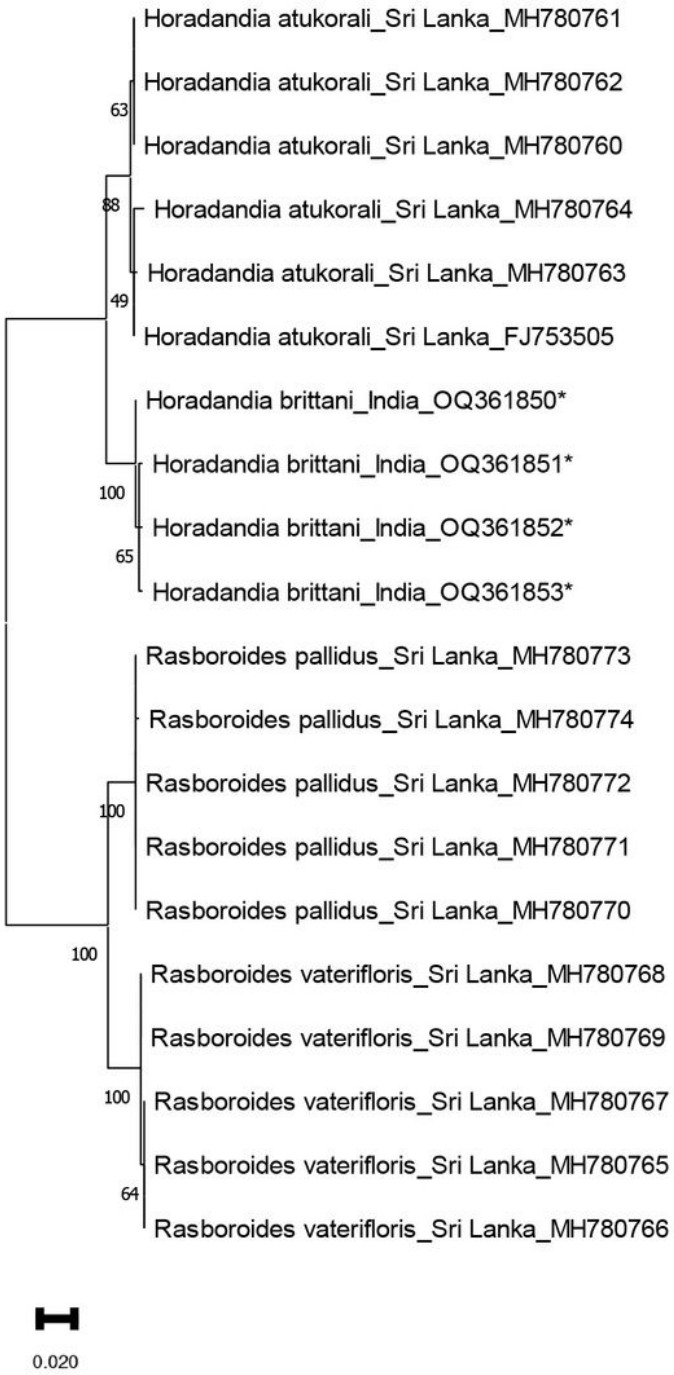

Figure 4

COI-based phylogenetic tree of *Macrognathus sp.* using maximum likelihood method. Node values represent bootstrap values. Asterisks indicate sequences from this study.