



A New Record of the Myristica Swamp Treefrog, *Mercurana myristicapalustris* (Anura: Rhacophoridae), from the Vazhachal Reserve Forest, Kerala, India

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The Western Ghats are a hotspot for biodiversity (Gunawardene et al. 2007). The Asian treefrog or gliding frog family Rhacophoridae comprises 431 species in 20 genera (AmphibiaWeb 2020), of which 43 species have been reported from the state of Kerala, which includes portions of the Western Ghats (Das 2015). The Myristica Swamp Treefrog (*Mercurana myristicapalustris*) is endemic to the Western Ghats (Abraham et al. 2013). This frog exhibits a unique reproductive behavior, which is documented to be a combination of both explosive and prolonged breeding strategies, making it widely adapted to life in the swamps (Abraham et al. 2018). These frogs are known to occur on the periphery of the Kulathupuzha Reserve Forest, which is outside the protected area network, and from the Shendurney Wildlife Sanctuary in Kollam District (8.868°N, 77.094°E), the Peppara Wildlife Sanctuary at Thiruvananthapuram (8.670°N, 77.120°E), Ammayambalam of the Kulathupuzha Reserve Forest (8.841°N, 77.035°E), and the Sasthanada Swamp in the Aripa Forest 3.8 km southeast of Ammayambalam (Abraham et al. 2013, 2018). To date, the known range of the species has been restricted to the lowland Myristica Swamps south of the Shencottah Gap. Herein we report the species from the Vazhachal Reserve Forest north of the Shencottah Gap (Fig. 1).

During a field study conducted at the Vazhachal Forest Division from June 2018 to August 2019, we identified an active breeding site of an unidentified rhacophorid in the Sukthae Pocket Swamp, Vazhachal Reserve Forest, on 8 June 2018 (10.298°N, 76.658°E). We collected an adult male (Fig. 2), which we tentatively identified as *Mercurana myristicapalustris* based on descriptions in Abraham et al. (2013). To confirm the identity of the frog, we removed a toe clip, which we preserved in 100% ethanol and stored at -20 °C. The specimen was vouchered in 70% ethanol.

Total genomic DNA was isolated from the toe clip using a DNeasy Blood and Tissue Kit (Qiagen). Fragments of mitochondrial 16S rRNA were amplified via PCR with the primers 16SF (F-5'-CGCCTGTTTATCAAAAACAT-3') and 16SR (R-5'-CCGGTCTGAAGTCTCAGATCACGT-3') (Palumbi et al. 1991). PCR was performed in 25 µl reactions consisting of 2.5 µl each of 10x PCR buffer, MgCl₂ (25 mM)

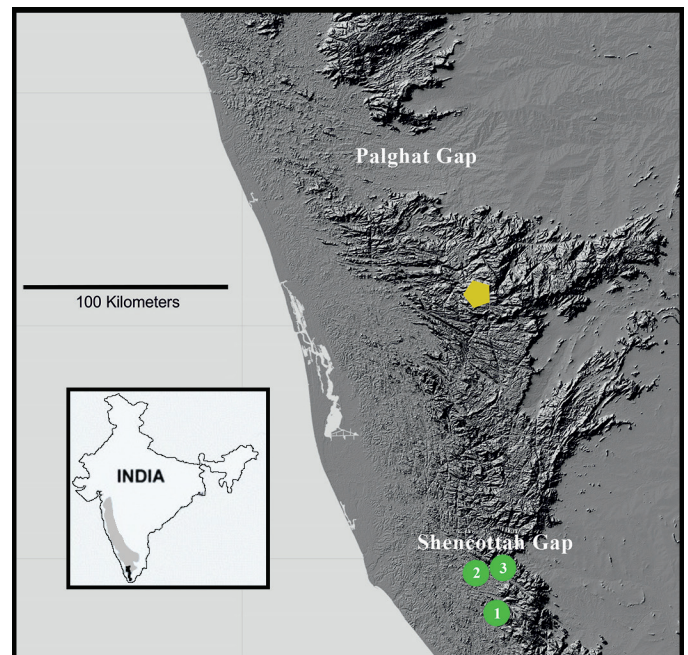


Fig. 1. Map showing known localities for the Myristica Swamp Treefrog (*Mercurana myristicapalustris*) in the southern Western Ghats, Kerala, India. Records previously published by Abraham et al. (2013) are marked by green dots: 1 = Peppara Wildlife Sanctuary, 2 = Kulathupuzha Reserve Forest, 3 = Shendurney Wildlife Sanctuary. The new locality in the Vazhachal Reserve Forest is indicated by the yellow pentagram. The approximate extent of the Western Ghats is shown in the inset map in gray; the smaller black area corresponds to the area shown in the larger map.



Fig. 2. The Myristica Swamp Treefrog (*Mercurana myristicapalustris*) in natural habitat: Male vocalizing from a branch perch (A & B), forest floor in the swamp in the early monsoon season (C), Gowrimara (*Polyalthia fragrans*), the predominant plant species in the swamp (D). Photographs by Sujith V. Gopalan.

and 0.5 μ l dNTPs (2 mM), 0.25 μ l of each primer (10 μ M), 1 μ l of Taq DNA polymerase (Phire Hot start DNA polymerase), 14 μ l of dH₂O and 4 μ l of template DNA (10–20 ng). The following thermocycling conditions were used for amplifications: 95 °C for 5 min, followed by 32 cycles of 95 °C for 30 sec, 58 °C for 40 sec, 72 °C for 90 sec, followed by a final extension step at 72 °C for 5 min. The PCR products were visualized on 1% agarose gels and purified using Exo Sap IT (USB). Sequencing was performed using the PCR primers and products were labeled with BigDye Terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc) and sequenced in an ABI 3730 capillary sequencer following manufacturer's instructions. The new sequence is deposited in GenBank under accession number MT913017.

We used 16S rRNA reference sequences of *M. myristicapalustris* (KC594294.1), *Beddomixalus bijui* (KC594290.1), *Rhacophorus malabaricus* (KU169992.1), *Raorchestes jayarami* (KU169989.1), *Raorchestes ponmudi* (KM596576.1), *Pseudophilautus amboli* (KU169982.1), *Pseudophilautus kani* (EU449994.1), and *Pseudophilautus wynaadensis* (KU169983.1) available in GenBank along with the sequences

generated in the present study for comparison. As outgroups, we used additional sequences of three sister taxa (*Ghatixalus asterops*, *Polypedates pseudocruciger*, and *Polypedates maculatus*) from GenBank (accession nos: KT359626.1, KU169984.1, and EU449995.1, respectively). Sequences were aligned using ClustalW, built-in BIOEDIT (Thompson et al. 1994; Hall 1999). For both genes, the best-fit nucleotide substitution model was selected as GTR + G + I using the model test implemented in MEGA ver. 5 (Tamura et al. 2011). The phylogenetic position of the species was determined using the maximum likelihood method as implemented in MEGA ver. 5 (Tamura et al. 2011). The branch support was evaluated using 1,000 bootstrap replicates (Felsenstein 1985). The pairwise genetic distances between the species were calculated using the Kimura 2-parameter method in MEGA ver. 5 (Tamura et al. 2011).

Morphological measurements of the adult male collected among many actively vocalizing males from an active breeding site at the Sukthae Pocket Swamp agree with those provided by Abraham et al. (2013) for *M. myristicapalustris*. Further, the 16S rRNA sequence generated from the sample

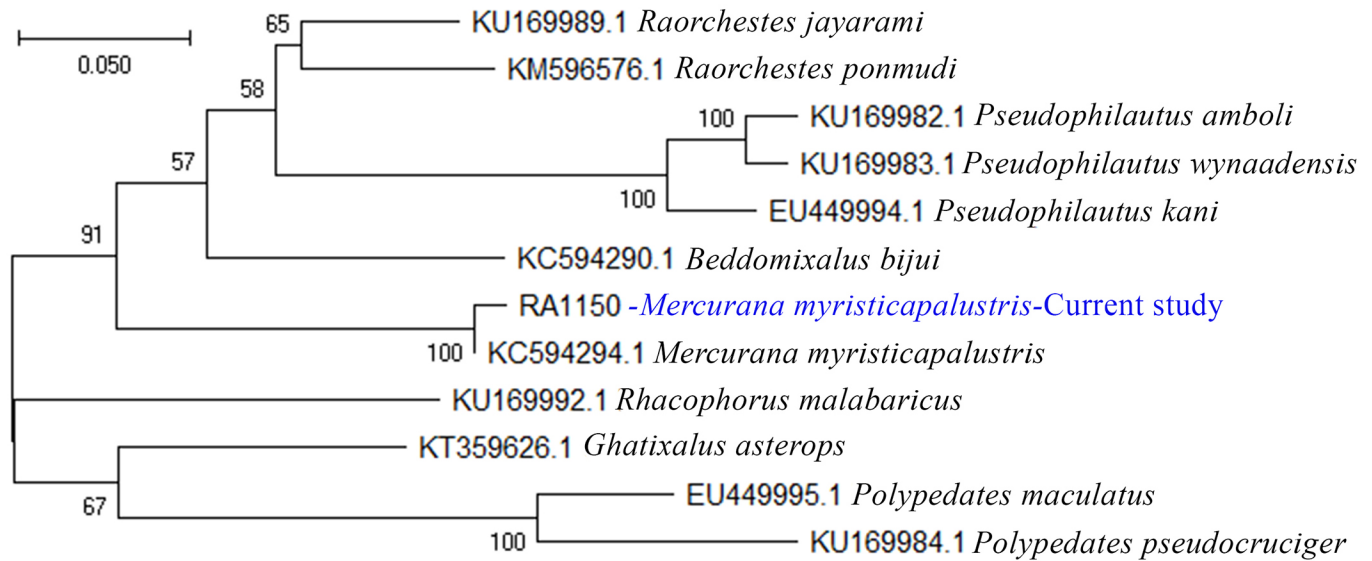


Fig. 3. Phylogenetic tree (ML) showing relationships of the Myristica Swamp Treefrog (*Mercurana myristicapalustris*) and related species constructed based on 16S rRNA sequence. The phylogenetic position of the species was determined using the maximum likelihood method as implemented in MEGA ver. 5 (Tamura et al. 2011).

clustered with the *M. myristicapalustris* voucher sequence (KC594294.1) with a high bootstrap value (Fig. 3). Since the 16S rRNA sequence showed a high degree of homology (>99%; Table 1), we confidently identified the sample as *M. myristicapalustris*. However, unlike populations south of the gap, in which activity was limited to the breeding season of two weeks to a month coinciding with the pre-monsoon in May to early June (Abraham et al. 2013, 2018), in both 2018 and 2019 we found frogs in the Vazhachal Reserve Forest active throughout June and into early July.

The discovery of *M. myristicapalustris* from the Vazhachal Reserve Forest extends the range of the species more than 170 km north of the Shencottah Gap (at 9°N), one of two gaps in the Western Ghats that play major roles in speciation in the region (Robin et al. 2010). The species could well be distributed in swampy pockets throughout the Western Ghats from the Shencottah Gap to Vazhachal and we cannot rule out the possibility that the species occurs in suitable habitat north of the Vazhachal Reserve Forest. Hence this study emphasizes the need for systematic surveys of the unexplored portions

Table 1. Pairwise genetic distances (%) between the Myristica Swamp Treefrog (*Mercurana myristicapalustris*) and related species calculated using the Kimura 2-parameter method in MEGA ver. 5 (Tamura et al. 2011).

	1	2	3	4	5	6	7	8	9	10	11
1 RA1150 (This study)											
2 <i>Mercurana myristicapalustris</i>	0.0102										
3 <i>Beddomixalus bijui</i>	0.1641	0.1636									
4 <i>Pseudophilautus amboli</i>	0.1965	0.2009	0.1697								
5 <i>Pseudophilautus kani</i>	0.1741	0.1645	0.1732	0.0527							
6 <i>Pseudophilautus wynaadensis</i>	0.1880	0.1922	0.1598	0.0252	0.0527						
7 <i>Polypedates maculatus</i>	0.2120	0.1944	0.1939	0.2414	0.2266	0.2286					
8 <i>Polypedates pseudocruciger</i>	0.2403	0.2215	0.2106	0.2480	0.2253	0.2195	0.0931				
9 <i>Rhacophorus malabaricus</i>	0.1909	0.1835	0.1771	0.2291	0.2093	0.2253	0.1941	0.2022			
10 <i>Ghatixalus asterops</i>	0.1865	0.1824	0.1611	0.2298	0.2146	0.2195	0.1647	0.1727	0.1689		
11 <i>Raorchestes jayarami</i>	0.1417	0.1421	0.1122	0.1263	0.1260	0.1227	0.1808	0.2069	0.1420	0.1656	
12 <i>Raorchestes ponmudi</i>	0.1281	0.1340	0.1181	0.1517	0.1419	0.1517	0.1981	0.2328	0.1746	0.1336	0.0773

of the Western Ghats to increase our limited knowledge of amphibians in the region.

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