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Morphological and molecular identification of the freshwater crab, *Barytelphusa cunicularis* from Kanniyakumari, India

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Abstract

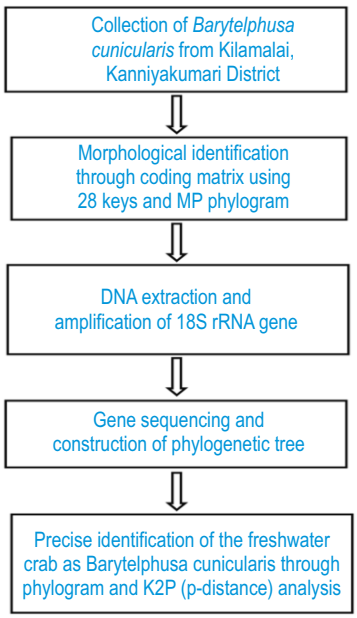
Aim: The present study aims to assess the taxonomical identification of commercially important freshwater crab, *Barytelphusa cunicularis* from Kumba River, Kanniyakumari using morphological and molecular tools.

Methodology: Samples of *B. cunicularis* were collected from Kumba River flowing in Kilamalai, Kanniyakumari district. The species identification was carried out through morphological keys constructed out of a coding matrix and phylogenetic tree (Maximum Parsimony) using Mesquite and PAUP4 software. 18S rRNA sequence was subjected to BLAST analysis, and the phylogenetic tree was constructed through Maximum Likelihood method using MEGA 11 software. Pairwise genetic distance of the species (p-distance,p = nd/n) was also assessed by comparing the K2P values involving phylogenetically close and distant relatives.

Results: Coding matrix prepared using the morphological keys, raised out of 28 distinctive characteristics of *B. cunicularis* and comparison with its phylogenetic relatives, have brought in valuable information on status of the species. These findings were further established by Maximum Likelihood Analysis, using the PCR amplicons of 18S rRNA. The phylogram prepared out of the sequence clearly reveals the candidate species' phylogenetic proximity to other members of the genus *Barytelphusa*. Further, the species' monophyletic status (with a BS value of 81%) suggests its early divergence from its congeners. The K2P pairwise genetic 'p' distance analysis (p = nd/n) of the 18S rRNA has helped us not only to further ascertain the extent of its genetic identity with its congeners, but as well has clearly provided us with the valuable cues per precise identification of the species.

Interpretation: Along with the morphological parameters, the present study, using molecular tools, provides valuable information for precise identification of the commercially important freshwater crab.

Key words: *Barytelphusa cunicularis*, Crustacea, Freshwater crab, Gecarcinucidae, Phylogram, Taxonomy



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Introduction

Freshwater crabs (Brachyura: Decapoda) are consumed in many parts of the world, as they are potential sources of vital nutrients such as vitamins, proteins, carbohydrates, assimilable fats, calcium, antioxidants, and anti-cancer components (Pradhan *et al.,* 2015; Chavan, 2022). Over 1500 freshwater species have been reported among Brachyurans thus far, housing them in five families such as Pseudothelphusidae, Potamonautidae, Potamidae, Gecarcinucidae and Trichodactylidae (Pati and Pradhan, 2020). Out of these, 89 species have been included in the family Gecarcinucidae (Mitra, 2020). The data on the Western Ghats has revealed the occurrence of 62 species distributed in 18 genera, under the family Gecarcinucidae. Among brachyuran crabs, the genus *Barytelphusa* (Gecarcinucidae) has so far been known to encompass three species such as *B. cunicularis, B. guerini* and *B. mccanni;* of late, however, a 4th species, *B. choprai* has been reported from the Eastern Ghats of India (Mandal *et al.,* 2022).

Having been identified as one of the largest freshwater crab varieties, and the only species distributed all over the biogeographic zone of Indian sub-continent (Pati, 2020), *B. cunicularis* has attained considerable importance not only from academic stand points, but also from economic perspectives, as it has been widely used as a source of food and medicine in several parts of India. The body extract of this crab variety has strongly been suggested to be effective in the treatment of cold, fever, headache, hepatitis, backache, joint pains, and sexual impotency like erectile deficiency (Padghane *et al.,* 2016; Rajesh *et al.,* 2017). Being remarkably diverse, the crustacean taxonomy has become an active field with researchers consistently making new discoveries. The number of brachyurans identified thus far, has increased phenomenally during the past years, causing serious concerns among carcinologists in their efforts to systematically accommodate all the newly identified ones (Schubart *et al.,* 2001; Ng and Castro, 2007; Yeo *et al.,* 2008; Teeni *et al.,* 2021). This situation has led to revisiting and re-assessing many of the taxa, mostly at generic and specific levels. From the taxonomic perspectives, ever since the inception of the name *Barytelphusa,* this genus has been either misidentified or erroneously classified under another taxa.

Significantly, Alcock (1910) and Roux (1931) have misidentified a brachyuran crab as *B. cunicularis* which was later correctly identified by Bott (1970) as *Travancoriana schirnerae.* Further, *Paratelphusa* (*Barytelphusa*) *jacquemontii* was originally described under Potamon by Rathbun (1905). Later, this has been placed as a distinct species under the genus *Barytelphusa* (Ng *et al.,* 2008). During these years, there have been several instances where the members of the genus *Barythelphusa* have been subjected to "inclusion-exclusion" changes and synonymizations, contributing to inconsistencies in the phylogenetic status of the genus. In a recent account, Pati and Yeo (2022) have commented on the "confusing" (taxonomic) history of *B. cunicularis* in as much as this species has been differently referred at various times as *Thelphusa cunicularis*, *Paratelphusa* (*Barytelphusa*) *jacquemontii* and *Thelphusa indica*. These ambiguities prevailing in the taxonomic nomenclature of these taxa have prompted us to conduct the present study, which addresses the question of precise and accurate taxonomic identification of this commercially important species (*B. cunicularis*) through an integrated approach using morphological and molecular tools.

Materials and Methods

Adults of *B. cunicularis* were collected by hand-picking and/or trap nets from Kumba river flowing in Kilamalai Reserved Forest area, Kanniyakumari District, Tamil Nadu, India, situated at the Southern tip of the Western Ghats region. Live specimens were brought to the laboratory and maintained in cement cisterns laid with wet sand. The crabs were fed *ad lib* on egg white and tender paddy shoots. Care was taken to maintain the cisterns clean; the left-over food was removed every day. The specimen has deposited in the Zoological Survey of India, Chennai and in the Zoology Museum, Department of Zoology, Holy Cross College, Nagercoil, maintained as the Prototype No. HCZB07.

Morphological analysis: In all, 28 distinct morphological characters (18 multivariate and 10 bivariate) were used to identify the species, followed by the confirmation of its taxonomic status by comparing it with 8 taxa, including out-groups, through a character coding matrix using Mesquite 2.75 (Maddison and Maddison, 2011) and the Maximum Parsimony (MP) analysis using PAUP 4.0 software. The parameters used to prepare the coding matrix were:

(1) Carapace outline; (2) carapace postero-lateral hind margin; (3) median cleft of the frontal margin; (4) post-orbital crest; (5) frontal median triangle; (6) first epibranchial tooth; (7) carapace postero-lateral region; (8) epibranchial crest; (9) epibranchial tooth; (10) ex-orbital angle; (11) rhomboidal gap in 3rd maxilliped; (12) 3rd maxilliped exopod length; (13) lateral margin of epistomial median; (14) cheliped dactylus dorsal margin; (15) cheliped dactylus; (16) pereiopods 2-5 merus; (17) pereiopods 2-5 dactyl spines; (18) sterno abdominal cavity; (19) male abdomen; (20) 6th abdominal segment; (21) suture between male abdominal segment a3-a4; (22) gonopod; (23) articulating joint of Gonopod 1 (G1) terminal article; (24) tip of G1; (25) G1 distal article with stuff of seta; (26) basal part of subterminal segment of G1; (27) gonopod 2 (G2) basal region; (28) G2 apex (Table 1).

Molecular analysis: The genomic DNA was extracted from vas deferens by ethanol precipitation method (after Sambrook and Russell, 2001). The purity and intactness of the extracted DNA were tested through UV-VIS spectrophotometric analysis using Nanodrop spectrophotometer (Thermo Fisher Scientific) and agarose (1%) Gel Electrophoresis. The genomic DNA was PCR-amplified using the published primers (Giribet *et al.,* 1996) (181f TACCTGGTTGATCCTGCCAGTAG and 184r GAATTACCGCGGCTGCTGG) designed to target the18S rRNA

gene sequence. The reaction cycles carried out for the PCR amplification were: the initial denaturation cycle at 96ºC for 2 min followed by 30 cycles of denaturation (96ºC, 30 s), annealing (50ºC, 40 s), and elongation (60ºC, 4 min), followed by a final extension (60ºC, 10 min). PCR amplicons were subjected to Sanger sequencing using a GeneAmp PCR System 9700 (Applied Biosystems) sequencer. The sequences were BLAST searched (Table 2) and CLUSTAL W (Thompson *et al.,* 1994) aligned for precise understanding of the phylogenetic status of the candidate specimen. The Maximum Likelihood (ML) phylogram (Kimura, 1980) and the genetic p-distance were constructed using the software MEGA 11.

Results and Discussion

Body colour, carapace nature, chelate legs and nature of gonopods G1 and G2, were used for the cladistic diagnosis of the candidate specimen.

Characters pertaining to family Gecarcinucidae under superfamily Gecarcinucoidea: The glabrous carapace with transversely sub-ovate shape, widest at the gently convex anterolateral margin and gradually sloped posteriorly; branchial region and postorbital cristae raised; epigastric cristae and postorbital cristae making a concave curve; epibranchial tooth separated from external orbital angle by a small cleft; 6th abdominal somite broader than long; notably broad frontal margin; slightly concave postero-lateral margin, distinct epigastric cristae and a deep cervical groove; a three-segmented first gonopod (G1) and a thin second gonopod (G2) with its proximal and distal parts being separated by a spoon-like protrusion (Klaus *et al.,* 2006).

Affinity to genus *Barytelphusa*: Carapace, broader than long (CW/CL 1.4–1.5), and relatively low (CH/CW 0.4); carapace gently convex anteriorly to flat posteriorly; the external orbital angle broadly triangular with a short outer margin 2-3 times the length of the inner margin (Bahir and Yeo, 2007). Frontal margin narrow (FW/CW 0.2–0.3); postorbital and epigastric cristae strongly developed; groove separating epigastric from postorbital cristae indiscernible; postorbital cristae well developed, reaching epibranchial tooth, but separated by deep cervical groove; epibranchial tooth relatively distinct and blunt, at the level of postorbital cristae, with distinct cleft; cervical grooves deep, relatively long, reaching the level of postorbital cristae; incomplete frontal medial triangle, with only dorsal margin, while the lateral margins were indistinguishable; relatively long antennae, subequal in length to eyestalks. Third maxilliped exopod with well-developed flagellum, equal or exceeding in length to merus. G1 relatively short, with short terminal segment, ~0.6× length of subterminal segment. G2 distinctly shorter than G1, with the distal segment conspicuously short, ~0.2× length of basal segment (Pati and Yeo, 2022). The morphometric cues with respect to the CW:CL and CW:CH ratio given by Pati and Sharma (2014) have also been relevant to distinctly identify the genus.

Characters pertaining to the species cunicularis: The genus *Barytelphusa* is hitherto being represented by only four species such as *Barytelphusa cunicularis, B. guerini, B. jacquemontii* and *B. pulvinata.* In *B. cunicularis,* the carapace generally flat, but slightly convex at its anterior part, with antero-lateral margin well pinched off from the general surface; relatively large and sharp epibranchial tooth lying just above the postorbital cristae, with a small but distinct gap (Bahir and Yeo, 2007). The gonadal characters such as long and slender disposition of G1, with its moderate base with the curved terminal segment, and G2 was slightly pointed outwards (Alcock, 1909, 1910; Mandal *et al.,* 2022) are potential tools for distinct identification of the species cunicularis from its congeners. For the precise assessment of the phylogeny, Parsimony Analysis was also employed in this study. It is a powerful tool for understanding biological evolution through the construction of phylogenetic trees (Stewart, 1993). The coding matrix, prepared using 28 distinct morphological parameters (Table 1), and its comparison with other species in its phylogenetic neighbourhood using the bioinformatics software PAUP4.0, has enabled us not only to define the phylogenetic status of the candidate species (*B. cunicularis*), but it could also bring to light, its phylogenetic proximity with the other members of

Table 1: Coding matrix of *B. cunicularis* based on morphological parameters and in comparison, with other brachyuran species

Taxon	Characters
Barytelphusa cunicularis	2010010010100131101511100204
Barytelphusa guerini	2000010000100111010321110214
Barytelphusa mccanni	2000011022100111000321110214
Cylindrotelphusa steniops	0120102001101031011021110100
Lamella lamellifrons	2101111022200131002100110100
Sundathelphusa orsoni	0010112020101031202101500210
Pulcratis amabilis	3120201011201130211611140120
Hemigrapsus takanoi	1001021111110030102411001O??
Sestrostoma toriumii	1000000011?1003020212015100??

Table 2: Pairwise alignment of 18S rRNA partial gene sequence of *B. cunicularis* (data from NCBI database)

Species 18S rRNA	Year	Accession Number	References
*Barytelphusa cunicularis**	2021	MN933631	-
Barytelphusa cunicularis	2020	MT829233	-
Barytelphusa cunicularis	2006	AY919101	Daniels *et al.* (2006)
Barytelphusa cunicularis	2006	AY919097	Daniels *et al.* (2006)
Barytelphusa sp.	2006	AY919103	Daniels *et al.* (2006)
Barytelphusa sp.	2006	AY919104	Daniels *et al.* (2006)
Menippe nodifrons	2013	HM638014	Lai *et al.* (2013)
Menippe mercenaria	2013	HM638013	Lai *et al.* (2013)

Table 3: The K2P Pairwise genetic distance of 18S rRNA gene sequence of *B. cunicularis* and the following brachyuran species, genetic distance (below diagonal).

	1	2	3	4	5	6	7
Barytelphusa cunicularis MN933631							
Barytelphusa cunicularis MT829233	0.690						
Barytelphusa cunicularis AY919101	0.690	0.000					
Barytelphusa cunicularis AY919097	0.690	0.000	0.000				
Barytelphusa sp. AY919103	0.687	0.016	0.019	0.016			
Barytelphusa sp. AY919104	0.701	0.036	0.032	0.036	0.049		
Menippe nodifrons HM638014	0.732	0.764	0.759	0.764	0.764	0.773	
Menippe mercenaria HM638013	0.732	0.764	0.759	0.764	0.764	0.773	0.252

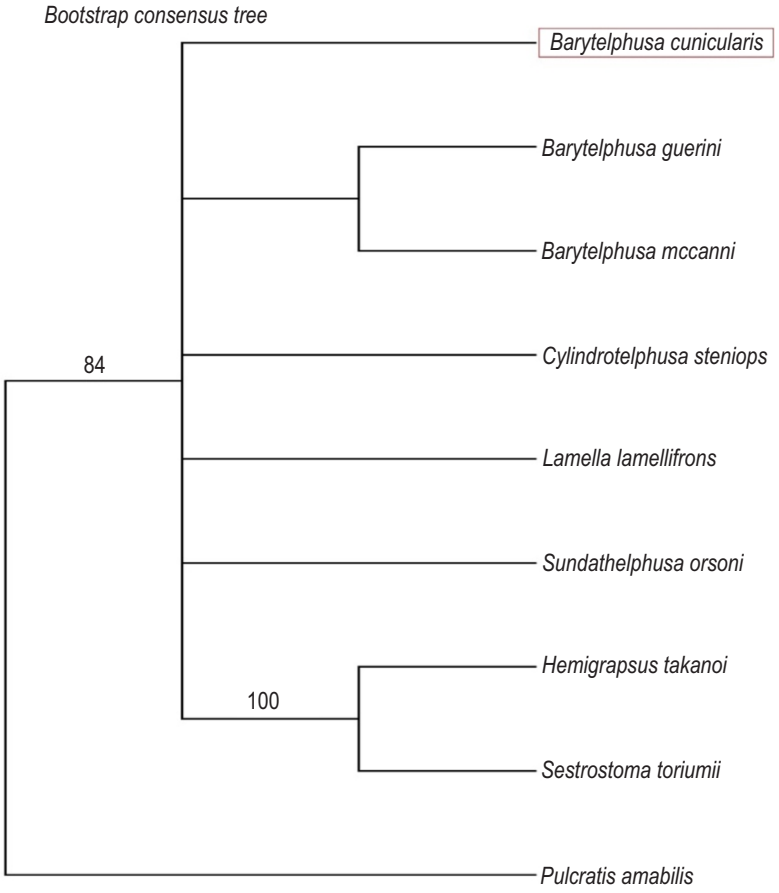

Fig. 1: Maximum Parsimony (MP) tree constructed from the coding matrix of morphological characters. Number indicates the Bootstrap (BS) from a heuristic search of 1000 replicates. The scores: Best tree score= 74; Consistency index (CI) = 0.585; Homoplasy index (HI) = 0.415; Retention index (RI) = 0.370; Rescaled consistency index (RC) = 0.217.

the genus (Fig. 1); the phylogram, also reveals the degree of divergence of the candidate species from distant taxa (shown as out-group). Further, the MP phylogram with 1000 bootstrap replicates, revealed the existence of a monophyletic lineage for *B. cunicularis* (Fig. 1). The character coding matrix of the selected 9 taxa (Table 1), revealed 2 variables as parsimony–uninformative, and 26 variables as parsimony–informative. The parsimony analysis further reveals close phylogenetic proximity existing among the members of the genus *Barytelphusa,* judged from having been positioned in the same cluster. *B. guerini* and *B. mccanni* holds a robust phylogenetic relation, judged from a bootstrap (BS) value 84% (Fig. 1). Pertinently, previous

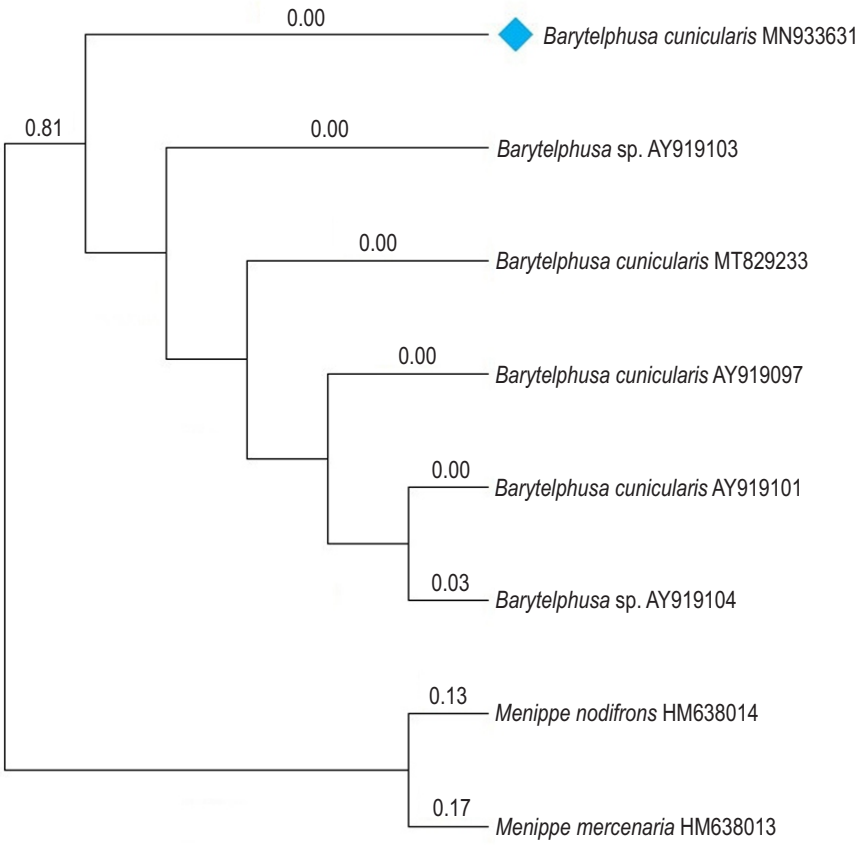

Fig. 2: Maximum Likelihood (ML) phylogeny of *B. cunicularis* based on 18S rRNA gene displaying the distinct clade of *B.cunicularis;* members of Menippidae are used as the out-group.

phylogenetic studies (Hillis and Bull, 1993; Soltis and Soltis, 2003) suggest that a BS value >70% would correspond to a probability of >95%, reinforcing that the extent of phylogenetic relation as shown in the phylogram, is highly significant. Accordingly, *B. cunicularis,* the candidate specimen of the present study, in spite of belonging to the same clade, seems to have diverged from the ancestral stock much earlier than that of its congeners, *B. guerini* and *B. mccanni.* This result is reminiscent of a recent report from Mandal *et al.* (2022), wherein *B. cunicularis* is shown to have a distinct cladistic path, well separated from its congeners (3 strains of *B. guerini* and 5 strains of *B. choprai*).

Maximum Likelihood analysis using 18S rRNA reveals the candidate species having been clustered with other members of *Barytelphusa* (Fig. 2), signifying its phylogenetic proximity to the genus. Further, the species 'monophyletic status (with a BS value of 81%) suggests its early divergence from the cladistic stock of Barytelphusids, as also evinced from the coding matrix analysis (Fig. 1). This observation not only reiterates the complementarity of the present Maximum Likelihood analysis, but also asserts the species' distinctiveness in the clade. With a view to obtain accurate estimates of genetic differences with closely allied species and the distant ones, the present study had employed the Kimura-two-parameter (K2P) model (Kimura, 1980). The results of comparison of the nucleotide sequences from the conserved regions (18S rRNA) of the candidate species (*B. cunicularis* NCBI, MN933631), with those of seven other brachyurans (both from within the family Gecarcinucidae and from outside) are represented in Table 3. Significantly, the p-distance of species within the genus *Barytelphusa* exhibited closeness with those of the out-groups from family Menippidae (*Menippe nodifrons,* HM638014 and *Menippe mercenaria*, HM638013), in consonance with the results of Maximum Likelihood analysis (Fig. 2).

The Kimura two-parameter (K2P) model is one of the most profusely used models for nucleotide substitution with a view to estimate genetic differences (called genetic distances, p-distance) and phylogenetic relationships. The robustness of p-distance as a potential tool to estimate the genetic distance between various genera and within the same genus, and to precisely assess the phylogenetic status of a species in question, has been successfully proven by its use in animals from various taxa, including decapod crustaceans. K2P model essentially works by considering the gaps (insertions and/or deletions), and

introduce a new measure for estimating genetic difference between two nucleotide sequences in terms of nucleotide changes that have occurred during the evolutionary process. Nishimaki and Sato (2019) in their studies using the nuclear ribosomal DNA ITS2 region from the genus *Physalis* (Family Solanaceae) demonstrated the role of K2P in species identification. More recently, a new species of freshwater crab of the genus *Nanhaipotamon* Bott, 1968 was described from Chengxiang Town, Fujian Province, China. Molecular evidence derived from the pair-wise distance based on K2P model using COI gene sequences, has been instrumental in identifying the new species (Cai *et al.,* 2021). Further, two new species of freshwater crabs inhabiting the high altitudes of Guangdong Province, China were precisely identified using the pair-wise estimates of K2P distances using MEGA software (Huang *et al.,* 2021). More recent examples are available from the results of investigations that depict K2P analysis as an ideal tool, not only for precise identification of species, but also for clearer understanding of genetic distance both within the taxa and between them (Tan *et al.,* 2021; Awas *et al.,* 2023). Taking cues from the aforementioned studies, we are encouraged to suggest that the use of p-distance is an enabling technique as much as a zero p-value will ensure 100% precision in identification of not only the species, but the strain as well.

Significantly, the results of the present study, through morphological and molecular evidences in an integrative fashion, would help us to assess the exact phylogenetic status of the species, and it as well affords quantitative (p-distance) cues for precise species/strain-level identification of *Barytelphusa cunicularis*.

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