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ANIMAL SCIENCE

From rivers to museums: 28S rDNA as a tool for molecular identification of Amazon palaemonids prawns

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Abstract: Morphological identification of Palaemonidae prawns often proves challenging due to the overlap in their characteristics. In this study, we evaluated the utility of 28S ribosomal DNA (rDNA) sequences as a molecular tool for accurately identifying prawns within this family. Specimens were sourced from scientific collections in the Brazilian Amazon, and sequences obtained from these samples were prioritized for constructing a reference sequence database. The efficacy of the 28S rDNA was then assessed by analyzing sequences within the reference database and applying it to identify samples from the Amazonas and Tapajós rivers. Our species delimitation analyses provided accurate molecular identification, revealing the potential of 28S rDNA sequences in addressing taxonomic inconsistencies. Notably, the results uncovered misclassifications among some specimens deposited in scientific collections, emphasizing the importance of molecular tools in refining Palaemonidae taxonomy in the Amazon.

Key words: Museum Collections, Ribosomal Markers, Species Identification, Tropical Crustaceans.

INTRODUCTION

The family Palaemonidae Rafinesque, 1815 comprises prawns widespread throughout tropical and temperate regions of all continents (Magalhães et al. 2016, De Grave et al. 2015), totaling about 1100 valid species distributed into 227 genera (DecaNet 2025). In the Brazilian Amazon, the diversity of Palaemonids has been well documented ever since the first taxonomic surveys in the 50's (Holthuis 1951, 1952). In particular, the genera *Macrobrachium* (Bate 1868), *Palaemon* (Weber 1795), and *Pseudopalaemon* (Sollaud 1911) stand out because of their higher number of species, respectively (Kensley & Walker 1982, Barros & Pimentel 2001, Iketani et al. 2011, Maciel et al. 2011, Pileggi et al. 2013, Pimentel & Magalhães 2014, Dos Santos et al. 2018, Carvalho et al. 2014, 2020).

Furthermore, phenotype plasticity is a common trait reported in *Macrobrachium* and *Palaemon* because both genera include species that occupy habitats of distinct environmental conditions (Taddei et al. 2017). This feature accounts for their intraspecific variability, besides favoring the genetic divergence among populations as described in *M. amazonicum* (Heller 1862) (Vergamini et al. 2011), *M. jelskii* (Miers 1878) (Vera-Silva et al. 2016), and some species of *Palaemon* (Carvalho et al. 2017, 2020). On the other hand, *Pseudopalaemon* is an endemic genus from freshwater habitats in South America, being closely associated with aquatic

systems (clear, white, and black waters) from the Amazon region and occupying microhabitats such as flooded areas and aquatic macrophytes (Magalhães 1986, Pimentel & Magalhães 2014). However, recent multigene phylogenetic analyses revealed that *Pseudopalaemon* is now formally considered a junior synonym of *Macrobrachium* (Mota et al. 2025a).

Actually, the literature about the morphological identification and taxonomy of Palaemonidae is rich and updated (Pileggi & Mantelatto 2012, Vera-Silva et al. 2017, Carvalho et al. 2014, 2017, 2020, Dos Santos et al. 2018, Mota et al. 2025a, and many others). Nonetheless, the identification of species based only on morphology is often complex because of their highly conserved interspecific traits along with a remarkable intraspecific variation according to differences in habitat, behavior, and reproductive stages (Vera-Silva et al. 2017).

In addition, a precise identification of specimens deposited in scientific collections represents one of the first and most important steps in biological studies since this material is used to further comparative analyses of organisms. All material and the information available in scientific collections compose a valuable reference dataset, often evaluated by experts. However, as highlighted by Fearing et al. (2025), the associated collection metadata can contain inaccuracies or missing information, underscoring the need for robust methods to assess their confidence and reliability rather than inherently assuring accuracy. In order to complement morphological studies, molecular analyses have proved to be essential in resolving species delimitation conflicts and phylogenetic relationships. Accordingly, the sequencing of a mitochondrial DNA portion from the cytochrome C oxidase subunit I (COI) gene became popular as a tool for species identification after the DNA barcode initiative (Hebert et al. 2003).

While extensive molecular work using traditional markers like COI has significantly advanced the understanding of *Macrobrachium* species (Liu et al. 2007, Pileggi & Mantelatto 2010, Matzen da Silva et al. 2011, Vergamini et al. 2011, Pileggi et al. 2014, Vera-Silva et al. 2016, Mantelatto et al. 2022, and numerous others), molecular characterization for *Palaemon* (Carvalho et al. 2014, Jordán-Hernández et al. 2019, Carvalho et al. 2020, Fanning et al. 2024) and especially for *Pseudopalaemon* has been comparatively limited. Studies involving *Pseudopalaemon* often present DNA sequences as secondary findings within broader research on *Macrobrachium* or *Palaemon* (Botello & Álvarez 2013, Carvalho et al. 2017), and only recently have there more comprehensive molecular analyses (Mota et al. 2025a). This disparity creates a significant knowledge gap, particularly in establishing robust molecular identification tools and clarifying taxonomic uncertainties concerning *Palaemon* and the *Macrobrachium* group, especially given the recent reevaluation of *Pseudopalaemon*.

Given these challenges and the potential limitations of COI in some crustacean groups, such as the overestimation of species due to heteroplasmy or pseudogenes (Lopez et al. 1994, Sorenson & Quinn 1998, Bensasson et al. 2001), with pseudogenes specifically reported in Palaemonidae species like *M. ferreirai* (Robe et al. 2012) and *M. amazonicum* (Iketani et al. 2021). Therefore, the analyses of nuclear markers can be particularly useful to support the results based on mitochondrial DNA (mtDNA) markers in phylogenetic or species delimitation studies (Sonnenberg et al. 2007).

In the case of the Palaemonid prawn, the 28S ribosomal RNA gene from the nuclear genome (28S rDNA) represents a potential marker to infer phylogenetic relationships and to delimitate species. For instance, Chen et al.

(2009) compared the tree topologies obtained from 16S (mtDNA) and 28S rRNA sequences in species of *Macrobrachium* from Taiwan, revealing a higher confidence in the latter than that based on 16S sequences. Wowor et al. (2009), using a multilocus approach (16S and COI mitochondrial genes and 18S, 28S, and Histone H3 nuclear genes) in *Macrobrachium* representatives from Asia, were able to build gene trees to determine their phylogenetic interrelationships and correlate the origin of species according to their habitats and modes of life. In addition, studies based on 28S rDNA and 16S mtDNA carried out by Castelin et al. (2017) in *Macrobrachium* prawns from the Indo-Pacific revealed that specimens of *M. australe* (Guérin-Méneville 1838) encompassed two taxa, including a cryptic form previously identified as *M. ustulatum* (Nobili 1899), but regarded as a junior synonym of *M. australe*.

Considering that body variation and the scarcity of distinctive morphological traits represent a challenge for the proper identification of Palaemonid prawns by curators, the inclusion of additional molecular markers represents a potential tool to distinguish species in this group. The main aim of this study was to establish a reference database for DNA barcode identification and subsequently assess its effectiveness by testing it with samples of unknown origin collected from the Amazon and Tapajós rivers in Santarém, Pará, Brazil.

MATERIALS AND METHODS

Sampling

The collection of specimens was carried out in the confluence of the Amazonas and Tapajós rivers close to the municipality of Santarém, in the western portion of the state of Pará in northern Brazil (Figure 1). Furthermore, we also analyzed stored samples of *Macrobrachium* (33 specimens from eight species) and *Palaemon* (seven specimens from two species) donated by scientific collections from distinct institutions in northern Brazil, as follows: Institute of Scientific and Technological Researches from the State of Amapá (IEPA), National Institute of Researches in Amazon (INPA) and Emílio Goeldi Museum (MPEG) (Table I). Finally, samples of *M. amazonicum*, *M. acanthurus* (Wiegmann 1836), and *M. carcinus* (Linnée 1758) available in the tissue collection from the Laboratory of Education and Evolution Professor Horacio Schneider (LEDEVO) at Federal University of Western Pará (UFOPA) were also included.

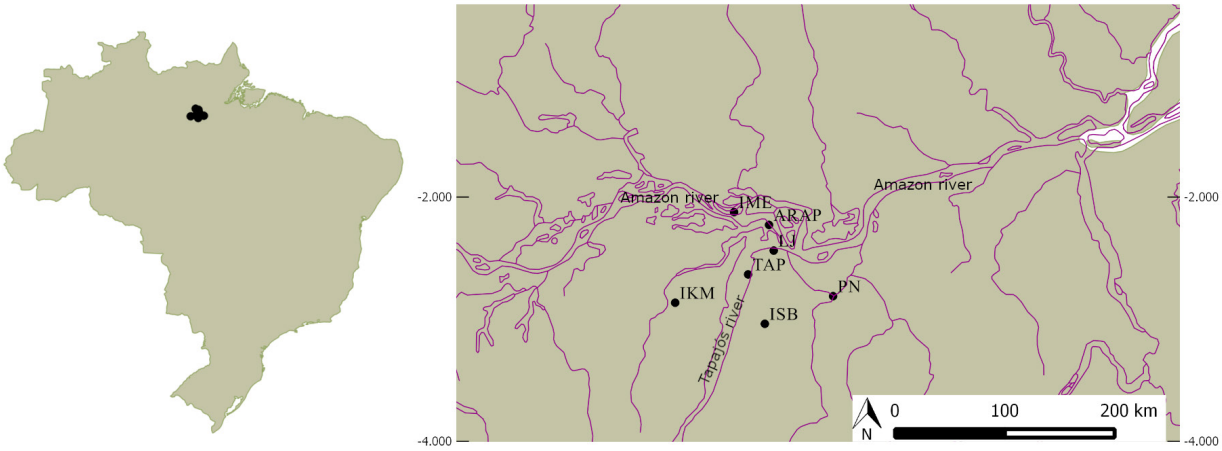

Figure 1. Collection sites of new specimens of shrimp of the family Palaemonidae used in the present study.

Table I. List of Palaemonidae shrimp species used in the present study. Laboratory collection: LEDEVO - Professor Horacio Schneider Education and Evolution Laboratory. Scientific Collections: IEPA- Amapá Institute of Scientific and Technological Research; INPA - National Institute of Researches in Amazon; MPEG - Emílio Goeldi Museum. The acronyms refer to the names of the institutions in Portuguese.

Morphological identification	Source	Specimen ID	Collection Voucher	Collection Date	Locality	GenBank accession numbers
Macrobrachium acanthurus (Wiegmann 1836)	LEDEVO	Mac01	-	sept/2015	Ilhéus – Bahia	OQ023198
Macrobrachium acanthurus	LEDEVO	Mac10	-	sept/2015	Ilhéus – Bahia	OQ023199
Macrobrachium acanthurus	LEDEVO	Mac32	-	may/2012	Bragança – Pará	OQ023200
Macrobrachium acanthurus	LEDEVO	Mac35	-	may/2012	Bragança – Pará	OQ023201
Macrobrachium amazonicum (Heller 1862)	LEDEVO	02-51	-	may/2013	Juruti - Pará	OQ023202
Macrobrachium amazonicum	LEDEVO	GO01	-	mar/2011	Bela vista – Goiás	OQ023203
Macrobrachium amazonicum	LEDEVO	MS01	-	mar/2011	Mato Grosso do Sul	OQ023204
Macrobrachium brasiliense (Heller 1862)	IEPA	84	537	08/24/2004	Braço Creek	OQ023205
Macrobrachium brasiliense	IEPA	85	537	08/24/2004	Braço Creek	OQ023206
Macrobrachium brasiliense	IEPA	86	537	08/24/2004	Braço Creek	OQ023207
Macrobrachium carcinus (Linnée 1758)	LEDEVO	McaAf15	-	may/2012	Afuá – Pará	OQ023208
Macrobrachium ferreirai (Kensley & Walker 1982)	INPA	51	189	07/17/1985	Uatumã River, Balbina Waterfall	OQ023209
Macrobrachium iheringi (Ortmann 1897)	IEPA	107	716	01/19/2005	Mapaoni River - Tumucumaque II	OQ023210
Macrobrachium iheringi	IEPA	109	716	01/19/2005	Mapaoni River - Tumucumaque II	OQ023211
Macrobrachium iheringi	IEPA	110	716	01/19/2005	Mapaoni River - Tumucumaque II	OQ023212
Macrobrachium iheringi	IEPA	111	716	01/19/2005	Mapaoni River - Tumucumaque II	OQ023213
Macrobrachium inpa (Kensley & Walker 1982)	INPA	61	663	05/17/1994	Ducke Reserve	OQ023214
Macrobrachium inpa	INPA	62	663	05/17/1994	Ducke Reserve	OQ023215
Macrobrachium jelskii (Miers 1877)	MPEG	16	731	11/10/1999	Flona de Caxiuanã, Melgaço, Pará	OQ023216
Macrobrachium jelskii	MPEG	18	731	11/10/1999	Flona de Caxiuanã, Melgaço, Pará	OQ023217
*Macrobrachium jelskii**	IEPA	124	619	07/23/2004	Índios Lagoon	OQ023218
*Macrobrachium nattereri**	IEPA	128	612	11/15/2004	Iratapuru River Sustainable Development Reserve	OQ023219
Macrobrachium nattereri (Heller 1862)	IEPA	129	612	11/15/2004	Iratapuru River Sustainable Development Reserve	OQ023220
Macrobrachium nattereri	IEPA	137	893	07/30/2005	Cupixi River	OQ023221
Macrobrachium nattereri	IEPA	138	893	07/30/2005	Cupixi River	OQ023222
Macrobrachium nattereri	IEPA	139	893	07/30/2005	Cupixi River	OQ023223
Macrobrachium nattereri	IEPA	140	893	07/30/2005	Cupixi River	OQ023224
Macrobrachium nattereri	IEPA	141	893	07/30/2005	Cupixi River	OQ023225
Macrobrachium surinamicum (Holthuis 1948)	MPEG	26	520	10/18/1997	Lomeiro do Ajiru, Marajó-Pará	OQ023226
Macrobrachium surinamicum	INPA	57	284	mar/85	Tocantins River, in Cametá - Pará	OQ023227

Table I. Continuation.

*Macrobrachium surinamicum**	IEPA	80	595	April/ May/2004	TABACO - IBAMA Headquarters	OQ023228
Macrobrachium surinamicum	IEPA	92	185	06/20/1999	Maniva River	OQ023229
Macrobrachium surinamicum	IEPA	95	185	06/20/1999	Maniva River	OQ023230
Macrobrachium chryseus (Kensley & Walker 1982)	MPEG	01	749	04/14/2002	Monte Dourado, Reserva Genética Felipe, Pará	OQ023238#
*Macrobrachium chryseus**	MPEG	03	749	04/14/2002	Monte Dourado, Reserva Genética Felipe, Pará	OQ023239#
*Macrobrachium chryseus**	MPEG	42	765	03/18/2001	Flona de Caxiuanã, Melgaço, Pará	OQ023240#
*Macrobrachium chryseus**	MPEG	43	765	03/18/2001	Flona de Caxiuanã, Melgaço, Pará	OQ023241#
Macrobrachium sp. 2*	IEPA	98	856	03/14/2005	Santo Antônio River Creek - Tributary of the Araguari River	OQ023242
Macrobrachium sp.1*	IEPA	131	869	05/04/2005	Baliza Creek - Campsite	OQ023243
Macrobrachium sp.1*	IEPA	132	869	05/04/2005	Baliza Creek - Campsite	OQ023244
Palaemon carteri (Gordon 1935)	MPEG	06	644	12/22/1999	Mocambo, Belém, Pará	OQ023231
Palaemon carteri	MPEG	07	644	12/22/1999	Mocambo, Belém, Pará	OQ023232
Palaemon carteri	MPEG	08	644	12/22/1999	Mocambo, Belém, Pará	OQ023233
Palaemon carteri	MPEG	09	644	12/22/1999	Mocambo, Belém, Pará	OQ023234
Palaemon carteri	MPEG	10	644	12/22/1999	Mocambo, Belém, Pará	OQ023235
Palaemon pandaliformis (Stimpson 1871)	MPEG	12	196	04/25/1997	Furo do Taici, Bragança, Pará	OQ023236
Palaemon pandaliformis (Stimpson 1871)	MPEG	13	196	04/25/1997	Furo do Taici, Bragança, Pará	OQ023237
Euryrhynchus sp.	LEDEVO	Eury01	-	2017	Santarém - Pará	OQ023245

***Different molecular identification.**
#This sequence is still shown in Genbank as *Pseudopalemon.*

Molecular data

The total DNA was isolated using the extraction protocol based on ammonium acetate with slight modifications (Bruford et al. 1992). When isolation of DNA samples was not successful by this method, we used the QIAamp® DNA Micro kit (Qiagen) according to the manufacturer's instructions. The target DNA sequence was amplified via PCR (Polymerase Chain Reaction) using 4.0 µL of dNTPs (1.25 mM), 2.5 µL of $MgCl_2$, 2.0 µL of buffer solution (10x), 0.25 µL of each primer (10 µM), 0.1 µL of Taq polymerase (5 U/ µL), 2.0 µL of template DNA, and ultrapure water to a final volume of 25 µL.

For the amplification of 28S rDNA sequences, we used the primers 28S A (5'-GACCCGTCTTGAAGCACG-3') and 28S B (5'-TCGGAAGGAACCAGCTAC-3') (Whiting et al. 1997). Each PCR encompassed a first denaturation step at 94°C for 3 min, followed by 35 cycles (30 s of denaturation at 94°C, 30 s of annealing at 51°C, and extension at 72°C for 1 min) plus a final extension step at 72°C for 5 min. After DNA isolation and amplification via PCR, the samples were visualized after electrophoresis in 1% agarose gel stained with GelRed (Biotium). The amplified fragments were purified and sequenced in an ABI 3500 (Applied Biosystems) automatic sequencer using a Big Dye 3.1 kit (Applied Biosystems) according to the manufacturer's instructions.

Data analysis

The sequences were visualized, edited (when necessary), and automatically aligned using the software CodonCode Aligner v. 8.0.2 (CodonCode Corporation) and organized into two datasets: (1) specimens from scientific collections (Table I) and samples of *M. amazonicum*, *M. acanthurus*, and *M. carcinus* from the tissue collection at LEDEVO and (2) recently collected samples (Figure 1) and single representatives from the previous tissue bank. The decision to separate the sequences into distinct datasets was taken to evaluate the reliability of identification in the samples available in scientific collections and to double-check the efficiency of 28S rRNA sequences for species identification. Duplicate sequences from both datasets were removed using the online tool ElimDupes (available at: https://www.hiv.lanl.gov/content/sequence/elimdupesv2/elimdupes.html). In addition, hypervariable regions and non-aligned gaps were excluded using the online software GBLOCKS v. 0.91b (Castresana 2000, Talavera & Castresana 2007).

The dataset from scientific collections (bank 1) was used to build a Neighbor-Joining (NJ) phylogenetic tree, while the significance of clusters was estimated using a bootstrap test based on 1000 pseudoreplicates (Felsenstein 1985). The evolutionary distances were calculated based on the Kimura-2-parameter (K2P) method (Kimura 1980). All analyses were performed in the software MEGA X (Kumar et al. 2018). A DNA sample from the genus *Euryrhynchus* was included as an outgroup due to its consistently basal phylogenetic position relative to the remaining Palaemonidae, as indicated by De Grave et al. (2015).

To identify the species in the recently collected samples, we started by selecting a single representative from each species according to the analyses from bank 1 and unique samples from creeks as estimated using ElimDupes. Three methods of species delimitation were subsequently carried out: Barcode gap, GMYC – General Mixed Yule Coalescent (Pons et al. 2006), and PTP – Poisson Tree Processes (Zhang et al. 2013). The barcode gap analysis was performed using the software ABGD - Automatic Barcode Gap Discovery (Puillandre et al. 2012) according to the following parameters: Pmin 1/4 0.001 and Pmax 1/4 0.1; steps 1/4 10; NBins 1/4 20; Relative gap 1/4 1 and Jukes-Cantor (JC69) evolutionary model.

The trees used as input in the GMYC approach were obtained in the software Beast v.2.3.1 (Bouckaert et al. 2014), including 10 million steps sampled at every 1,000 generations, a burn-in of 10% under three distinct priors (Yule, Constant coalescent, and Relaxed clock). The software TRACER v. 1.6 was used to evaluate the convergence of trees, assuming values of ESS (effective sample size) above 200. The GMYC was run in the package Splits (Species Limits by Threshold Statistics) (Ezard et al. 2009), available in the software R. At last, the PTP – Poisson Tree Processes (Zhang et al. 2013) was carried out using as input a Maximum Likelihood (ML) phylogenetic tree obtained in RaxML, provided by the web server CIPRES Science Gateway v. 3.3 (Miller et al. 2010), with bootstrap values based on 100 pseudoreplicates.

RESULTS

Molecular analysis of specimens from scientific collections

The dataset comprised 48 sequences of 383 base pairs (bp) from 14 species. The phylogenetic tree revealed well-defined clades for the genus *Palaemon*. For the genus *Macrobrachium*, while the overall genus-level clade showed moderate support (57%, Figure 2), most species-defining derived clades exhibited strong support,

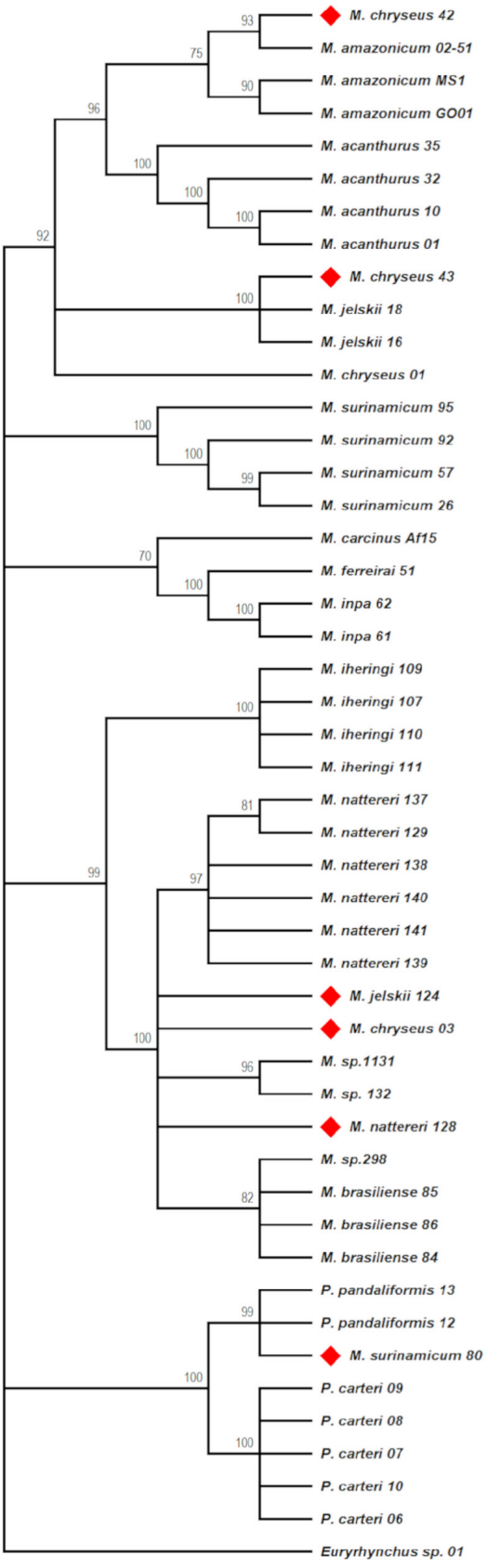

Figure 2. Neighbor-Joining tree based on 28S rDNA sequences using Kimura-2-parameter (K2P) for species of *Macrobrachium* and *Palaemon* deposited in the scientific collections of IEPA (Amapá Institute of Scientific and Technological Research), INPA (National Institute of Researches in Amazon), and MPEG (Emílio Goeldi Museum). The acronyms refer to the names of the institutions in Portuguese. The numbers on branches indicate the bootstrap values (only values above 70 are shown). Red diamonds highlight putative cases of incorrect morphological identification.

except for *M. nattereri* and *M. brasiliense*. The intergeneric genetic divergence was higher between *Palaemon* and *Macrobrachium* (15,98 to 24,38%).

The *Macrobrachium* samples with intraspecific variation were represented by *M. brasiliense* and *M. amazonicum*. For *M. brasiliense*, genetic divergence was 2.54%, while *M. amazonicum* showed 0.9% variation considering only samples from the Amazon basin. When samples collected in the states of Goiás and Mato Grosso do Sul (Central Brazil) were included, the intraspecific divergence in *M. amazonicum* reached 3.3%. The lowest value of interspecific variation was observed between *M. nattereri* and *M. brasiliense* (1.61%), followed by *M. ferreirai* x *M. inpa* (3.01%). The highest genetic differentiation was detected between *M. carcinus* and *M. iheringi* (20.11%). The estimated interspecific differentiation between *P. carteri* and *P. pandaliformis* was equal to 6.33%. The genetic divergence between *M. chryseus 01* (identification confirmed by 16S rDNA sequencing; BLAST 98.84% similarity to sequence from Carvalho et al. 2017) and the other species of *Macrobrachium* ranged from 7.93% (*M. amazonicum*) to 18.31% (*M. surinamicum*).

The specimens from scientific collections lacking species-level identification - *Macrobrachium* sp. 2-98 (voucher 856 – IEPA), M. sp. 1-131, and *Macrobrachium* sp.1-132 (voucher 869 – IEPA) were genetically assigned to *M.*

brasiliense, with distance values ranging from 0.0 to 1.39%. Furthermore, the tree topology revealed six nominal species that diverged from their expected clusters, indicating putative cases of morphological misidentification (Table II). In fact, the sample referred to as *M. chryseus* 42 (voucher 765 – MPEG) grouped along with *M. amazonicum* with a genetic distance value of 0.94%. On the other hand, *M. chryseus* 43 (voucher 765 – MPEG) proved to be closely related to *M. jelskii* with 0.46% of genetic divergence. In addition, M. jelskii 124 (voucher 619 – IEPA), M. nattereri 128 (voucher 612 – IEPA), and *M. chryseus* 03 (voucher 749 – MPEG) were recovered within the cluster composed of *M. brasiliense*, with values of genetic distance between 0.0 and 1.61%. Similarly, the sample identified as *M. surinamicum* 80 (voucher 595 – IEPA) was genetically identical to *P. pandaliformis* (Table II). The estimated genetic divergence for all species can be viewed in the supplementary files.

Molecular analysis of samples collected in creeks and species delimitation

A total of 154 samples collected from creeks were sequenced in the present study, resulting in 27 haplotypes (Supplementary Material - Table SI). This dataset, along with the 15 sequences of specimens from scientific collections with congruent molecular and morphological identification (bank 1), composed a final dataset formed by 42 sequences of up to 444 bp.

According to the distance-based ABGD species delimitation method, 21 groups (putative species) were identified using the initial threshold. After recursive partitions, the number of groups ranged from 20 to 21 potential species. The GMYC method partitioned the dataset into 20 species of similar identification for two of the used priors (Yule and Constant coalescent). Finally, the PTP approach delimited 39 potential species being 16 of them characterized by support values of 50%. Comparatively, ABGD and GMYC discriminated the same potential species (Figure 3).

Based on the clusters generated from species delimitation algorithms, the haplotypes 1 to 15 encompassed a single species close to *M. amazonicum* 02-51 in two out of the three methods (ABGD and GMYC). Inversely, these haplotypes were differentiated from each other by PTP, resulting in 13 putative species. In

Table II. List of specimens showing discrepancies between molecular and morphological identifications as reported in scientific collections. IEPA: Amapá Institute of Scientific and Technological Research; MPEG: Emílio Goeldi Museum. The acronyms refer to the names of the institutions in Portuguese.

Morphological Identification	Scientific Collection	*Voucher*	Molecular Identification	Genetic divergence (%)
*Macrobrachium sp.*2-98	IEPA	856	*Macrobrachium brasiliense*	0.0 to 0.46
*Macrobrachium sp.*1-131	IEPA	869	*Macrobrachium brasiliense*	0.91 to 1.39
*Macrobrachium sp.*1-132	IEPA	869	*Macrobrachium brasiliense*	0.91 to 1.39
Macrobrachium jelskii 124	IEPA	619	*Macrobrachium brasiliense*	1.83 to 2.32
Macrobrachium nattereri 128	IEPA	612	*Macrobrachium brasiliense*	0.45 to 0.92
Macrobrachium surinamicum 80	IEPA	595	*Palaemon pandaliformis*	0.00
Macrobrachium chryseus 03	MPEG	749	*Macrobrachium brasiliense*	1.61 to 2.09
Macrobrachium chryseus 42	MPEG	765	*Macrobrachium amazonicum*	0.94
Macrobrachium chryseus 43	MPEG	765	*Macrobrachium jeslkii*	0.46

addition, the samples of *M. amazonicum* 02-51 from Juruti and *M. amazonicum* GO01 from Goiás are also separated.

The haplotypes 16 and 19 composed a highly supported clade by the three methods, being closely related to *M. chryseus* 01. The PTP indicated that these five samples should correspond to *M. chryseus*, while these haplotypes would belong to *M. amazonensis* or *M. bouvieri* based on the ABDG and GMYC methods.

The haplotype 20 was identified as belonging to *M. nattereri* by ABGD and GMYC, while PTP placed this sample as a distinct species closely related to *M. brasiliense* and *M. nattereri*. The haplotypes 21, 22, 23, and 24 formed a group of high support values along with *M. brasiliense* and *M. nattereri* by the three algorithms, being

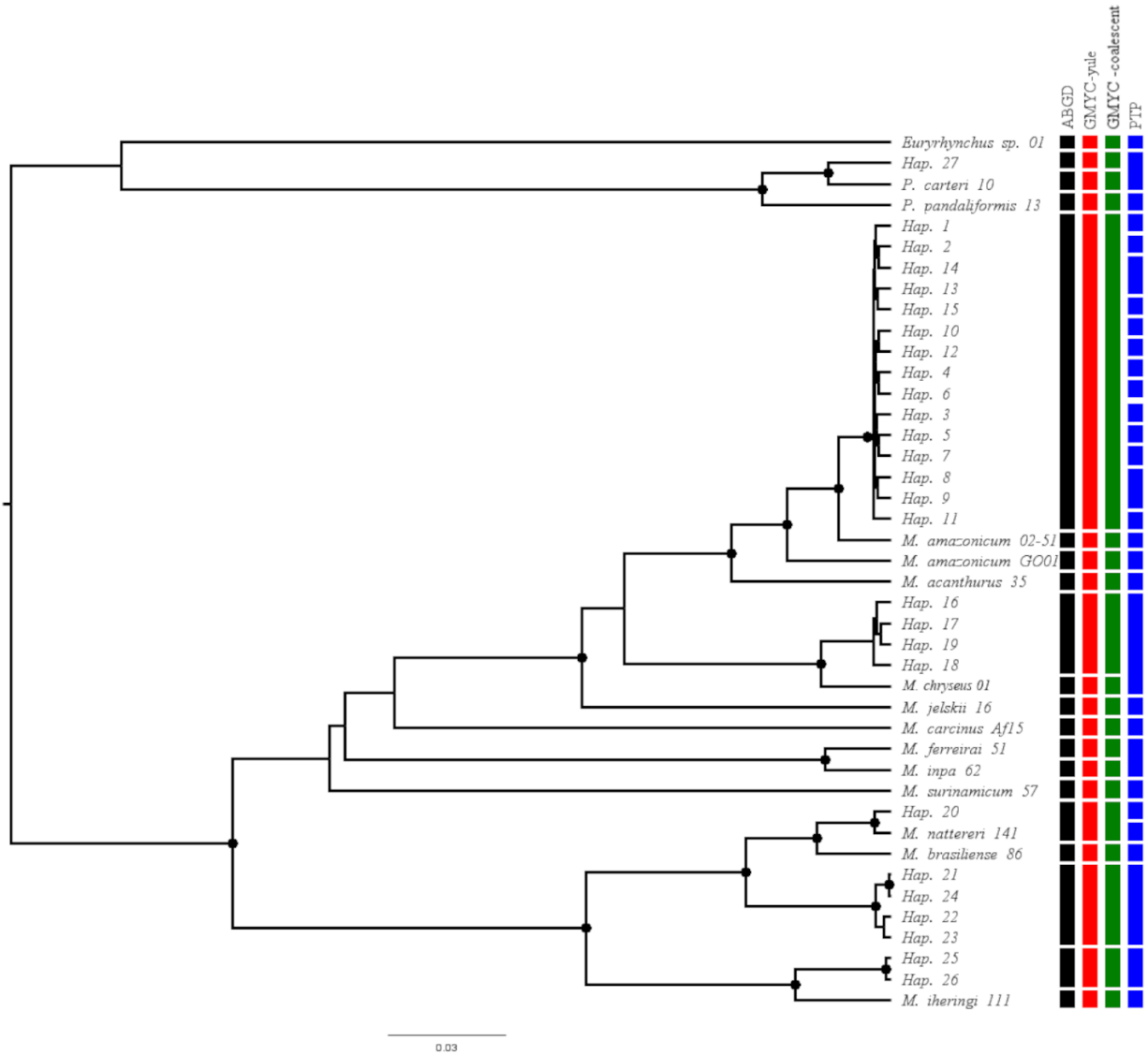

Figure 3. Ultrametric tree based on 28S rDNA along with the results inferred from species delimitation methods. The vertical bars correspond to the species identified by each method (ABGD in black, GMYC-Yule in red, GMYC-Coalescent constant in green, PTP in blue). The points above the branches correspond to the values of posterior probabilities (>0.9).

thus identified as *Macrobrachium* sp. On the other hand, the haplotypes 25 and 26 were recovered as a highly supported cluster with sequences of *M. iheringi*, independently of the method used. The haplotype 27 was identified as *P. carteri* by PTP, while ABGD and GMYC results indicated it should belong to a distinct species.

DISCUSSION

The molecular identification based on DNA barcodes has been successfully applied in crustaceans (Lefébure et al. 2006, Raupach et al. 2015, Matzen da Silva et al. 2011, Costa et al. 2007). In the case of the family Palaemonidae, COI data from 93 species proved to be efficient for their molecular identification in most cases in spite of the lack of a clear barcode gap (Robe et al. 2012). Similarly, the analyses of 28S rDNA sequences revealed no barcode gap among samples, but the present methodology allowed identifying the samples based on the correspondence between sequences inferred from distinct species delimitation algorithms. As a matter of fact, even potential cases of morphological misidentification of specimens from scientific collections were indicated.

Molecular analysis of specimens from scientific collections

Natural history collections are essential for genetic studies because they preserve specimens representing biological diversity across space and time. Sequencing the DNA of these samples allows for the confirmation of identifications, the correction of morphological classification errors (such as in the case of cryptic species), and the integration of molecular and morphological data. Moreover, museum specimens provide access to populations that are extinct or difficult to collect, enabling evolutionary, phylogeographic, and conservation research (Puillandre et al. 2012). However, to ensure these benefits, data accuracy is crucial. Incorrect morphological identifications of deposited specimens can trigger a cascade of errors, and if the DNA of these samples is sequenced, incorrect results are perpetuated and amplified in online databases such as GenBank and BOLD. In this context, analyzing DNA sequences can not only confirm identifications but also reveal potential errors, as demonstrated in the present study involving 28S rDNA sequences from major collections of the Brazilian Amazon.

The results of our analysis revealed incongruencies in six samples previously identified by morphological traits (Table II). While *M. chryseus* 01 identification was genetically confirmed (as detailed in Results), the molecular data for other samples initially assigned morphologically as *Pseudopalaemon chryseus* 03, 42 and 43 (now *M. chryseus*) showed clustering with distinct *Macrobrachium* species (*M. amazonicum*, *M. brasiliense*, and *M. jelskii*). These findings are fully consistent with the recent taxonomic revision by Mota et al. (2025a), which formally recognized *Pseudopalaemon* as a junior synonym of *Macrobrachium*. As demonstrated by Mota et al. (2025a) and corroborated by our phylogenetic inferences, species formerly placed in *Pseudopalaemon* are not monophyletic and are entirely nested within *Macrobrachium*. This collectively underscores significant historical misidentifications and the limitations of solely morphological characterization, supporting the re-evaluation of specific taxonomic boundaries within the genus.

The inherent difficulties in morphological identification probably contributed significantly to the observed incongruencies. Notably, specimens identified as *M. chryseus* may correspond to juvenile or immature individuals, which is known to hinder precise identification. Furthermore, the historical scarcity of studies

focusing on species previously grouped under *Pseudopalaemon* (De Oliveira et al. 2017, Mota et al. 2025b) likely contributed to taxonomic challenges that are now reflected within the broader *Macrobrachium* genus. Similarly, the complexities in morphologically identifying juveniles, coupled with the noted external similarities between closely related species such as *M. amazonicum* and *M. jelskii* (Guerra et al. 2014, Vera-Silva et al. 2017, Dos Santos et al. 2018), *M. brasiliense* and *M. nattereri* (Rossi et al. 2020), and *Palaemon* species like *P. carteri* and *P. yuna* (Carvalho et al. 2014, García-Dávila et al. 2005), collectively explain the remaining incongruent identifications (Table II).

Molecular analysis of samples collected in creeks and species delimitation

The tree topology (Figure 3) showed a lack of consensus among the species delimitation methods. While ABGD and GMYC (Yule and Constant coalescent) discriminated 20 groups, the PTP algorithm identified 30 potential species. The highest divergence was observed in the delimitation of samples clustered along with *M. amazonicum*, inasmuch as ABGD and GMYC converged to a large cluster composed of a single species, whereas PTP discriminated 13 putative species. Zhang et al. (2013) point out that PTP might overestimate the number of species when the amount of sampled taxa is uneven, a common issue in groups characterized by subtle variation. In this case, the increased partition of species indicated by PTP seems unlikely since most of the haplotypes were sampled from a single locality (Tucumatuba Creek).

The sequence of *M. amazonicum* from Juruti was recovered as a clade closely related to the large group composed of haplotypes 1 to 15. Species delimitation algorithms indicate that these samples are genetically close, displaying a genetic divergence of 3.3% relative to *M. amazonicum* from Goiás and Mato Grosso do Sul, where *M. amazonicum* is now recognized as *M. pantanalense* (Dos Santos et al. 2013, Lima et al. 2025). Previous molecular studies based on mtDNA (Vergamini et al. 2011, Iketani et al. 2021) had already identified three main lineages (inner Amazon region, coastal areas along the Amazon, and Pantanal wetlands). Recently, Lima et al. (2025), using SNP markers, not only confirmed the status of M. *pantanalense* as a distinct species from *M. amazonicum* but also provided evidence for a putative process of ongoing speciation among the Amazonian lineages of *M. amazonicum*, reinforcing the need for additional studies in these populations, as previously suggested by Dos Santos et al. (2013).

The 28S rDNA sequences allowed identifying the haplotype 20 as *M. nattereri* and the samples *Macrobrachium* sp. 2-98, *Macrobrachium* sp. 1-131, and *Macrobrachium* sp. 1-132 from scientific collections as *M. brasiliense*. On the other hand, four unique samples (haplotypes 21, 22, 23, and 24) were closely related to *M. nattereri* and *M. brasiliense*. In fact, phylogenetic analyses have already shown a close evolutionary relationship between both species, forming a clade along with *M. ferreirai*, *M. inpa*, *M. depressimanum* (Pereira 1993), and *M. aracamuni* (Rodríguez 1982) (Pileggi & Mantelatto 2010). Along the Brazilian Amazon, *M. depressimanum* has been recorded only in the states of Acre, Amazonas, and Rondônia (Pileggi et al. 2013), while M. *aracamuni* is supposed to be restricted to the state of Amazonas (Mantelatto et al. 2008). Therefore, we suggest that these haplotypes could belong to related species not included in the reference dataset (such as *M. depressimanum* or *M. aracamuni*), thus extending the distribution range of any of these species. Alternatively, these unique haplotypes could represent new, undescribed species.

The samples of the genus *Palaemon* were low (two species), and only a specimen

(haplotype 27) was closely related to *P. carteri*, being named as *Palaemon* sp. Indeed, the abundance of *Palaemon* in the wild is reduced, while *M. amazonicum* might represent up to 80% of macrocrustacean biomass in wetlands and flooded areas of the Amazon (Odinetz-Collart & Enriconi 1993). Therefore, the present results corroborate previous reports, as about 20% of the collected samples referred to *Palaemon*.

Based on molecular data, Carvalho et al. (2014) stated that populations of P. *carteri* are restricted to the eastern Amazon, while the populations from the central and western Amazon are represented by *P. ivonicus* (Holthuis 1950), *P. yuna*, and P. *mercedae* (Pereira 1986). Moreover, these authors revealed that the samples from central and western Amazon previously identified as *P. carteri* by morphological studies (Odinetz-Collart & Enriconi 1993, García-Dávila & Magalhães 2003, García-Dávila et al. 2005) actually corresponded to either *P. ivonicus* or *P. yuna*. Later, Carvalho et al. (2020) reported genetic structure among populations of *P. carteri* throughout the Amazon region. Considering this information and some ecological aspects, such as the hydrological system (white water environments), the haplotype 27 could not be identified at the species level.

The application of the 28S rDNA nuclear gene was efficient in discriminating the prawn species from our database. The use of this gene in combination with other nuclear (Littlewood 1994, Taylor et al. 2007, Hirai et al. 2013) or mitochondrial (Page et al. 2008, Landstorfer & Schubart 2010, Castelin et al. 2017, Wood et al. 2019, Bloom et al. 2019) markers has been helpful to the reconstruction of both phylogenies and taxa delimitation. Hirai et al. (2013) carried out an evaluation combining ITS2 and 28S nuclear regions as a molecular tool for the identification of North Pacific copepod crustaceans and concluded that this region is easily amplified and sufficiently variable to identify distinct species. Other reports have also successfully used 28S rDNA to identify fungi (Pinnoi et al. 2007), trematodes (Hernández-Mena et al. 2016, Orelis-Ribeiro et al. 2017), wasps (Campbell et al. 1994), myriapods (Gai et al. 2006), Niphargus crustaceans (Gai et al. 2006, Hekmatara et al. 2013), and krill species 28S rDNA evolution in the Eumalacostraca and the phylogenetic position of krill (Jarman et al. 2000).

Nonetheless, the reduced coverage of sequenced species, particularly within the genus Palaemon, represents a limiting factor, since some haplotypes could only be identified at the genus level. Moreover, the clade composed of haplotypes 25 and 26 plus *M. iheringi* represented an unexpected result. The freshwater prawn *M. iheringi* is endemic to Brazil, whose distribution seems to be restricted to the states of Espírito Santo, Rio de Janeiro, Minas Gerais, São Paulo, and Paraná (southeastern and southern Brazil), with no records for the Amazon region (Pileggi et al. 2013). The samples of this species derived from scientific collection are identified as originating from the Mapaoni River, a tributary of the left margin of the Jari River, in the Tumucumaque hills, in the State of Amapá (northern Brazil). In addition, molecular phylogenetic inferences revealed that *M. iheringi* would be closely related to *M. candango* (Mantelatto et al. 2021) (Pileggi & Mantelatto 2010) and *M. potiuna* (Müller 1880) (Rossi et al. 2020), species with no occurrence along the Amazon basin. Based on this information, it is possible that the present identification of samples as *M. iheringi* was performed incorrectly. Thus, this clade may also represent a new species of *Macrobrachium*.

It should be pointed out that haplotypes 16, 17, 18, and 19 clustered along with the sample identified as *M. chryseus* 01 based on the PTP approach, but they were placed as a distinct species by ABGD and GMYC algorithms. Based on

distribution data (Pileggi et al. 2013, Pimentel & Magalhães 2014), only *M. chryseus* is recognized for the collection site of these haplotypes (São Benedito creek - Tapajós River and Mentai stream - Arapiuns River), indicating potential cryptic diversity requiring further investigation.

On the other hand, previous studies on distribution and species records (e.g., Kemenes et al. 2010, Montoya et al. 2014, Magalhães et al. 2018) already recommended more detailed investigations into the occurrence of putative new species, which at the time were referred to as *Pseudopalaemon*, from localities such as the Jaú River (Amazon), the Iriri River (Xingú River basin, Pará), and the Cinaruco River (Orinoco River basin, Venezuela). With the recent synonymization of *Pseudopalaemon* with *Macrobrachium* (Mota et al. 2025a), these suggestions now apply to potential new species within *Macrobrachium*. The genus *Pseudopalaemon*, now *Macrobrachium*, comprised seven species; of these, only three (now *M. amazonensis*, *M. bouvieri*, and *M. chryseus*) have DNA sequences for the 16S rRNA, Histone H3, 18S rRNA (Mota et al. 2025a), and 28S genes (*M. chryseus* only, from this study). Most likely, this scenario is related to the difficulties in obtaining samples from this group, associated with their small size and similarities with *Macrobrachium* juveniles (De Oliveira et al. 2017, Magalhães et al. 2018, Mota et al. 2025b), thus hindering reliable morphological identification.

Pimentel & Magalhães (2014), for example, reported new records of Palaemonid species in the states of Amapá and Pará based on scientific collections from the Amazon, but their morphological analyses diverged from the molecular data presented herein. The specimens from the collections identified as MPEG 749 and MPEG 765, previously identified as *Pseudopalaemon chryseus* by these authors, have now been genetically identified as other species of the genus *Macrobrachium*. Such incongruencies highlight that cases of incorrect identification deposited in scientific collections can result in taxonomic uncertainties, leading to misleading records regarding occurrence and distribution. Therefore, a detailed morphological analysis, combined with reliable molecular data, is necessary to support the classification of this group of prawns.

CONCLUSIONS

The present study successfully validates 28S rDNA sequences as a reliable molecular marker for Palaemonidae, effectively revealing genetic variation and intrafamilial relationships. Our analyses identified significant taxonomic inconsistencies, including morphological misidentifications within scientific collections, and indicated the potential for undescribed species. Consequently, we recommend expanding 28S sequencing to additional vouchers and a broader range of Palaemonid specimens to enhance taxonomic resolution and identification reliability.

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Data availability

All data used in this study, including DNA accession numbers (see Table I), are available within the manuscript or may be requested from the corresponding author via email. All sequences have been made available through the GenBank database.