

Article

Record of *Caromiobenella* (Copepoda, Monstrilloidea) in Brazil and Discovery of the Male of *C. brasiliensis*: Morphological and Molecular Evidence

Judson da Cruz Lopes da Rosa ^{1,*} , Cristina de Oliveira Dias ² , Eduardo Suárez-Morales ^{3,*},
Laura Isabel Weber ⁴ and Luciano Gomes Fischer ^{1,4} 

- ¹ Programa de Pós-Graduação em Ciências Ambientais e Conservação (PPG-CiAC), Universidade Federal do Rio de Janeiro (UFRJ), Macaé, Rio de Janeiro, RJ 27965-045, Brazil; luciano.fischer@gmail.com
 - ² Laboratório Integrado de Zooplâncton e Ictioplâncton or Programa de Engenharia Ambiental-PEA, Departamento de Zoologia, Instituto de Biologia, Escola Politécnica, Universidade Federal do Rio de Janeiro (UFRJ), Cidade Universitária, Rio de Janeiro, RJ 21941-590, Brazil; crldias@hotmail.com
 - ³ El Colegio de la Frontera Sur (ECOSUR), Av. Centenario Km. 5.5, Chetumal, Quintana Roo 77014, Mexico
 - ⁴ Instituto de Biodiversidade e Sustentabilidade (NUPEM), Universidade Federal do Rio de Janeiro (UFRJ), Macaé, Rio de Janeiro, RJ 27965-045, Brazil; lauraweberufrj20@gmail.com
- * Correspondence: judsoncruz@yahoo.com.br (J.C.L.R.); esuarez@ecosur.mx (E.S.-M.)

Abstract: Monstrilloid copepods are protelean parasites with a complex life cycle that includes an endoparasitic juvenile phase and free-living early naupliar and adult phases. The monstrilloid copepod genus *Caromiobenella* Jeon, Lee and Soh, 2018 is known to contain nine species, each one with a limited distribution; except for two species, members of this widespread genus are known exclusively from males. Hitherto, members of *Caromiobenella* have not been recorded from tropical waters of the South Western Atlantic (SWA). The nominal species *Monstrilla brasiliensis* Dias and Suárez-Morales, 2000 was originally described from female specimens collected in coastal waters of Espírito Santo and Rio de Janeiro (Brazil), but the male remained unknown. The failure to reliably link both sexes of monstrilloid species is one of the main problems in the current taxonomy of the group, thus leading to a separate treatment for each sex. New zooplankton collections in coastal waters and intertidal rocky pools of the SWA yielded several male and female monstrilloid copepods tentatively identified as *Monstrilla brasiliensis*. Our results of both morphologic and molecular (mtCOI) analyses allowed us to confirm that these males and females were conspecific. We also found evidence suggesting that *Caromiobenella* is not a monophyletic taxon. Our male specimens are morphologically assignable to *Caromiobenella*, therefore, females of the nominal species *Monstrilla brasiliensis*, are matched here with the aforementioned males and, thus, the species should be known as *C. brasiliensis* comb. nov. (Dias and Suárez-Morales, 2000). This finding represents the third documented discovery of a female of *Caromiobenella*, the first record of the genus in the Southwestern Atlantic, and the first documented record of monstrilloids from coastal tidepools. With the addition of *C. brasiliensis*, *Caromiobenella* now includes 10 valid species worldwide. This work represents the second successful use of molecular methods to link both sexes of a monstrilloid copepod. The male of *C. brasiliensis* is herein described, and a key to the known species of *Caromiobenella* and data on the habitat and local abundance of *C. brasiliensis* are also provided.

Keywords: Brazil; integrative taxonomy; monstrilloid copepods; new record; parasitic copepods tropical zooplankton



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1. Introduction

Monstrilloid copepods are protelean parasites of benthic invertebrates, including polychaetes, molluscs, and sponges [1–3]; most juvenile stages are endoparasitic and free-living adult individuals lacking mouthparts are non-feeding reproductive forms that briefly become part of the zooplankton community [1]. As endoparasites they can cause

strong inflammatory processes to their hosts [4]. Because of their rarity in the plankton and taxonomic and nomenclatural complexity [5,6], there are large geographic areas in which the monstilloid copepod fauna remain largely unknown [2,7]. According to a previous analysis of their known diversity and distribution [2], the regions with the highest number of monstilloid records are the Northeastern Atlantic (32 species), the northwestern Tropical Atlantic (including the Caribbean Sea and the Gulf of Mexico) (24), and the Indonesia-Malaysia-Philippines, and Japan Seas region (23+). The Brazilian-Argentinean coasts are known to harbor a relatively low monstilloid diversity (16 species).

The Brazilian monstilloid copepods have been studied for more than 20 years [4,8–12]. Several new species of Monstilloidea have been described from Brazilian coastal waters, some of them from females only (i.e., *Monstilla pustulata* Suarez-Morales and Dias, 2001, *M. satchmoi* Suárez-Morales and Dias, 2001, *M. careli* Suarez-Morales and Dias, 2000, *M. brasiliensis* Dias and Suárez-Morales, 2000, and *M. bahiana* Suarez-Morales and Dias, 2001), others from males (i.e., *Monstillopsis fosshageni* Suárez-Morales and Dias, 2001, *Cymbasoma rochai* Suárez-Morales and Dias, 2001). These taxonomic works [8–10] also include a new geographic record of *M. brevicornis* Isaac, 1974 in the Atlantic Ocean [9,10], ecological aspects of a species of *Cymbasoma* [11], and the discovery and description of the female of *C. rochai* [12]. The male specimens collected in the surveyed area were tentatively identified as *Monstilla* and presumed to belong to one of the Brazilian species previously known only from females. A more detailed analysis allowed us to recognize our males as members of *Caromiobenella*. Both morphologic and molecular analyses were performed on our male and female specimens to reveal if they are conspecific.

Matching both sexes of monstilloid species has been raised as one of the main obstacles to determine the true diversity of the group [2,13]. Individuals of both sexes are mixed with those of other species in the plankton samples. Reliable methods to link the sexes of a species include: (1) particular autapomorphies shared by males and females of a species, (2) finding them emerging from the same host and mating, and (3) the use of molecular tools.

The goals of this survey were to: (1) to reveal the taxonomic identity of the male specimens collected from the Rio de Janeiro area, and reliably link them to the female through morphological and molecular analyses; (2) present data about their habitat and local abundance; (3) provide an updated identification key to the known species of *Caromiobenella*.

2. Materials and Methods

2.1. Sampling

Zooplankton samples were collected monthly between August 2017 and December 2018 in marine coastal waters and rocky tidepools at five localities in the State of Rio de Janeiro, Southeastern Brazilian coast (Figure 1). Some of these samples contained male and female monstilloid copepods that were taxonomically examined. Monstilloids were firstly found in three rocky tidepools of the municipality of Rio das Ostras (Areias Negras beach: 22°31′47.60″ S, 41°55′32.22″ W, Remanso beach: 22°31′40.57″ S, 41°55′21″ W) and Armação de Búzios (Rasa beach: 22°43′59.6″ S, 41°57′26.2″ W), and subsequently in a shallow costal area in the municipality of Rio das Ostras (Cemitério beach: 22°31′52.21″ S, 41°56′32.18″ W) (Figure 1, Table 1).

The water in the rocky tidepools was drained with an electric bilge pump (12 V) and the zooplankton was retained in a 100 µm mesh filter attached to the end of the water pipe. The drained water volume was recorded to calculate the zooplankton densities (individuals/m³). The water temperature and salinity were recorded with a thermosalinometer (YSI Yellow Spring Pro 2030). The samples used in taxonomic examination and descriptions were fixed in 4% formaldehyde, analyzed in a stereomicroscope, and quantified in a Dollfus chamber.

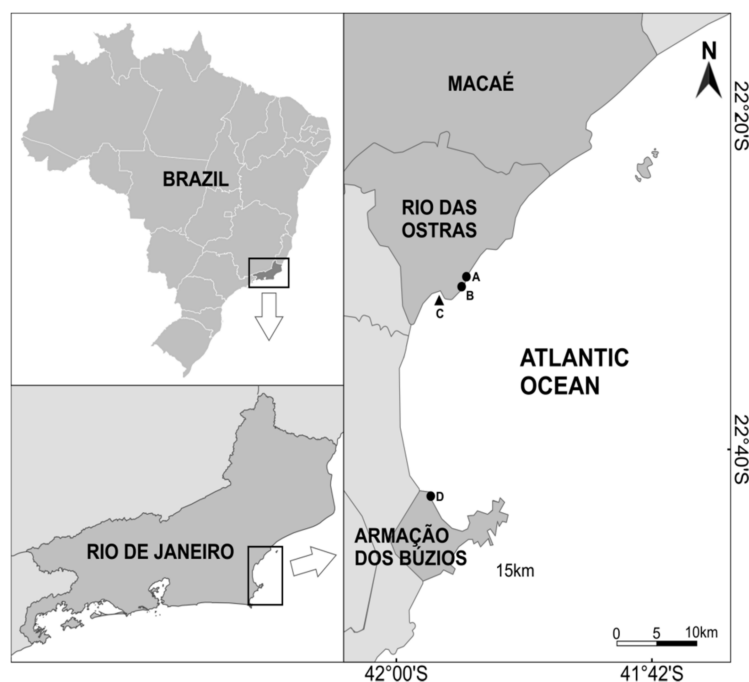


Figure 1. Map showing zooplankton sampling sites in the Rio de Janeiro coast, SW Atlantic, with the coastal municipalities where monstrilloid copepods were collected. Dots: collection in tidepools of rocky shores. Solid triangle: collection in shallow coastal zone (<5 m depth). (A) Remanso beach, (B) Areias Negras Beach, (C) Cemiterio beach, (D) Rasa beach.

To complete the genetic analysis, five additional collections were carried out between September and December 2018 (Table 1) in Cemiterio beach, coastal region beyond the surf zone, in an area without rocky shores. These collections were made with a 100 µm mesh plankton net towed by a kayak without using a flowmeter, thus without density estimates. These samples were preserved in 92.8° GL ethanol and monstrilloids were sorted and taxonomically examined in the laboratory.

2.2. Morphologic Analysis

A few male specimens, including those presented here, were tentatively assigned to the genus *Monstrilla* Dana, 1849. A reexamination of these male individuals allowed us to recognize them as a species of *Caromiobenella*, largely known from males and with distinctive morphological characters [3]. One of these specimens (MNRJ30136) was selected to be described. Our description of this male followed the current upgraded descriptive standards in monstrilloid taxonomy [1,13,14] and included the distinctive characters of *Caromiobenella* [3,15]. The morphologic terminology follows Huys and Boxshall (1991) [16]. The Brazilian male specimens were deposited in the collections of the Museu Nacional-Federal University of Rio de Janeiro (MNRJ) and Invertebrate Collection of the Instituto de Biodiversidade e Sustentabilidade (NUPEM/UFRJ) (CIN-NPM), where they are available for inspection.

2.3. Molecular Analysis

Total DNA was extracted from four females and two male copepods by the Chelex resin protocol [17,18] as follows. Ethanol-preserved copepods were picked up with plastic pipettes under a stereomicroscope and then transferred to 0.6 mL Eppendorf vials. The excess of ethanol was then removed and left to dry at room temperature for at least 1 h. Afterwards, 10 µL proteinase-k (10 mg/µL) was added directly over the copepod body, followed by 75 µL lysis buffer 2x (0.1 mM Tris, 0.01 mM EDTA, pH 8.0), 75 µL 12% chelating resin (Chelex 100), SIGMA, shaken in vortex, and incubated overnight at 55 °C.

Table 1. Collection sites data. TD = Tidepool drainage, PN = Plankton net in shallow coastal area, M = male, F = female, temp = water surface temperature (°C), sal = surface water salinity. NA = no data available. Collections at Cemiterio beach were not made in tidepools, but in the coastal zone without using a flowmeter, thus without volume and density estimates.

Date	Site	Collection Type	<i>C. brasiliensis</i>		Zoopl. Density (ind. m ³)	Tidepool Volume (L)	Drained Volume (L)	Catalog Number	Temp	Sal
			N°, Sex	Density (ind. m ³)						
08.08.17	A.Negras	TD	3M	15.0	155	345	200	NPM00020 (1M *), (2M * lost%)	24.2	30.0
21.09.17	Remanso	TD	2F	5.0	206	1900	400	NPM00021 (1F), (1F lost%)	20.4	34.3
16.10.17	Remanso	TD	1 M	5.0	991	1900	200	1M (dissected)	21.5	33.2
16.10.17	Remanso	TD	2M	10.0	335	600	200	NPM00022 (1M *), MNRJ30136 (1M *)	21.2	33.6
31.10.17	Rasa	TD	1F	2.5	77	1440	400	NPM00023 (1F)	22.6	28.1
03.02.18	Remanso	TD	1M	2.5	870	1900	400	1M * (dissected)	28.8	32.0
19.09.18	Cemiterio	PN	1F/2M	NA	NA	NA	NA	MNRJ28990 (1M), 1M #, 1F \$ *	-	-
20.10.18	Cemiterio	PN	1F/1M	NA	NA	NA	NA	1M #, 2F \$	-	-
21.10.18	Cemiterio	PN	2F/1F	NA	NA	NA	NA	2F #, 1F \$ *	-	-
28.10.18	Cemiterio	PN	1F	NA	NA	NA	NA	1F # *	-	-
24.11.18	Cemiterio	PN	2F	NA	NA	NA	NA	MNRJ28991 (1F), 1F #	-	-
25.11.18	Cemiterio	PN	1F	NA	NA	NA	NA	NPM00024 (1F)	-	-
02.12.18	Cemiterio	PN	1F	NA	NA	NA	NA	NPM00025 (1F)	-	-

* used in morphometrics, # used in DNA analysis with success; % lost in handling.

The anterior region of the mitochondrial cytochrome c-oxidase, subunit I (COI) was amplified by the polymerase chain reaction (PCR) from 5 and 10 µL of extracted DNA, using Folmer et al. (1994) [19] primers, HCO2198 (TAAACTTCAGGGT GACCAAAAAATCA) and LCO1490 (GGTCAACAAATCATAAAGATATTGG) in a 25 µL final reaction volume containing 1x reaction buffer, 3 mM MgCl₂, 0.24 µM of each dNTP, 0.12% Triton-X-100, 0.4 µM of each primer, 2 U of DNA polymerase. PCR was performed in a thermocycler (Gradient Mastercycler, Eppendorf) as follows: 1 cycle at 94 °C for 4 min; 35 cycles at 94 °C, 48 °C and 72 °C for 1 min each; and a final cycle at 72 °C for 7 min. DNA fragments were visualized after electrophoresis over ultraviolet (UV) light using the fluorescent stain UniSafe dye (Uniscience) and PUC19 ladder for fragment size determination. Amplicons were sequenced by the automation system of capillary electrophoresis sequencing (ABI 3730xl System; Sanger method). Sequences were edited using the Chromas Pro, v. 2.1.8 software and then aligned with Clustal W online tool. Edited and revised sequences were analyzed by the Basic Local Alignment Search Tool (BLAST) to find similar sequences deposited in GenBank and verify their proximity to the expected taxonomic group. Multiple alignment and gap insertions were performed retaining conserved coding regions at the same positions.

Pair-wise *p*-distances and Tamura-Nei distances were obtained for the Brazilian group. For comparisons with species from different genera, Tamura-Nei and Kimura-2 parameters distances were used. Maximum likelihood (ML) trees were obtained using Tamura-Nei distance and using 1000 bootstrap iterations for the branch confidence. A parsimony tree was also obtained. Phylogenetic and molecular evolutionary were conducted using MEGA version 6 [20,21]. Sequences of copepod species of other genera obtained from GenBank were used for comparisons and to verify the position of sequences of local copepods. Some of the sequences of other monstilloid genera available in GenBank were shorter than those found in the present study for the Brazilian copepods. Therefore, to include a greater number of species in the comparison, it was necessary to work with a smaller region, but present in all of them. These comparisons were only possible at over 582 bp DNA fragment of the Brazilian copepods. It was not possible to include *Caromiobenella ohtsukai* (MH638358) and *Monstillopsis longilobata* (MF447160; MF447163) because they were shorter at the 5' end.

3. Results

3.1. Taxonomy

Subclass Copepoda Milne-Edwards, 1840
Order Monstilloidea Thorell, 1859

Family Monstrillidae Dana, 1849

Genus *Caromiobenella* Jeon, Lee and Soh, 2018

Caromiobenella brasiliensis (Dias and Suárez-Morales, 2000) comb. nov.

3.1.1. Material Examined

Eight adult males: 08/08/2017 (1♂NPM-00020, 2♂lost in shipping), 10/16/2017 (1♂NPM-00022, 1♂MNRJ30136, and 1♂dissected), 02/03/2018 (1♂dissected), and 09/19/2018 (1♂MNRJ28990). Specimens NPM-00020, NPM-00022, and MNRJ28990 fixed in 4% formaldehyde and 1 ♂ undissected, mounted on semi-permanent slide with glycerin, sealed with acrylic varnish.

3.1.2. Type Locality

Camburi, Baía do Espírito Santo (20°16.383' S, 40°15.900' W) [8].

3.1.3. Diagnosis (Female and Male)

Female *Monstrilla* with medium-sized, robust body, cephalothorax with dorsal and ventral scattered fields of striae. Urosome 4-segmented, ovigerous spines short: fifth legs elongate, bilobed, divergent; outer lobe long, slender, with three setae, inner lobe short, cylindrical, with single seta. Antennule 4-segmented, first segment partially fused to cephalothorax, segments 3–4 fused; combined third segment with outer surface produced, forming proximal rounded process furnished with field of coarse cuticular ridges. Caudal rami with 6 setae. Male: *Monstrilla*-like, medium-sized, body segmentation as in *Caromiobenella* (body length ~0.98 mm), cephalothorax representing ~49% of total body length. Pedigerous somites 2–4 representing 39% of total body length. Oral papilla at 34% of way back along ventral surface of cephalothorax. Cephalothorax with dorsal and ventral scattered pores and fields of cuticular striae. Antennules 5-segmented, representing ~35.6% of total body length, geniculated between segments 4–5; first segment partially fused to cephalothorax; second segment with outer margin produced into rounded process ornamented with field of deep transverse wrinkles. Distal antennular segment with usual armature of genus. Fifth pedigerous somite with reduced fifth legs represented by pair of knob-like processes. Preanal somite with medial pair of small keel-like acute processes on postero-ventral surface. Genital complex of type I, represented by short robust shaft with short, thick distal lappets, branches separated by deep longitudinal slit. Caudal rami armed with 6 setae including short, slender biserially plumose innermost seta.

3.1.4. Description of Adult Male

Body shape and tagmosis as in *Caromiobenella* (see Jeon et al., 2018) (Figure 2A–C). Body robust, total body length of examined individual = 0.98 mm, measured from anterior end of cephalothorax to posterior margin of anal somite. Additional measurements in Table 2. Body short, robust, cephalothorax incorporating first pedigerous somite representing ~49% of total body length. Succeeding pedigerous somites 2–4 each bearing pair of biramous swimming legs; pedigerous somites 2–4 combined accounting for 39% of total body length in dorsal view. Dorsal surface of pedigerous somites 2 and 3 each with pair of “crater-like” cuticular processes; those on third somite being smaller (Figure 2A,C). Cephalic region of cephalothorax wide, smooth, bilaterally protuberant in dorsal view, slightly narrower than cephalothorax; outer margin of cephalic protuberances weakly corrugate. Pair of small dorsal pit setae between antennular bases. Forehead moderately produced, weakly rounded, with transverse striation fields on dorsal anterior and lateral surfaces; no other cephalic ornamentation was observed on dorsal anterior surface (Figure 2A). Cephalothorax robust, 0.36 mm long, representing almost 37% of total body length; dorsal surface with scattered dorsal pores (Figure 2A). Anterior ventral surface with rounded preoral projection (Figure 3C) between antennule bases and oral papilla, visible in lateral view. Oral papilla at 34% of way back along ventral surface of cephalothorax, with adjacent field of transverse cuticular striae (Figure 3C). Cephalothorax with eyes consisting of

relatively small, unpigmented paired lateral cups separated medially by length of less than one eye diameter plus medial cup slightly larger than lateral cups. One pair of relatively small nipple-like cuticular processes present on anterior ventral surface between antennule bases and oral papilla (arrowed in Figure 2B); nipple-like cuticular processes surrounded by field of wrinkles (Figure 2A).

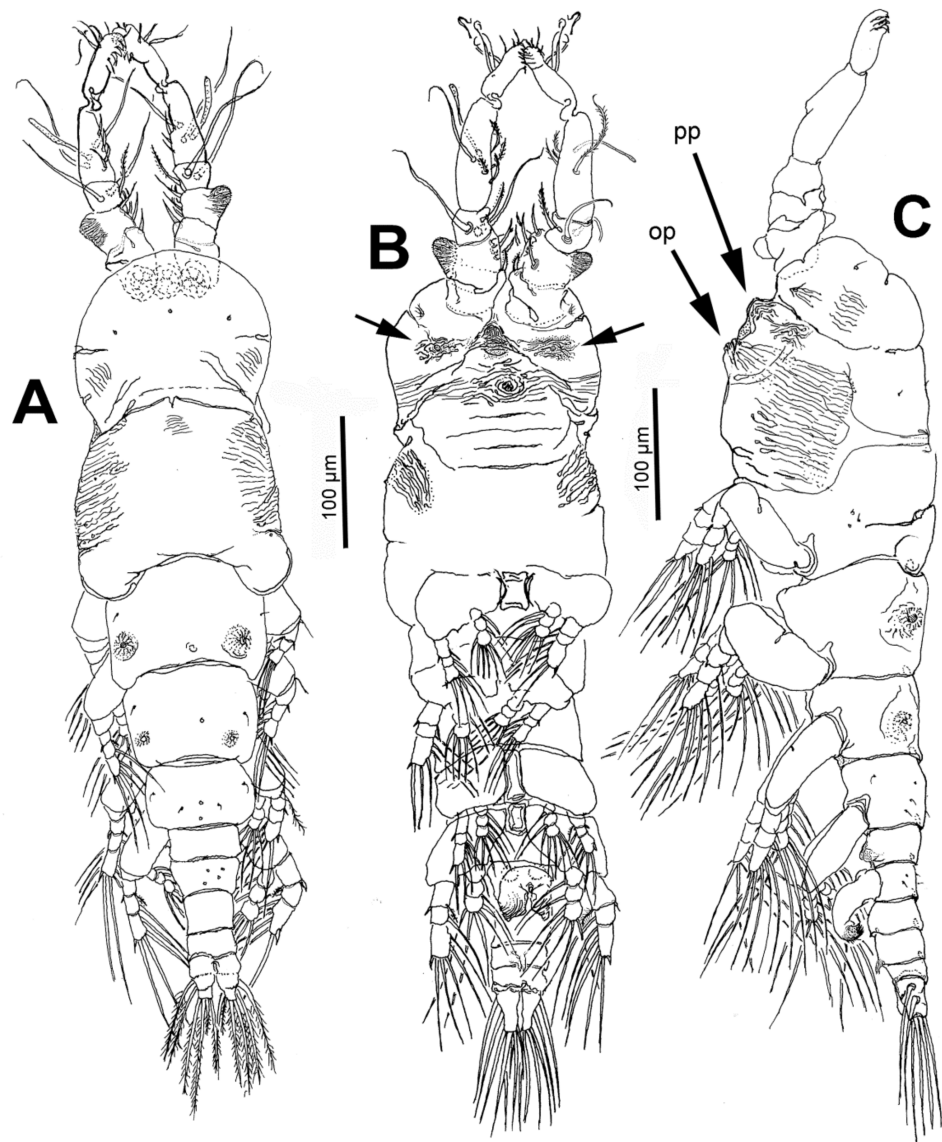


Figure 2. *Caromiobenella brasiliensis* (Dias and Suárez-Morales, 2000) comb. nov., from Rio de Janeiro area, adult male (MNRJ30136). (A) Habitus, dorsal view; (B) Habitus, lateral view; (C) Same, ventral view. Arrows indicate antero-ventral nipple-like cuticular processes. Legend: pp = preoral process, op = oral papilla.

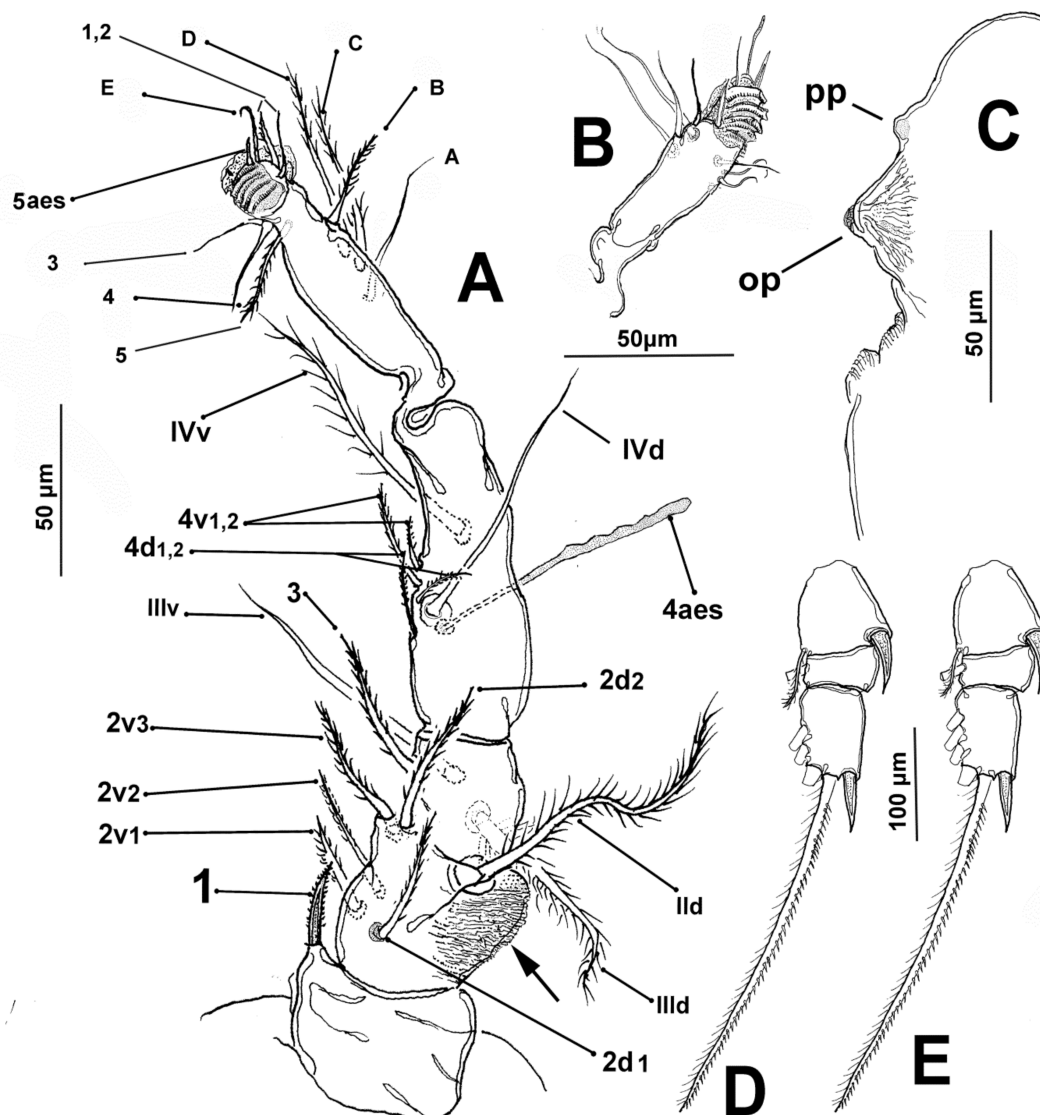


Figure 3. *Caromiobenella brasiliensis* (Dias and Suárez-Morales, 2000) comb. nov., from Rio de Janeiro area, adult male (MNRJ30136). (A) Right antennule, dorsal view showing setal elements (segments 1–4 [14], segment 5 [1]; (B) Left fifth antennular segment; (C) Anterior part of cephalothorax, lateral view (pp = preoral process, op = oral papilla); (D) Leg 1 exopod; (E) Leg 3 exopod. Arrow indicates wrinkled outer process on second segment. Legend: pp = preoral process, op = oral papilla.

Table 2. Additional measurements of male specimens of *Caromiobenella brasiliensis* observed in this study (1♂NPM-00020, 1♂NPM-00022, 1♂MNRJ30136, 1♂03/02/2018 (dissected)).

Measure	Min (mm)	Max (mm)	Mean (mm)
Total length	0.816	1.170	1.004
Antennule length	0.187	0.373	0.323
Cephalothorax height	0.186	0.269	0.240
Cephalothorax length	0.339	0.475	0.413
Cephalothorax width	0.283	0.456	0.381
Metasome length	0.172	0.239	0.211
Urosome length	0.816	1.170	1.004
Incorporated pediger	0.180	0.256	0.225
Free pediger 1	0.211	0.294	0.266
Free pediger 2	0.164	0.220	0.200
Free pediger 3	0.131	0.203	0.178
Genital somite	0.082	0.810	0.273
Preanal somite	0.065	0.660	0.216
Caudal ramus length	0.053	0.066	0.059
Caudal ramus width	0.036	0.058	0.046

Antennule length = 0.35 mm, representing ~35.6% of total body length. Antennule relatively short, 5-segmented, type III [16] representing 36% of total body length, and 73% of cephalothorax length; antennules indistinctly 5-segmented, segments 1–4 separated by incomplete sutures. First antennular segment subrectangular, partially fused to cephalothorax proximally and to second segment distally. Second segment with bulging lateral process on outer proximal half; process furnished with deep transverse cuticular wrinkles (arrow in Figure 3A). Fourth segment being longest, representing 37% of total antennular length. Geniculation between segments 4 and 5 (Figure 3A). In terms of the pattern described by Grygier and Ohtsuka (1995) [14] for antennular armature of segments 1–4 and complemented with Huys et al.'s (2007) [1] nomenclature for elements on the male fifth antennule segment, element 1 present on first segment; element spiniform, lightly pinnate, relatively short, barely reaching midlength of succeeding second segment. Second segment armed with long, lightly plumose elements 2d_{1,2}, 2v_{1–3}, and slender seta IId. Third segment partially fused with second, subquadrate, armed with setiform elements 3, IIIId, and IIIv. Setal element 3 lightly pinnate, reaching proximal 1/3 of succeeding fourth segment. Fourth segment subrectangular, elongate, about 3.5 times as long as wide, bearing normally developed elements 4d_{1,2} and 4v_{1,2} as well as long setae IVd and IVv; long, slender aesthetasc 4aes on mid-ventral position. Elements of group 4v short, setiform, lightly pinnate; element 4v₃ not discernible in the specimen examined. Distal segment with conspicuous proximal geniculation, armed with 11 setal elements (sensu Huys et al., 2007) including elements 1–5 and A–E plus apical aesthetasc 5aes.

Intercoxal sclerites of legs 1–4 subrectangular, smooth. As in other members of *Caromiobenella* [3,15], basis with inner margins produced, forming rounded expansions (arrows in Figure 4A–D); outer margin of basipods of legs 1–4 with lightly setulose basipodal seta; on leg 3, basipodal seta about twice as long as and slightly thicker than in other legs (Figure 4C). Endopodites and exopodites of legs 1–4 triarticulated, outer margins of exopodites smooth. Third exopodal segment of legs 1–4 with 6 setal elements (Figure 3E) except for leg 1 with only 5 elements (Figure 3D). Ramus setae all biserially plumose except for robust apical spiniform seta on exopodal segment 3 (Figure 3D,E); spiniform apical seta on legs 1–4 long, with inner margin spinulose and outer margin lightly setulose (Figure 3D,E). Intercoxal plates of legs 1–4 rectangular, smooth. Armature of legs 1–4 as in Table 3.

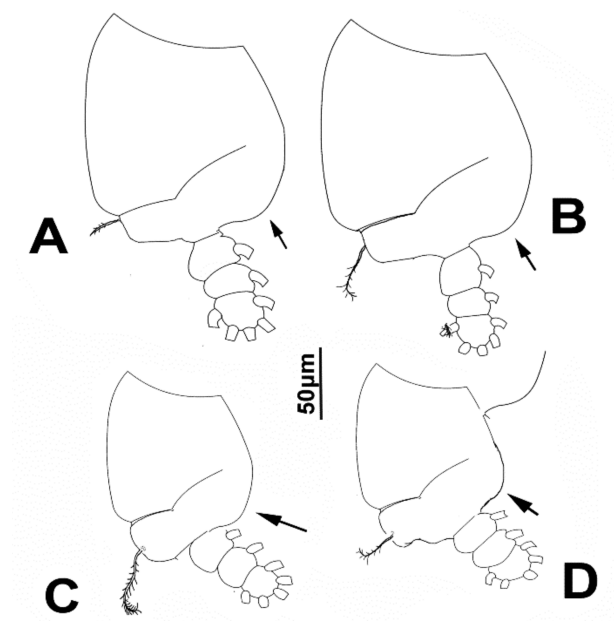


Figure 4. *Caromiobenella brasiliensis* (Dias and Suárez-Morales, 2000) comb. nov., from Rio de Janeiro area, adult male (MNRJ30136). (A) Leg 1; (B) Leg 2; (C) Leg 3; (D) Leg 4. Arrows indicate expanded inner margins of basipod.

Table 3. Armature of legs 1–4 including basis, exopods and endopods. Roman numerals indicate spiniform elements, Arabic numbers indicate setiform elements [13,14].

Leg	Basis	Endopod	Exopod
Leg 1	1–0	I-1; 0–1; 2, 2, 1	I-1; 0–1; 2, 2, 1, I
Legs 2–4	1–0	0–1; 0–1; 2, 2, 1	I-1; 0–1; 2, 2, 1, I

Urosome relatively short, representing about 19% of total body length, consisting of fifth pedigerous somite, genital somite (carrying genital complex), and two short free post-genital somites (Figure 5A–C). Anal somite short, with straight outer margins. Fifth pedigerous somite ventrally produced, carrying reduced fifth legs represented by pair of knob-like processes (Figure 5B,C) and proximal half (Figure 5C). Genital somite slightly shorter than preceding fifth pedigerous somite; genital complex of type I [15,20], represented by short, robust, ventrally expanded shaft; genital complex with short, medially conjoined lappets, both tapering distally into apical subtriangular opercular process (Figure 5C–E). Lappets with rugose anterior surface and coarsely striated distal half, branches parallel, separated medially by deep smooth slit (Figure 5E). Preanal somite slightly longer than anal somite, furnished with pair of small keel-like processes on postero-ventral surface, visible in lateral view (arrow in Figure 5C). Caudal rami subrectangular, approximately 1.3 times as long as wide, about 1.3 times as long as anal somite. Each ramus armed with five subequally long caudal setae (setae I–V) [16] plus short, slender, lightly setose caudal seta VI (Figure 5A,B).

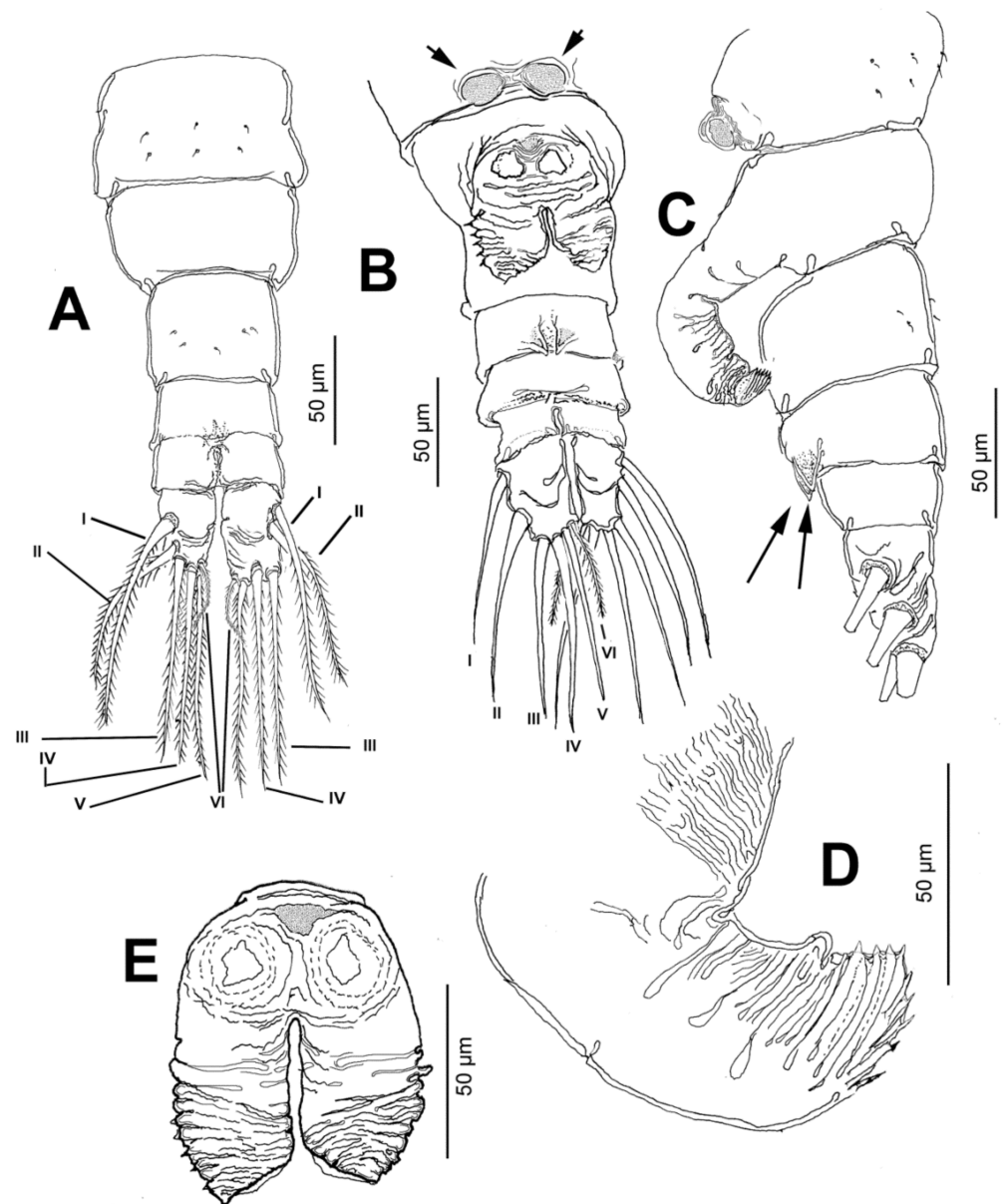


Figure 5. *Caromiobenella brasiliensis* (Dias and Suárez-Morales, 2000) comb. nov., adult male (MNRJ30136). (A) Urosome, dorsal view; (B) Urosome, ventral view showing reduced knob-like fifth legs (arrows); (C) Urosome, lateral view showing genital complex with ventral keel-like processes arrowed; (D) Detail of genital lappets, lateral view; (E) Genital lappets, ventral view.

3.1.5. Additional Male Specimens Measured

Five specimens: 3♂ collected in 08/08/2017 (NPM-00020), 1♂10/16/2017 (NPM-00022), 1♂02/03/2018 (dissected). Body length ranged from 0.878 to 1.170 mm. Cephalothorax length ranged from 0.339 to 0.475 mm representing between 38.6% to 44.2% of the total body length (see Table 2). Antennule length between 0.330 to 0.373 mm representing 51.8% to 97.3% of the cephalothorax and between 22.9% to 37.6% of the total body length. Urosome representing 19.4–22.6% of total body length.

3.1.6. Female Specimens Measured

Based on the examination of the additional specimens: 1♀10/21/2018, 1♀09/19/, 1♀10/28/2018. Total body length was measured from anterior end of cephalic somite to posterior margin of anal somite. The body length of three females ranged between 2.08 and

2.35 mm. Cephalothorax length ranged between 0.863 and 1.044 mm, thus representing 41.6–44.7% of total body length. Antennule length representing 41.2–52.2% of cephalothorax length and 18.3–21.7% of total body length. Urosome representing 20.5–23.5% of total body length. Caudal rami length between 0.11 and 0.13 mm.

3.2. Molecular Analysis

Six monstilloid copepods including four females (identified as *M. brasiliensis*) and two males (tentatively assigned to *M. brasiliensis*), underwent molecular analysis to verify that they belong to the same species. We amplified all specimens' 5' end of the COI gene successfully, corresponding to a fragment of 681 bp (Table S1). The region showed 21 variable sites including 12 that were phylogenetically informative (shared). These copepods are conspecific, as shown by the distances among them (Table 4) with mean distances of 0.014 ± 0.003 , characteristic of intraspecific individuals. The male MZ223434 clustered with MZ223430 female and the male MZ223435 clustered with the other females (Figure 6), confirming that the males belong to the same species of the studied females.

Table 4. Pair-wise genetic distances between monstilloid copepods from Rio das Ostras (State of Rio de Janeiro, Brazil) based on 681 pb COI (cytochrome c-oxidase, subunit I) fragment. Distances: (below diagonal) *p*-distance; (above diagonal) Tamura-Nei distance.

	Female	Female	Female	Female	Male	Male
GenBank Accession	MZ223430	MZ223431	MZ223432	MZ223433	MZ223434	MZ223435
MZ223430		0.018	0.019	0.023	0.015	0.006
MZ223431	0.018		0.010	0.010	0.009	0.018
MZ223432	0.020	0.010		0.006	0.010	0.019
MZ223433	0.022	0.010	0.006		0.013	0.019
MZ223434	0.015	0.009	0.010	0.013		0.015
MZ223435	0.006	0.018	0.019	0.019	0.015	

The branch in the ML tree (Figure 6) that contains exclusively the six Brazilian copepods (Rio das Ostras, RJ, Brazil) was 100% supported by bootstrap. This Brazilian branch appears as a sister group of *Monstrilla ilhoii* (see Table 5), followed by *Caromiobenella* branch, which includes also in its branch the species *Caromiobenella hamatapex* (*Monstrilla hamatapex* in Genbank and in Figure 6). More distantly is grouped *Caromiobenella helgolandica* (as *Monstrilla helgolandica* in Genbank and in Figure 6) and the branch of *Maemonstrilla*. The most distant group from the examined Brazilian copepods was the genus *Cymbasoma* and *Monstrillopsis longilobata* (KR049000 and KY553229).

Table 5. Mean pair-wise genetic distances over 582 pb COI fragment among different monstilloid genera or branches as shown in Figure 6 for the comparisons with Brazilian copepods from Rio de Janeiro coast. See Table S1 for sequences obtained from GenBank. Distances: (below the diagonal) Kimura-2 Parameter; (above the diagonal) Tamura-Nei distance.

Branches/Spp	1	2	3	4	5	6	7	8
(1) <i>C. brasiliensis</i>		0.305 ± 0.026	0.308 ± 0.023	0.470 ± 0.038	0.366 ± 0.031	0.464 ± 0.030	0.505 ± 0.041	0.535 ± 0.036
(2) <i>M. ilhoii</i>	0.299 ± 0.025		0.401 ± 0.028	0.459 ± 0.039	0.388 ± 0.033	0.446 ± 0.030	0.595 ± 0.054	0.576 ± 0.038
(3) <i>Caromiobenella</i>	0.302 ± 0.022	0.390 ± 0.027		0.443 ± 0.031	0.417 ± 0.031	0.503 ± 0.028	0.540 ± 0.041	0.545 ± 0.035
(4) <i>C. helgolandica</i>	0.452 ± 0.035	0.435 ± 0.035	0.428 ± 0.029		0.398 ± 0.033	0.542 ± 0.037	0.573 ± 0.054	0.620 ± 0.043
(5) <i>Maemonstrilla</i>	0.353 ± 0.028	0.374 ± 0.029	0.406 ± 0.029	0.385 ± 0.031		0.489 ± 0.032	0.550 ± 0.052	0.579 ± 0.041
(6) <i>Cymbasoma</i>	0.451 ± 0.028	0.434 ± 0.028	0.488 ± 0.027	0.517 ± 0.033	0.471 ± 0.030		0.596 ± 0.042	0.593 ± 0.034
(7) <i>M. longilobata</i>	0.481 ± 0.036	0.543 ± 0.041	0.515 ± 0.026	0.519 ± 0.040	0.500 ± 0.039	0.561 ± 0.034		0.397 ± 0.027
(8) External group	0.522 ± 0.034	0.554 ± 0.034	0.532 ± 0.033	0.588 ± 0.037	0.553 ± 0.035	0.573 ± 0.032	0.388 ± 0.025	

(1) Brazilian Copepods (females: MZ223430, MZ223431, MZ223432, MZ223433 and males: MZ223434, MZ223435); (2) *Monstrilla ilhoii* (KY553214); (3) *C. hamatapex* (KR048992), *C. castorea* (KY553209, type species) and *C. polluxea* (KY553211); (4) *C. helgolandica* (KT209330 and KT209379); (5) *Maemonstrilla* sp. (KY553231, KY553232) and *Maemonstrilla simplex* (KR049003); (6) *Cymbasoma* sp. (KR048989), *Cymbasoma reticulatum* (KR048990) and *Monstrillopsis longilobata* (KY553229); (7) *Monstrillopsis longilobata* (KR049000); (8) *Tigriopus japonicus* (KR049009), *Lepeophtheirus salmonis* (KR049053) and the planktonic [22] *Calanus helgolandicus* (AY604521), *Caromiobenella hamatapex* and *C. helgolandica* appear as *Monstrilla* in GenBank.

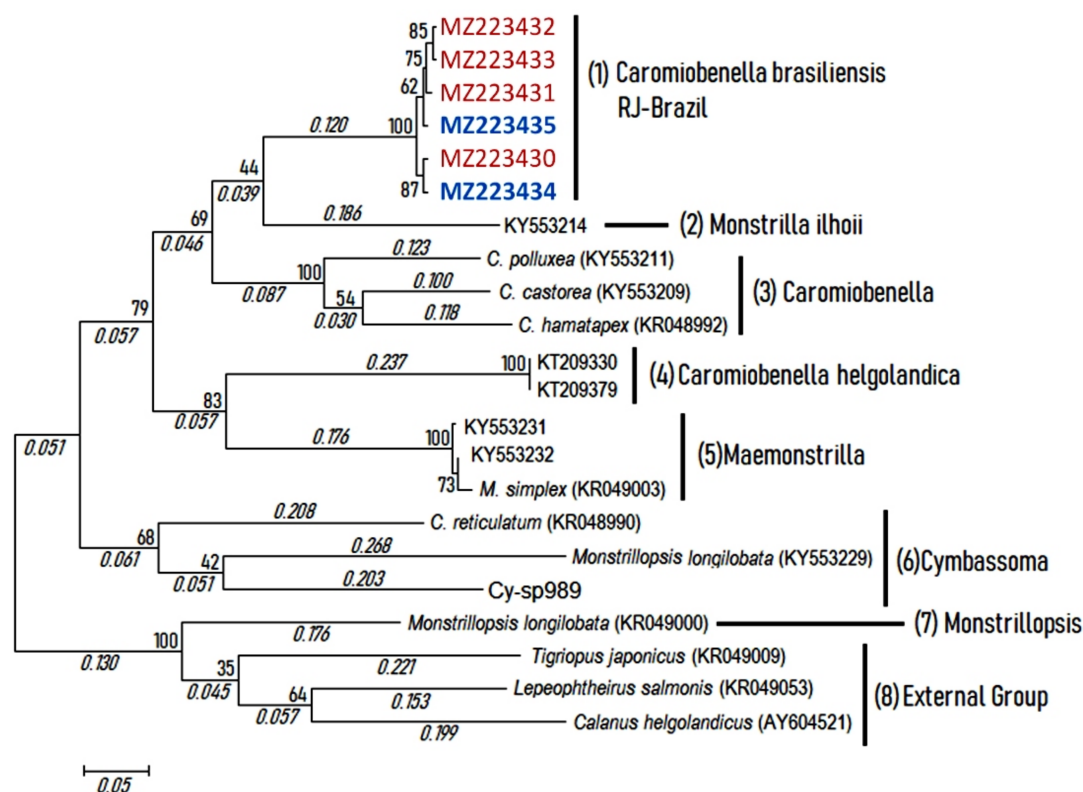


Figure 6. Maximum likelihood tree (Tamura-Nei) showing the position of Brazilian copepods from Rio de Janeiro coast in relation to other monstrellid genera and species obtained from GenBank. Female Brazilian copepods in red and males in blue. See Table S1 for specific names and GenBank accession numbers for the partial sequences of *C. brasiliensis* used. *Caromiobenella hamatapex* and *C. helgolandica* appear as *Monstrilla* in GenBank.

4. Discussion

The males examined herein can be morphologically identified as members of *Caromiobenella* Jeon, Lee and Soh, 2018 by their possession of distinctive genus characters [3], as follows: (1) the modified distal half of the male fifth antennular segment, with a series of brush-like processes is the main distinguishing character of *Caromiobenella* [3] and our specimens clearly have this character (Figure 3A,B); (2) the body shape and tagmosis and the urosome segmentation are also typical of *Caromiobenella* [3] (see Figure 1A–C); (3) the presence of 6 caudal setae with innermost apical caudal seta VI clearly shorter and slenderer than the others is another synapomorphic character shared with *Caromiobenella* ([3], Figure 5A,B); the male genital complex is also of the same type (type I) as that described for this genus ([3] Figure 5B–E). The results of our mtCOI analysis and comparison support the designation of our specimens as a species of *Caromiobenella*.

A reliable morphologic method to link the sexes of a monstrellid species consists of finding particular autapomorphies shared by both sexes [2]. Thus, having a complete description of both sexes of the nominal *M. brasiliensis* ([8], present data), it was possible to use this morphologic criterion. It was previously applied to designate a male preliminarily identified as a subspecies of *M. wandelli* Suárez-Morales and Islas-Landeros, 1993 as the true male of *M. mariauegeniae* Suárez-Morales, 1994 [23]. During our examination of the males and females from our samples, we were able to find a distinctive autapomorphy shared by both sexes of *Caromiobenella brasiliensis* comb. nov. This key character is the peculiar wrinkled protuberance on the male and female antennules; it is sexually dimorphic, present on segment 2 in the male and on segment 3 in the female ([8], Figure 4A,B); this character has not been observed in any other monstrellid species. In addition, both sexes of *C. brasiliensis* share a short, spiniform element 1 ([8,14], Figure 3A) and a first

antennular segment partially fused with the cephalothorax ([8], Figures 3B and 5A). Another option to link both sexes is by using molecular and genetic markers, a method that was successfully tested to match males and females of Korean monstrilloid copepods [24]. Overall, *Caromiobenella brasiliensis* can be morphologically distinguished from its known congeners by the distinctive process on the second antennular segment and by the paired keel-like processes on the ventral surface of the preanal somite.

Currently, there are nine species of *Caromiobenella* recorded from different geographic regions: *C. polluxea* Jeon, Lee and Soh, 2018, *C. castorea* Jeon, Lee and Soh, 2018, and *C. ohtsukai* Jeon, Lee and Soh, 2019 from South Korea, *C. helgolandica* (Claus, 1863) and *C. serricornis* (Sars, 1921) from Europe and Canada, *C. arctica* (Davis and Green, 1974) from northern Canada, *C. hamatapex* (Grygier and Ohtsuka, 1995) from Japan and Korea, *C. pygmaea* (Suárez-Morales, 2000) from the Mediterranean, *C. patagonica* (Suárez-Morales, Ramírez and Derisio, 2008) from the Beagle Channel, and now *C. brasiliensis* (Suárez-Morales and Dias, 2000) from off Southeast Brazil (Southwestern Atlantic).

4.1. Ecology

As far as we are aware of, monstrilloids have not been hitherto reported from tidal pools of rocky reefs. Rocky reefs are coastal shores made from solid rock and considered reef-like ecosystems. Monstrilloids parasitize of marine benthic invertebrates including benthic polychaetes, but also pyramidellid and vermetid gastropods [1,2] and mussel [4]. Interestingly, the largest known aggregations of monstrilloids have been recorded from reef-related areas [25–27].

In addition, the occurrence of monstrilloids in this coastal region, and particularly in the rocky tide pools, may be related to the abundance of host species. So far, the hosts of *C. brasiliensis* remain unknown, but a potential, locally abundant host species is the brown mussel *Perna perna* (Linnaeus, 1758), previously found, either directly [4] or indirectly [28], in association with *Monstrilla* sp. in southern Brazil.

Most (78.3%) individuals of *C. brasiliensis* from both coastal waters and rocky tidepools were collected during spring. In the rocky tidepools, the densities of *C. brasiliensis* ranged between 2.5 and 15 ind./m³ and contributed up to 6% of the total copepod community in August (Table 1). *Caromiobenella brasiliensis* was found in water with temperatures ranging between 20.4 and 28.8 °C and salinities between 28.1 and 32.0.

4.2. Molecular Remarks

The genetic analysis based on the 681 bp COI fragment confirmed that the six copepods from Rio das Ostras (State of Rio de Janeiro, Brazil) investigated here form a single intraspecific group, and confirm that males showing the described morphology are conspecific with the females found in the surveyed area, designated here as *Caromiobenella brasiliensis* comb. nov. The phylogenetic tree (Tamura–Nei), based on 582 pb COI fragment, showed that the Brazilian species clustered in a major branch (69% bootstrap confidence) containing *Monstrilla ilhoii*, the most closely related species (D = 0.299, Kimura-2-Parameter), followed by a distance of 0.302 (D, Kimura-2-Parameter) by the branch containing three species of *Caromiobenella*, *C. polluxea*, *C. castorea* (the type species), and *C. hamatapex*. All other groups showed distances over 0.450 (D, Kimura-2-Parameter) with respect to the Brazilian copepods. The obtained phylogenetic tree (ML) suggests that the genus *Caromiobenella* is not monophyletic.

4.3. Key to Known Species of *Caromiobenella* (Males)

1. Antennule with wrinkled protuberance on second antennular segment.....
C. brasiliensis (Dias and Suárez-Morales, 2000)
- 1A. Antennule lacking special processes on second segment 2
2. Element 1 (*sensu* Grygier and Ohtsuka, 1995) long, reaching well beyond midlength of succeeding second segment..... *C. polluxea* Jeon, Lee and Soh, 2018

- 2A. Element 1(*sensu* Grygier and Ohtsuka, 1995) not as long, not reaching halfway of second segment *C. ohtsukai* Jeon, Lee and Soh, 2019
3. Element 2d2 (*sensu* Grygier and Ohtsuka, 1995) remarkably long, reaching halfway of fourth segment..... *C. castorea* Jeon, Lee and Soh, 2018
- 3A. Element 2d2 (*sensu* Grygier and Ohtsuka, 1995) not as long, barely reaching distal margin of third segment or shorter..... 4
4. Body length (excluding caudal rami) less than 0.5 mm, 5 caudal setae..... *C. pygmaea* (Suárez-Morales, 2000)
- 4A. Body length (excluding caudal rami) more than 1.0 mm, 6 caudal setae..... 5
5. Elements 2v₁₋₃ (*sensu* Grygier and Ohtsuka, 1995) relatively long, elements 2v_{2,3} reaching beyond halfway of succeeding third segment 6
- 5A. Elements 2v₁₋₃(*sensu* Grygier and Ohtsuka, 1995) short, barely reaching halfway of third segment..... *C. serricornis* (Sars, 1921)
6. Antennulary segment 4 with proximal bulging process..... *C. patagonica* (Suárez-Morales, Ramírez and Derisio, 2008)
- 6A. Antennulary segment 4 lacking proximal bulging process.....7
7. Fifth leg present..... 8
- 7A. Fifth leg absent..... 9
8. Fifth leg 1- lobed, inner margin straight..... *C. helgolandica* (Claus, 1863)
- 8A. Fifth leg 1-lobed, with two setae, inner margin produced..... *C. hamatapex* (Grygier and Ohtsuka, 1995)
9. Antennulary setae A–D (*sensu* Huys et al., 2007) branched..... *C. arctica* (Davis and Green, 1974)

5. Conclusions

The integrative taxonomy approach proved to be useful in solving this particular case among the Monstrilloidea by allowing us to: (1) confirm its identity as a member of *Caromiobenella* and (2) reliably match females and males of *C. brasiliensis*. This nominal species has remained as *Monstrilla brasiliensis* for the last 21 years, but the molecular analysis does not support *Caromiobenella* as a monophyletic genus. Therefore, our findings of the males and those from molecular analysis, allowed us to place the Brazilian species in *Caromiobenella*, thus representing the first discovery of this genus in the Southwestern Atlantic Ocean and the first record of monstrilloids from a restricted habitat (i.e., coastal rocky tidepools). This work represents the second successful use of integrative taxonomy (molecular + morphologic) methods to link both sexes of a monstrilloid copepod. *Caromiobenella* now includes 10 species worldwide.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/d13060241/s1>, Table S1: GenBank sequences used for comparisons with Brazilian copepods sequences (present study) and for the construction of Maximum Likelihood Tree.

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