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# Hidden but not Forgotten: Molecular Diversity and Species Delimitation of Proseriata (Platyhelminthes: Rhabditophora) across the Brazilian Coastline, with Focus on Genus *Kata*

Thainá Cortez[1],*, Taynara Cândido[1], Maikon Di Domenico[2], Sónia C. S. Andrade[1]

[1]*Departamento de Genética e Biologia Evolutiva, Instituto de Biociências, Universidade de São Paulo, São Paulo, Brazil. *Correspondence: E-mail: thainacortez@usp.br (Cortez)*
*E-mail: taynara.candido1996@gmail.com (Cândido); soniacsandrade@ib.usp.br (Andrade)*
[2]*Centro de Estudos do Mar, Universidade Federal do Paraná (CEM-UFPR). E-mail: maik2dd@gmail.com (Di Domenico)*

ORCiD:
Thainá Cortez https://orcid.org/0000-0003-2312-5087
Taynara Cândido https://orcid.org/0000-0003-1473-3830
Maikon Di Domenico https://orcid.org/0000-0002-4232-3962
Sónia C. S. Andrade https://orcid.org/0000-0002-1302-5261

The Proseriata (Platyhelminthes) order represents one of the most abundant meiofauna groups in swash zones. Predicting the diversity and distribution of these organisms is challenging due to the presence of cryptic species. For this reason, molecular data have been increasingly integrated into phylogenetic and population analyses to support reliable species identification. Here, we aimed to assess proseriates diversity along the Brazilian coastline and explore connectivity patterns within sandy beaches. To achieve this, we used partial sequences of the 28S rRNA gene from 32 Proseriata specimens collected in Brazil and Florida (USA). We then performed species delimitation and phylogeographic analyses, focusing on the *Kata* genus. The four delimitation methods were consistent, with slight variation in the number of evolutionary lineages, ranging from seven to eight. Molecular identification revealed species from the genera *Nematoplana*, *Meidiama*, *Parotoplana*, *Kata*, and *Archotoplana* present in the samples. Here, two species of *Kata* were identified in the Brazilian samples, corresponding to *K. leroda* Marcus, 1950, and a yet unidentified species. For both, the analyses revealed that despite the lack of a larval stage, there might be some gene flow across distant locations due to shared haplotypes among populations separated by ~900 km. Here, we provided the first molecular diversity record of Proseriata along the Brazilian coast. Our findings offer significant contributions to the molecular taxonomy field and our understanding of the evolutionary dynamics within this relatively understudied meiofauna group.

**Keywords:** Meiofauna, Molecular taxonomy, Phylogenetics, Population genetics

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## BRACKGROUND

Metazoans with body lengths ranging from 50 to 500 μm are called meiofauna, which inhabit the interstitial spaces between sediment grains (Higgins and Thiel 1988; Giere 2019; Schmidt-Rhaesa 2020). Due to their small size and the lack of diagnostic features in some groups (Giere 2009; *e.g.*, Egger et al. 2020), they are traditionally believed to have a wide distribution (Finaly 2002; Fenchel and Finlay 2004). However, recent studies have shown that predicting the geographic distribution of meiofauna is a complex task since species with broad geographic distributions comprise multiple cryptic species with a limited distribution (*e.g.*, Andrade et al. 2011; Jörger et al. 2012; Leasi et al. 2016; Mendes et al. 2018). The integration of molecular data alongside morphological data has improved taxonomic identification. Nonetheless, there is still a lack of molecular studies for many meiofaunal groups, which hinders investigations into cryptic species and, consequently, major phylogenetic relationships and genetic diversity.

Within the meiofauna, the Proseriata Meixner, 1938, an order of free-living Platyhelminthes, is characterized by its tubiform, plicatus-type pharynx and often elongated, relatively large bodies. These intriguing flatworms are predominantly marine, with over 400 species (WoRMS Editorial Board, August 2023) showing a high diversity in the number of species and morphological traits (Martinez et al. 2024). However, the actual species diversity within this taxon is vastly underestimated (Curini-Galletti et al. 2012; Balsamo et al. 2020). The systematics and phylogeny of Proseriata were built mostly on morphological features alone (*e.g.*, Martens and Curini-Galletti 1989; Lanfranchi and Melai 2008; Delogu and Curini-Galletti 2009). Molecular studies of the group became more frequent in the last decade (*e.g.*, Scarpa et al. 2015 2016a b 2017) but are still particularly scarce in the context of population-level studies.

In Brazil, meiofauna proseriate species have received attention since E. Marcus described several new species (Marcus 1946 1948 1949 1950 1951 1952 1954), followed by the subsequent redescriptions and new records by Curini-Galletti (2014). Among these, the genus *Kata* Marcus, 1949, stands out as one of the most abundant on Brazilian sandy beaches. Its description and subsequent records, primarily from the Southeast coast (São Paulo), are concentrated in the swash zones of reflective beaches—highly energetic environments (Curini-Galletti 2014). Historically, the swash zone of reflective beaches has been referred to as the 'Otoplana-zone,' dominated by

*Otoplana* species, particularly on Atlantic–Mediterranean coasts (Gerlach 1953, Ax 1956). In the western Atlantic, however, *Kata* serves as the ecological counterpart to *Otoplana*.

Until the present study, there were no other records of *Kata* species outside of this area. Two species can be found on the Brazilian coast: *Kata leroda* Marcus, 1950, and *Kata evelinae* Marcus, 1949, which are differentiated by the presence of two vaginae in *K. evelinae*, along with some other distinct sclerotized structural details (Curini-Galletti 2014). Additionally, the species seem to have habitat preferences: *K. leroda* is common in medium sand, while *K. evelinae* inhabits coarser and highly energetic sandy beaches (Curini-Galletti 2014).

Currently, no studies are addressing the genetic diversity and population structure of proseriates in Brazil. Hence, this work aims to assess the Proseriata genetic diversity along the Brazilian coastline. To achieve this goal, we analyzed partial sequences of the 28S rRNA gene from proseriate specimens collected from five locations in Brazil and one location in the United States (USA). We also performed delimitation methods and phylogeographic analyses for the genus *Kata*.

## MATERIALS AND METHODS

### Biological sampling

Thirty-two Proseriata individuals were collected across five Brazilian locations within the Southern and Southeastern regions between December 2021 and July 2022 (Fig. 1 and Table 1) under the Instituto Chico Mendes de Conservação da Biodiversidade license nº 77439. Two additional samples were collected on one sandy beach in Florida, USA. Samples were collected by scooping up the superficial layer of sandy sediments from the lower intertidal level. The worms were extracted from the sand in the laboratory using $MgCl_2$ decantation (Schockaert 1996), and were analysed alive by slight squeezing under the coverslip. For the molecular analyses, all samples were fixed in 99% ethanol and kept at the Laboratório de Diversidade Genômica of the University of São Paulo (IB-USP, Brazil).

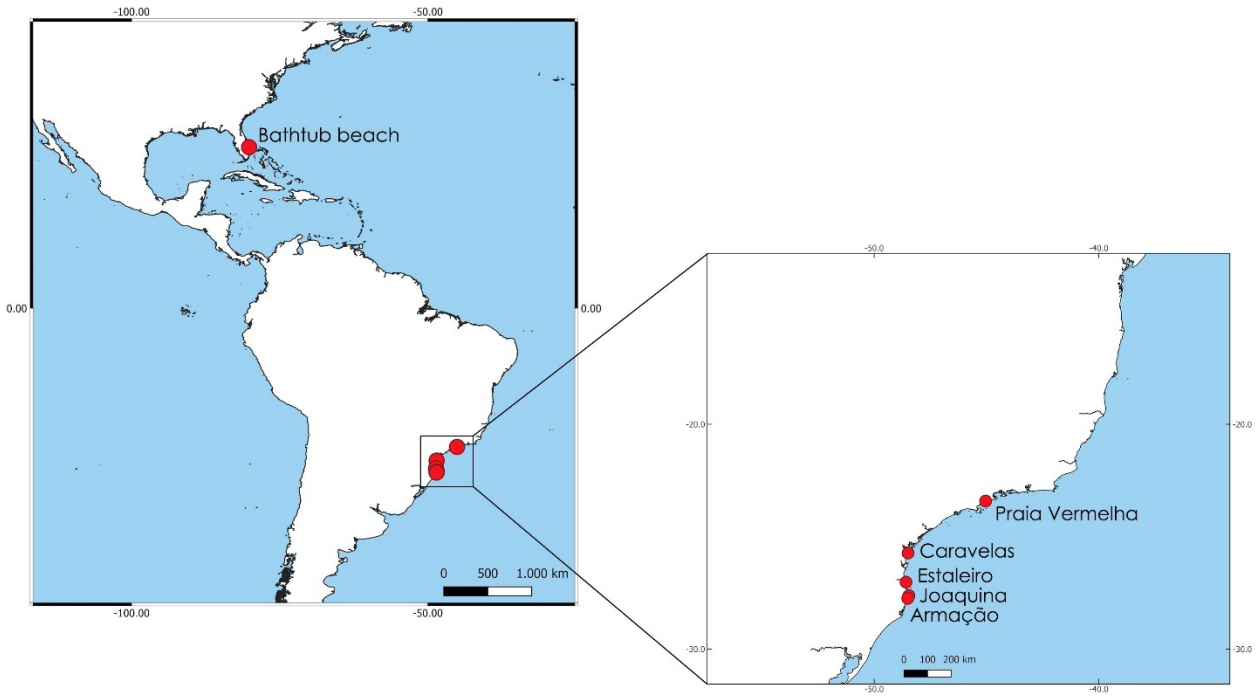


**Fig. 1.** Distribution of sampling locations along the Brazilian and Florida coastline.

**Table 1.** Sampling locations with geographic coordinates

| Location | Abbr | N | City (State), Country | Date | Latitude | Longitude |
|---|---|---|---|---|---|---|
| Armação | AR | 9 | Florianópolis (SC), BR | Dec 2021 | -27.747164 | -48.505924 |
| Joaquina | JO | 8 | Florianópolis (SC), BR | Dec 2021 | -27.625718 | -48.450281 |
| Estaleiro | ES | 5 | Florianópolis (SC), BR | Dec 2021 | -27.027092 | -48.580835 |
| Caravelas | CA | 1 | Matinhos (PR), BR | Dec 2021 | -25.731785 | -48.488227 |
| Praia Vermelha do Norte | VER | 7 | Ubatuba (SP), BR | Dec 2022 | -23.418995 | -45.035664 |
| Bathtub Beach | BB | 3 | Stuart (FL), USA | jul/22 | 27.186984 | -80.16069 |

The table presents the abbreviation (Abbr), the sample size (N) and date collection of each location.

**DNA extraction, amplification and sequencing**

The genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer protocol, and DNA integrity was checked by 1% agarose gel electrophoresis and quantified using the Thermo Scientific NanoDrop Spectrophotometer. The partial sequences of the nuclear ribosomal 28S (28SrRNA) were obtained for subsequent analyses using the primer pair 28Srd1a (5'-CCC SCG TAA YTT AGG CAT AT-3') and 28Srd4b (5'-CCT TGG TCC GTG TTT CAA GAC-3') (Edgecombe and Giribet 2006). The polymerase chain reactions (PCR) were carried out in a 20 µl total volume containing 1 × PCR Buffer, 2.5 U *Taq* DNA Polymerase, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.20 µM of each oligonucleotide, and

ultrapure water to achieve the reaction volume (*Taq* PCR Master Mix, Qiagen®). The cycling profile on the thermal cycler involved a denaturation step at 94°C for 1 min, 35 cycles of denaturation at 94°C for 1 min; annealing at 44ºC for 1 min, extension at 72°C for 1 min; and extension at 72°C for 10 min.

The PCR products were purified using a 15% polyethylene glycol (PEG) solution, followed by amplification for sequencing on both the forward and reverse strands at ACTGene Análises Moleculares (Rio Grande do Sul, Brazil). Chromatograms were analyzed using Geneious v.2023.1 software (Biomatters Limited). Individual consensus sequences were aligned using the automated algorithm (--auto) implemented in MAFFT v.7.520 (Katoh 2019).

**Phylogenetic relationship and species delimitation**

The phylogenetic relationships among taxa were explored using maximum likelihood analysis through RAxML v. 8.2.12 (Stamatakis 2014). The analysis incorporated 1,000 bootstrap replicates (BS), employing the GTRGAMMA model. Here, we used a Polycladida species from the USA as the outgroup (Table S1). The resulting consensus tree was visualized using FigTree v.1.4.4 software(http://tree.bio.ed.ac.uk/software/figtree/).

To delineate evolutionarily independent entities within Proseriata species from this study, we employed species delimitation approaches (Carstens et al. 2013). These approaches encompassed one distance-based method (ASAP) and three tree-based methods (GMYC, bPTP, and mPTP) for species delimitation. The ASAP (Puillandre et al. 2021) was performed using the aligned FASTA file and the Jukes-Cantor method. Both bPTP (Zhang et al. 2013) and mPTP (Kapli et al. 2016) analyses were conducted using the maximum-likelihood (ML) tree from RAxML. The recently introduced multi-rate PTP (mPTP) delimits species assuming a constant speciation rate with different intraspecific coalescent rates. For this analysis, we ran two independent Markov Chain Monte Carlo (MCMC) analyses for 100 million steps, sampling every 10,000 steps, to assess average support values and the confidence of the ML delimitation for each species. The optimal probabilistic model for sequence evolution applied for the Bayesian approach was determined using jModeltest v. 2.1.10 (Darriba et al. 2012), based on an ML optimized search, along with the Akaike information criterion (AIC) and the Bayesian information criterion (BIC). These methods converged to GTR + G model as the most suitable for the dataset. The GMYC method (Fujisawa and Barraclough, 2013) was applied based on the ultrametric gene tree constructed on BEAST v. 1.10.4 (Drummond and Rambaut 2007) under the uncorrelated relaxed clock (Drummond et al. 2006) and the GTR + GAMMA model of substitution. Analyses were performed for 10 million

generations, sampling every 10,000. The maximum clade credibility tree was created using TreeAnnotator v. 1.10.4 (Rambaut 2014) using mean heights for annotation.

After obtaining the delimitation results, all specimen sequences were submitted to BLAST+ v. 2.12.0 (Altschul et al. 1990) through the blastn tool using an e-value threshold of $10^{-3}$. Then, only evolutionary lineages identified as *Kata* were used for new delimitation tests, population, and genetic diversity analysis. The delimitation methods were the same as described above. To avoid misidentification of species and to provide support to our identification, we incorporated data from *Kata* species previously identified with both morphological and molecular data (Curini-Galletti 2014; Scarpa et al. 2015). In addition, a phylogenetic analysis was performed using all *Proseriata* 28SrRNA sequences available in the GenBank database up to January 2025 to determine the placement of our *Kata* specimens, following the same RAxML settings described above.

### Genetic diversity analyses

A parsimony haplotype network was built using the software PopART v. 1.7 (Leigh and Bryant 2015) following the TCS algorithm (Clement et al. 2002) for the evolutionary lineages identified as *Kata* (see results). Then, the following diversity measures were calculated for each cluster individually. The number of haplotypes (H), number of polymorphic sites (PS), and nucleotide diversity ($\theta_\pi$) were measured using Arlequin v. 3.5.2.2 (Excoffier and Lischer 2010). Finally, the haplotype diversity (Hd) was calculated using DnaSP v. 6.12.03 (Librado and Rozas 2009). To confirm the identity provided by blastn, a new phylogenetic analysis was performed using all of our *Kata* species, plus the four sequences available on GenBank, two from *K. leroda* and two from *K. evelinae*. Here, the same settings from RAxML were applied.

## RESULTS

In this study, we generated 32 sequences from Proseriata and one Polycladida species of the partial nuclear gene 28SrRNA (798bp). The localities and GenBank accession numbers of the analyzed specimens are listed in Table S1.

Overall, the methods of species delimitation produced consistent results regarding the number of evolutionary units: eight for ASAP, bPTP, and GMYC, and seven for mPTP (Fig. 2). mPTP grouped two distinct specimens from Joaquina, both identified as *Nematoplana* (Table S2), into a single unit. Three distinct clades were identified as *Kata*, with one of them consisting of a single specimen from the USA, and the other two distributed across the Brazilian coastline. Other

clades were identified by blastn as *Nematoplana* Meixner 1938, *Meidiama* Marcus 1946, *Parotoplana* Meixner 1938, and *Archotoplana* Ax 1956 (Table S2). The outgroup species was identified as Polycladida. In this tree, *Kata* appeared as a sister clade to *Archotoplana*, while *Nematoplana*, in our dataset, represented the basal taxon.

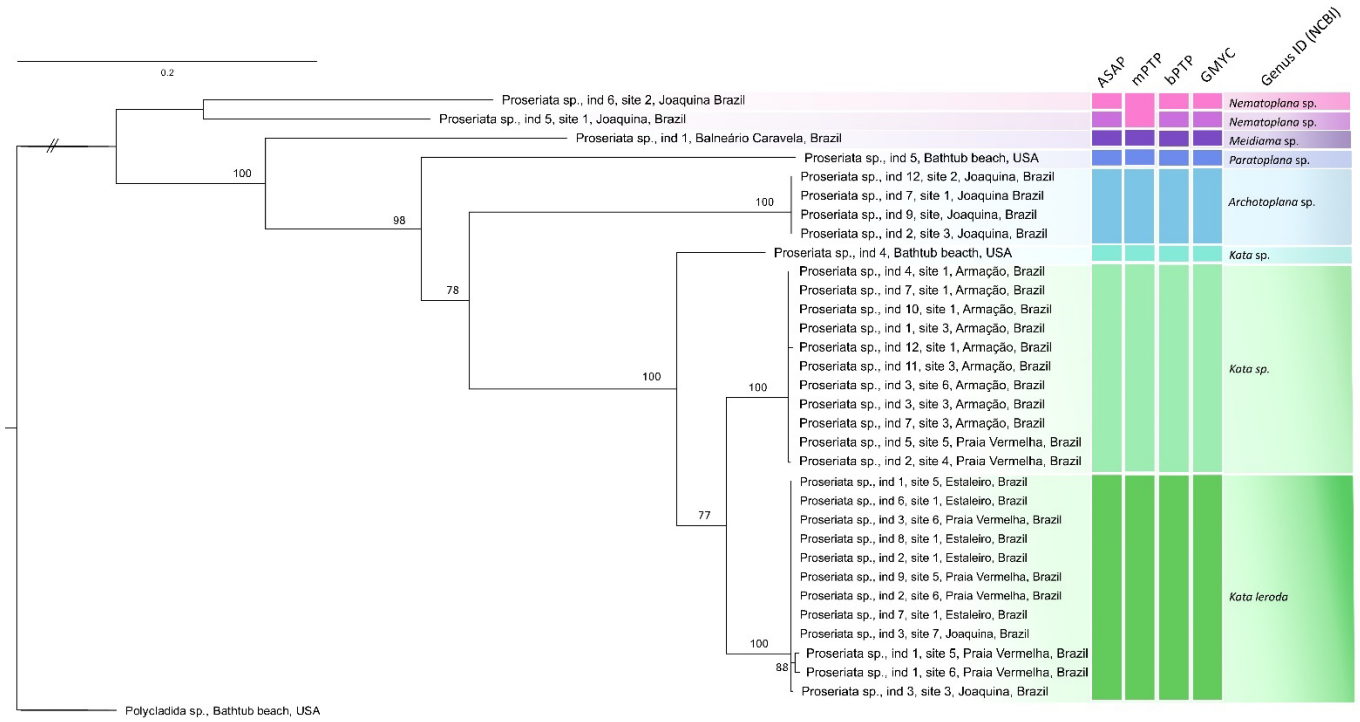


**Fig. 2.** Species delimitations estimated for a 28SrRNA dataset comprising 32 Proseriata specimens. The maximum likelihood tree is shown on the left. Vertical bars on the right represent evolutionary lineages determined by ASAP, mPTP, bPTP, and GMYC, respectively. The final column shows genus-level taxonomic identification based on blastn. Different-colored clades in the tree indicate consensus on species identification.

To provide a more robust molecular identification of the specimens obtained here, a phylogenetic tree was constructed using all 412 Proseriata 28SrRNA sequences available on GenBank (accession codes on Fig. S1). The recovered topology showed that the *Kata* genus is non-monophyletic (Fig. 3 and Fig. S1). A first branch contains three *Kata* sp. from Panama and multiple *Otoplana* sp (BS = 92). The second branch includes only specimens from Brazil and Panama, which form a clade with *Otoplana* (BS = 97). All Brazilian individuals pertain to a single clade (BS = 70) with three internal branches: *K. leroda* (BS = 99), *K. evelinae* (BS = 100), and a third unidentified *Kata* sp. (BS = 100). The *K. leroda* branch includes specimens from previous morphological and molecular studies (Curini-Galletti 2014; Scarpa et al. 2015). In contrast, *K. evelinae* is represented solely by species previously identified using morphological data (Curini-Galletti 2014), with no new specimens sampled here. Finally, the *Kata sp.* clade comprises individuals we sampled in Armação and Praia Vermelha. The specimen from the USA was placed at the base of the clade *Otoplana* + *Kata* together with two individuals from Panama (Fig. 3). The identification performed

with BLAST+ is presented in Table S2. In agreement with the phylogenetic position, the individual from the USA was identified as *Kata,* with a similarity rate of 94.7% (Table S2). The Brazilian specimens were identified as *K. leroda* with similarity rates ranging from 99.5% to 100%, supporting the molecular identification. The non-identified *Kata* sp. revealed about 94% similarity with *K. evelinae.*

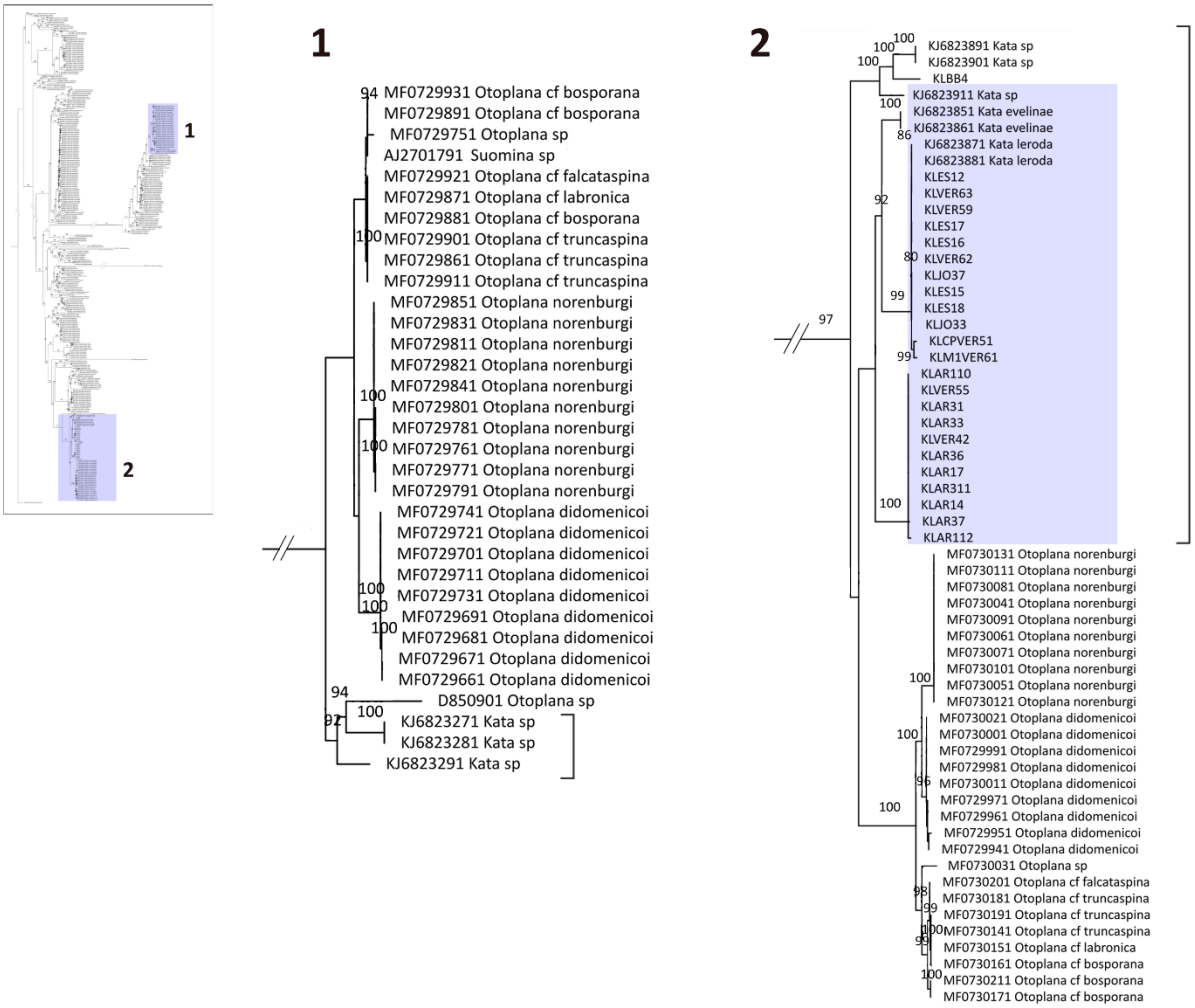


**Fig 3.** Maximum Likelihood phylogenetic tree of Proseriata highlighting the *Kata* genus clade based on the partial 28SrRNA using 1,000 bootstraps replicates (lnL = -40128.5342) The tree includes specimens from the present study, along with all *Kata* sequences available in GenBank. Bootstraps support (BS) values are indicated at each node. Only BS above 80 are shown. Specimens from Brazil are highlighted in the partial tree (2). The complete phylogenetic tree is indicated on the left and also provided in figure S1.

For the Brazilian specimens only, the delimitation tests supported the existence of three lineages (Fig. 4A), where population analyses revealed a pronounced geographic pattern in the distribution of these species. The haplotype network constructed for the two *Kata* species combined revealed that *K. evelinae* and *Kata* sp. are separated from *K. leroda* by 59 and 69 mutational steps, respectively (Fig. 4B). The cluster of the non-identified *Kata* species included 11 individuals (Table S2) from two locations, Armação and Vermelha, yielding only two distinct haplotypes.

Consequently, the haplotype diversity was low (Hd = 0.1818). In the population from Praia Vermelha ($n$ = 2), both individuals shared the same haplotype, resulting in a nucleotide diversity of zero (Table 2). In contrast, the Armação population comprised nine distinct specimens, yet it still exhibited only two haplotypes ($\theta_S$ = 0.0003). *Kata leroda* (Table S2) clade comprises 12 specimens from three locations (Joaquina, Estaleiro, and Praia Vermelha), encompassing just three haplotypes with seven polymorphic sites and four singletons (Fig. 4B). For this reason, the haplotype diversity was high (Hd = 0.4545), where the population from Praia Vermelha population ($n$ = 5) displayed a higher nucleotide diversity ($\theta_S$ = 3.00) with three distinct haplotypes (Table 2). Nevertheless, the Estaleiro population revealed five specimens entirely identical, resulting in a nucleotide diversity equal to zero. In contrast, the Joaquina population ($n$ = 2) had two specimens differing only by one polymorphic site, thereby yielding a nucleotide diversity of one.

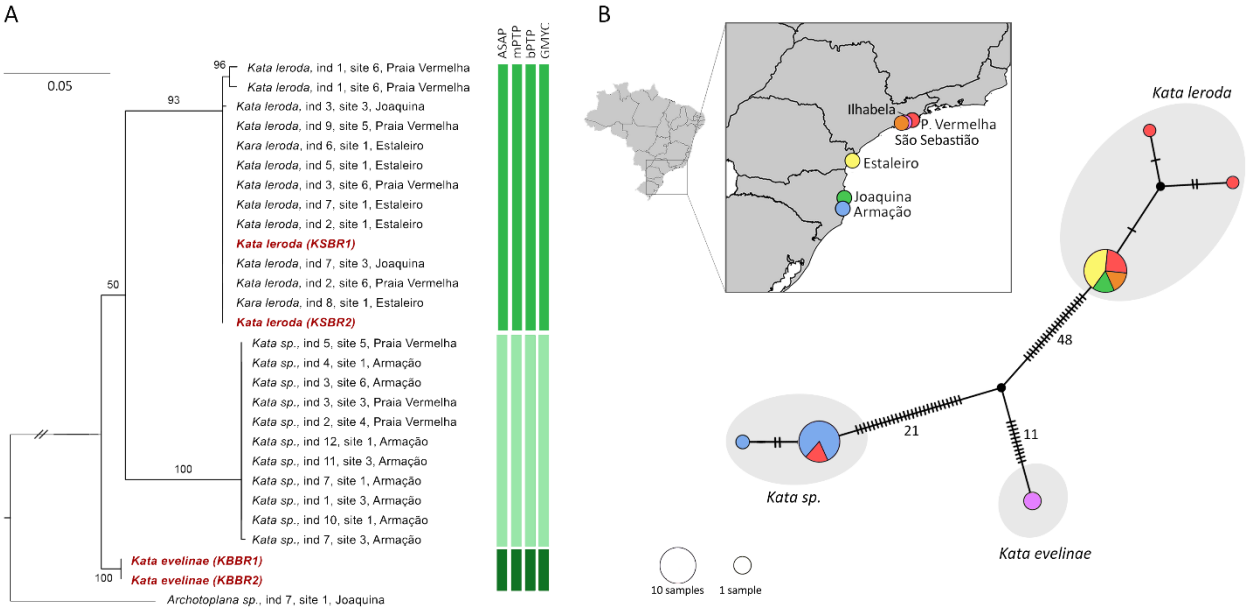


**Fig. 4.** Haplotype networks and a phylogenetic tree constructed from *Kata leroda*, *K. evelinae* and *Kata* sp. 28SrRNA partial genes. A) The maximum likelihood phylogenetic tree shows the relationships among *Kata* species. Vertical bars on the right represent evolutionary lineages determined by ASAP, mPTP, bPTP, and GMYC, respectively. Different-colored clades in the tree indicate consensus on species identification. Taxa in dark red were obtained from NCBI and previously identified based on morphology. B) Haplotype network built using the TCS model for the *Kata* species. Each node represents a haplotype, where its size indicates haplotype frequency, and colors indicate the locality. Black nodes represent inferred ancestral nodes and mutation steps are represented as hatch marks.

**Table 2.** Diversity indices based on 28SrRNA of two *Kata* species obtained from Arlequin software

| Population | n | H | PS | θπ |
|---|---|---|---|---|
| *Kata* sp. | | | | |
| Praia Vermelha (VER) | 2 | 1 | 0 | 0 |
| Armação (AR) | 9 | 2 | 1 | 0.0003 |
| *Kata leroda* | | | | |
| Praia Vermelha (VER) | 5 | 3 | 6 | 3 |
| Estaleiro (ES) | 5 | 1 | 0 | 0 |
| Joaquina (JO) | 2 | 2 | 1 | 1 |

The table presents the sample size (n), number of haplotypes (H), number of polymorphic sites (PS), nucleotide diversity (θπ), and segregating sites ($\theta_S$) of each location from both *Kata* clusters identified on the delimitation tests.

## DISCUSSION

In this study, we sequenced a partial region of the nuclear gene 28SrRNA from 32 proseriates collected from the Southern and Southeastern regions of the Brazilian coastline, as well as specimens from the USA, in order to describe the genetic diversity of the group, focusing on the *Kata* genus. This study presents the first molecular study at a population level of Proseriata along the Brazilian coast. Additionally, it contributes to the understanding of molecular taxonomy within this understudied group in meiofauna diversity investigations.

According to previous records of Proseriata from Brazil, the genera we found have already been documented in the Southeastern coast, including species such as *Meidiama lutheri*, *Nematoplana asita*, *Nematoplana mirabilis*, *Parotoplana, and Kata leroda* (Curini-Galletti 2014), providing support to our molecular identification. The relationship recovered with the ML method implemented here revealed a similar topology previously reported for proseriates using both 18SrRNA and 28SrRNA combined (Scarpa et al. 2017). This is likely because we used the 28S rRNA partial gene, a conserved region among different lineages. This marker has been previously employed for species delimitation and phylogeny investigations with Proseriata (Litvaitis et al. 1996; Scarpa et al. 2015 2016a b 2017), providing reliable results. Nonetheless, it is worth noting that, since our analysis is based on a single gene, both the phylogenetic relationships and the intrapopulation diversity results we observed reflect the evolutionary history of this specific marker.

A phylogenetic relationship assessment was conducted using specimens identified through morphology (Curini-Galletti 2014) and molecular data (Scarpa et al. 2015) to determine the placement of the Brazilian *Kata* specimens within Proseriata. In this matter, the tree topology obtained for all Proseriata sequences placed all Brazilian individuals within a clade alongside

specimens previously identified through refined morphological analyses as *Kata*. The presence of three well-supported clades of *Kata* species was observed along the Brazilian coastline: *K. leroda*, present in Joaquina, Praia Vermelha and Estaleiro, *K. evelinae* from Ilhabela reported in previous studies, and a still non-identified *Kata* sp., found in Armação and Praia Vermelha. This indicates either the existence of an undescribed *Kata* species or the occurrence of cryptic speciation within the genus. Both hypotheses are plausible and warrant further investigation, which should be tested through larger sample sizes, and additional comparative morphological analyses. This should be specifically tested with specimens from Praia Vermelha, where the apparent coexistence of the two *Kata* species provides a unique opportunity for detailed comparative analyses. The specimen from Florida was grouped with two other *Kata* from Panama, sister to a clade containing the Brazilian specimens and *Otoplana*. Due to the lack of more detailed ecological and morphological data on both specimens from Florida and Panama, the data is insufficient to provide a deeper discussion regarding its evolutionary implications.

Additionally, the presence of cryptic speciation between *Kata* and *Otoplana* genera is supported in the phylogeny. The *Kata* specimens from Panama, identified through morphology in previous studies, cluster with two distant *Otoplana* clades. These observations are noteworthy, as *Kata* acts as the ecological counterpart to *Otoplana* on opposite sides of the Atlantic in an area known as the 'Otoplana-zone' (Gerlach 1953, Ax 1956). Further investigation into their species diversity and morphological convergences is needed to enhance our understanding of the meiofauna functional traits in highly dynamic sandy beach environments (*e.g.*, Martinez et al. 2024).

For a long time, it was widely assumed that meiofauna would tend to exhibit wide distribution due to their small size, lack of larval stage, and lack of diagnostic features. Therefore, we would expect a low rate of gene flow and low genetic diversity. Our results seem to partially support this scenario. For *Kata* sp., the individuals from the same beach are entirely – or mostly – genetically identical, revealing very low diversity. For *K. leroda*, despite a higher number of individuals per location, the flatworms appear to be highly similar on a microgeographic scale. However, both species still might exchange migrants among distant locations, supported by the shared haplotypes among distinct locations. An alternative scenario is that these local populations originated from the same evolutionary lineage in the past, composing one single panmictic population. Similar cases have been observed in other direct-developing marine invertebrates (*e.g.*, Derycke et al. 2010; Kieneke and Nikoukar 2017), where the authors found long-distance migration evidenced by shared haplotypes. Despite lacking a dispersal larval stage, certain meiofauna taxa avoid entering the water column by adhering to the sediment, where these individuals may migrate through passive transport or be carried by currents in suspended sediments (Giere 2009; Di Domenico et al. 2014). In these cases, the dispersal of adults may play a more significant role than

the larval stage in explaining genetic diversity (Peres et al. 2018). The discovery of a distinct evolutionary lineage, suggesting the presence of a new, unidentified *Kata* species, should be further confirmed through comprehensive morphological and molecular analyses.

## CONCLUSIONS

In this study, we provided a molecular record of Proseriata and the first study at a population level of the genus *Kata* along the Brazilian coast. Our investigation contributes to the understanding of molecular taxonomy within this relatively understudied group in meiofauna research. We initially hypothesized that the lack of a larval stage in these organisms would result in low local genetic diversity and limited gene flow between distant populations. Instead, for *Kata leroda*, we found evidence of high genetic diversity and dispersal. Our research significantly contributes to the gap of knowledge regarding evolutionary processes at the molecular level in soft-bodied meiofauna and highlights the importance of the combination of morphological and molecular taxonomy for further studies.

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**Authors' contributions:** TCO, SCSA and MDD conceived and designed research. TCO and TCA conducted molecular experiments. TCO and MDD performed the sampling collections. TCO analyzed data and wrote the manuscript. All authors read and approved the manuscript.

**Competing interests:** The authors declare that they have no conflict of interest.

**Availability of data and materials:** Data supporting this study are openly available from GenBank database at accession codes OR526693-OR526725. More details are provided in Table S1.

**Consent for publication:** All of the authors declare that they have approved the manuscript submission.

**Ethics approval consent to participate:** Not applicable.

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**Supplementary materials**

**Fig S1.** Maximum Likelihood phylogenetic tree constructed based on 28SrRNA partial sequences of Proseriata species including those obtained in the present study (lnL = -40128.5342). The tree was built using 1,000 bootstrap replicates (BS). The major clades where *Kata* genus is included are highlighted in blue. Only BS greater than 80 are shown. (download)

**Table S1.** GenBank accession codes of individual 28SrRNA sequences. (download)

**Table S2.** Blast results for all specimens used in this study. (download)