Molecular Phylogeny of Weakfish Species of the Stellifer Group (Sciaenidae, Perciformes) of the Western South Atlantic Based on Mitochondrial and Nuclear Data

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Abstract

The phylogenetic relationships within the Stellifer group of weakfishes (Stellifer, Odontoscion, Ophioscion, and Bairdiella) were evaluated using 2723 base pairs comprising sequences of nuclear (rhodopsin, TMO-4C4, RAG-1) and mitochondrial (16S RNA and COI) markers obtained from specimens of nine species. Our results indicate a close relationship between Bairdiella and Odontoscion, and also that the genus Stellifer is not monophyletic, but rather that it consists of two distinct lineages, one clade containing S. microps/S. naso/S. brasilensis and the other, S. rastrifer/ S. stellifer/ Stellifer sp. B, which is closer to Ophioscion than the former clade. The O. punctatissimus populations from the northern and southern Brazilian coast were also highly divergent in both nuclear (0.8% for rhodopsin and 0.9% for RAG-1) and mitochondrial sequences (2.2% for 16S RNA and 7.3% for COI), which we conclude is consistent with the presence of two distinct species. The morphological similarities of the members of the Stellifer group is reinforced by the molecular data from both the present study and previous analyses, which have questioned the taxonomic status of the Stellifer group. If, on the one hand, the group is in fact composed of four genera (Stellifer, Ophioscion, Odontoscion, and Bairdiella), one of the two Stellifer clades should be reclassified as a new genus. However, if the close relationship and the reduced genetic divergence found within the group is confirmed in a more extensive study, including representatives of additional taxa, this, together with the morphological evidence, would support downgrading the whole group to a single genus. Obviously, these contradictory findings reinforce the need for a more systematic taxonomic revision of the Stellifer group as a whole.

Introduction

The family Sciaenidae includes approximately 70 genera and 270 species of demersal fishes found mainly over muddy or sandy bottoms of the continental shelf of the Atlantic, Indian, and Pacific oceans, as well as freshwater genera in the rivers of the Old and New Worlds [1,2]. In the western South Atlantic, sciaenids are abundant and highly diverse, encompassing approximately 50 species representing 19 genera [3,4].

Chao [5] evaluated the phylogenetic relationships of the 21 western Atlantic sciaenid genera and two freshwater genera based on morphological traits, and identified 11 suprageneric groups: Micropogonias, Nebrius, Pogonias, Sciaenops, Larimus, Sciaena, Umbrina, Menticirrhus, Lonchurus, Cynoscion, and Stellifer. Of these groups, Stellifer can be distinguished from all the others by the presence of two (rather than one) pairs of large otoliths and a swim bladder with two (rather than one) chambers.

The Stellifer group includes four genera – Stellifer, Ophioscion, Bairdiella, and Odontoscion – represented by 12 species in the western South Atlantic: Stellifer naso, S. griseus, S. venezuelae, S. brasiliensis, S. microps, S. rastrifer, S. stellifer, Stellifer. sp. A, Stellifer. sp. B, Odontoscion dentex, Ophioscion punctatissimus, and Bairdiella ronchus [5]. These species are characterized by a very strong second anal spine, two pairs of large otoliths, and a swim bladder with two chambers, a carrot-shaped posterior chamber, and the anterior one yoke-shaped with a pair of diverticula on the posterolateral surface [4,5].

Species of the Stellifer group are widely distributed in the western Atlantic, where they are abundant in coastal and estuarine waters with sandy or muddy bottoms [6,7], including the coast of Brazil [8–15]. This group is especially appropriate for studies of the genetic connectivity of populations because the species are widely distributed, and normally inhabit estuarine environments. Despite this, few studies have focused on the bio-ecological or phylogenetic characteristics of this group. Regarding the phylogenetic relationships, all the available studies [1,5,16,17] have emphasized the close relationships among Bairdiella, Stellifer, Ophioscion, and Odontoscion, although intergeneric and interspecific relationships have yet to be defined conclusively due to the limitations or inconsistencies found in the data, as described below.
The first phylogeny based on morphological traits was proposed by Chao [5], who concluded that Stellifer is most closely related to Ophioiscus, with Bairdiella appearing as a sister group to Odontoscion. In a subsequent morphological study, Sasaki [1] suggested that Ophioiscus and Stellifer are sister groups which form a clade with Bairdiella, whereas Odontoscion is related to the sciaenids of the eastern Pacific, Elattarchus and Corvula.

In a phylogenetic study based on 16S rRNA sequences, Vinson et al. [16] confirmed the close relationship between Stellifer and Bairdiella, although they did not include Ophioiscus or Odontoscion in their analyses, impeding the systematic assessment of the evolutionary relationships within the group. In a recent study based on both mitochondrial (COI and 16S rRNA) and nuclear markers (TMO-4C4), Santos et al. [17] concluded that Stellifer is a sister group of Ophioiscus and that Bairdiella is the basal taxon within the group, confirming the proposal of Sasaki [1]. However, as in Vinson et al. [16], the relationships between all of the taxa of the Stellifer group could not be defined because Odontoscion was not included in the analyses. Additionally, the relationships among the Stellifer species remain unclear, given that, in Vinson et al. [16], S. microops is a sister group to S. naso and S. rastrifer is closely related to S. stellifer, whereas in Santos et al. [17], S. rastrifer is a sister group to Stellifer sp., and S. stellifer is more closely related to O. punctatissimus.

In addition to the divergences in the conclusions of the morphological studies regarding the intergeneric relationships within Stellifer group, then, there are also disagreements among molecular phylogenies, especially with regard to the relationships among the Stellifer species. Given this, the present study evaluates the phylogenetic relationships within the Stellifer group, including all of its genera, using nuclear (TMO-4C4, RAG-1, and rhodopsin) and mitochondrial (16S rRNA and COI) markers, all of which have been widely used in phylogenetic reconstructions of fish taxa [17–27].

Materials and Methods

Ethics Statement

The species analyzed in the present study are not endangered or protected in the regions from which samples were obtained. The specimens were captured by artisanal fishermen and processed (collection, handling, transportation, and DNA extraction) with the authorization of the Brazilian Environment Ministry through permit number 12773-1 emitted in the name of Dr. Iciàlda Sampaio. All work was performed in compliance with and approved by the Ethics Committee of the Federal University of Pará.

Sampling

A total of 36 samples representing nine species of the four genera of the Stellifer group distributed in the western South Atlantic were collected along the Brazilian coast (Table 1). Most of the specimens were obtained from the Sciaenidae tissue bank of the UFPA Genetics and Molecular Biology Laboratory of the Institute of Coastal Studies in Bragança, Brazil. The species were identified using the specialized literature [3], and muscle tissue was extracted from each specimen and conserved in absolute ethanol and frozen until analysis in the laboratory.

DNA Extraction, PCR, and Genomic Sequencing

Total DNA was extracted by using the Wizard genomic DNA purification kit (Promega, Madison, Wisconsin, USA) following the protocol for extraction from muscle tissue as defined by the manufacturer. To evaluate the quality of the DNA, samples were electrophoresed in 1% agarose gel stained with GelRed (Biotium Inc., Hayward, California, USA) and analyzed under a UV transilluminator.

The mitochondrial (16S rRNA and COI) and nuclear (TMO-4C4, RAG-1, and rhodopsin) regions were amplified by PCR using the primers and amplification cycles described in Table 2. The RAG-1 region was amplified using a nested PCR, in which the primers 2510F [20] and RAG1R1 [32] were used first, followed by a second amplification using the primers RAG1F1 and RAG1R2 [32]. The reactions were conducted in a final volume of 25 μl containing 4 μl of dNTPs (1.25 mM), 2.5 μl of PCR buffer (10X), 1 μl of MgCl2 (50 mM), 1 μl of DNA (100 ng/μl), 1 μl of each primer (50 ng/μl), 0.2 μl of Taq DNA Polymerase (5 U/μL, Invitrogen, Carlsbad, California, USA), and sterile water to complete the final volume. The PCR products were run on an agarose gel (1%) stained with GelRed (Biotium Inc., Hayward, California, USA) to verify the quality of the amplification products under ultraviolet light.

The positive PCR products were purified with ExoSAP-IT (Affymetrix, Cleveland, Ohio, USA) following the manufacturer's instructions, and sequenced by the di-deoxyterminator method with reagents from the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, California, USA). Electrophoresis was conducted in an ABI 3500XL automatic sequencer (Applied Biosystems).

Phylogenetic and Nucleotide Divergence Analyses

The sequences obtained were manually edited, and aligned using the CLUSTAL W algorithm [33] implemented in the BioEdit 7.2.5 program [34]. Some of the 16S rRNA and TMO-4C4 sequences included in the analysis were obtained from GenBank (see Table 1). Nucleotide saturation of each set of data was evaluated by plotting transitions and transversions against genetic distances in DAMBE 4.0.65 [35].

Phylogenetic relationships were reconstructed based on both the individual data sets (per gene) and the concatenated data, using maximum parsimony, maximum likelihood, and normal and hierarchical Bayesian inference approaches. Two species of the family Lutjanidae, Ocyurus chrysurus and Lutjanus punctatus, the probable sister group of the Sciaenidae, were used as the outgroups for all analyses (Table 1). The evolutionary models used in the phylogenetic reconstructions were obtained in jModeltest 0.1.1 [36]. The maximum parsimony analysis was run using a heuristic search with 1,000 random step-wise additions, using the subtree pruning-regrafting (SPR) algorithm with branch-swapping in PAUP* 4.0b10 [37]. The maximum likelihood tree was constructed in PhyML v3.0 [38] using a heuristic search to find the most probable topologies based on the substitution models TIM2ef+I+G (for 16S rRNA), TIM2+I+G (COI), K80+I (TMO-4C4), TIM1+G (rhodopsin), and TrNef+I+G (RAG-1), and, TPM1uf+I+G for the concatenated data set. Statistical support for the maximum parsimony and likelihood analyses was determined using 1,000 bootstrap pseudoreplicates [39].

Bayesian inference analyses were run in MrBayes 3.1.2 [40] using the evolutionary models TPM2+G (for 16S rRNA), TrN+I+ G (COI), K80+I (TMO-4C4), TPM1+G (rhodopsin), and K80+I+ G (RAG-1). Metropolis-coupled Markov chain Monte Carlo (MCMCMC) sampling was conducted with two independent runs of 3,000,000 generations to estimate the posterior probabilities of the observed clades, using the parameters defined by the models as starting values. The Bayesian posterior probabilities for the clades were determined using the 50% consensus rule for trees sampled every 20 generations after removing the trees produced before the chains became stationary. The burn-in was empirically defined by
Table 1. Species and genomic regions used in the present study, including the samples used as outgroups.

| Family         | Species                  | N   | Brazilian state of origin | GenBank accession number          |
|----------------|--------------------------|-----|---------------------------|-----------------------------------|
|                |                          |     |                           | 16S rRNA | COI  | TMO-4C4 | RHOD | RAG-1       |
| Sciaenidae     | Ingroup                  |     |                           |          |      |         |      |             |
|                | Bairdiella ronchus       | 2   | Pará                      | JX903962, KJ907197                | KJ907229, KJ907230 | JX904028, KJ907267 | KJ907299, KJ907300 | KJ907335, KJ907336 |
|                | Bairdiella ronchus       | 2   | São Paulo                 | KJ907198, KJ907199                | KJ907231, KJ907232 | KJ907268, KJ907269 | KJ907301, KJ907302 | KJ907337           |
|                | Odontoscion dentex       | 5   | Espírito Santo            | KJ907200–KJ907204                | KJ907233–KJ907237 | KJ907270–KJ907274 | KJ907303–KJ907307 | KJ907338–KJ907342 |
|                | Ophioscion punctatissimus| 2   | Pará                      | JX903981, KJ907205                | KJ907238, KJ907239 | JX904047, KJ907275 | KJ907308, KJ907309 | KJ907343, KJ907344 |
|                | Ophioscion punctatissimus| 3   | São Paulo                 | KJ907206–KJ907208                | KJ907240–KJ907242 | KJ907276–KJ907278 | KJ907310–KJ907312 | KJ907345, KJ907346 |
|                | Stellifer brasiliensis    | 3   | São Paulo                 | JX903988, KJ907209, KJ907210    | KJ907243–KJ907245 | JX904054, KJ907279 | KJ907313–KJ907315 | KJ907347           |
|                | Stellifer microps        | 2   | Pará                      | KJ907211, KJ907212               | KJ907246, KJ907247 | KJ907281, KJ907282 | KJ907316, KJ907317 | KJ907348           |
|                | Stellifer naso           | 3   | Pará                      | KJ907213–KJ907215               | KJ907248–KJ907250 | KJ907283–KJ907285 | KJ907318, KJ907319 | -                 |
|                | Stellifer rastrifer      | 4   | Pará                      | KJ907216–KJ907219               | KJ907251–KJ907254 | KJ907286–KJ907289 | KJ907320–KJ907323 | KJ907349–KJ907352 |
|                | Stellifer rastrifer      | 1   | Santa Catarina            | KJ907220                        | KJ907255          | KJ907290          | KJ907324          | KJ907353           |
|                | Stellifer sp. B          | 5   | São Paulo                 | JX903992, KJ907221–KJ907223     | KJ907256–KJ907260 | JX904058, KJ907291–KJ907293 | KJ907325–KJ907328 | KJ907354–KJ907357 |
|                | Stellifer stallifer       | 3   | Pará                      | JX903991, KJ907224, KJ907225    | KJ907261–KJ907263 | JX904057, KJ907294, KJ907295 | KJ907329–KJ907331 | KJ907358, KJ907359 |
|                | Stellifer stellifer      | 1   | São Paulo                 | KJ907226                        | KJ907264          | KJ907296          | KJ907332          | KJ907360           |
|                | Outgroup                 |     |                           |          |      |         |      |             |
|                | Lutjanidae               |     |                           |          |      |         |      |             |
|                | Lutjanus purpureus       | 1   | -                         | KJ907227                        | KJ907265          | KJ907297          | KJ907333          | KJ907361           |
|                | Ocyurus chrysurus        | 1   | -                         | KJ907228                        | KJ907266          | KJ907298          | KJ907334          | KJ907362           |

GenBank accession numbers are listed. N is the number of individuals used, and the Brazilian state of origin is the site where the samples were collected. doi:10.1371/journal.pone.0102250.t001
evaluating the likelihood values. Convergence of the data was evaluated by verifying the parameters throughout the generations in Tracer 1.5 [41].

A species tree was constructed according to the hierarchical Bayesian inference principle in the BEAST 1.7.4 software package [42]. In this analysis, one tree was defined a priori, and each species of the group was considered to be a valid taxon. Markov chain Monte Carlo (MCMC) sampling was performed for 450 million of the group was considered to be a valid taxon. Markov chain Monte Carlo (MCMC) sampling was performed for 450 million generations with parameters sampled every 1,000 generations, and an initial burn-in of 10%. Convergence of the parameters was evaluated in Tracer 1.5 [41]. All of the trees obtained were viewed and edited in FigTree 1.4.0 [43].

Nucleotide divergence within and among the lineages for each set of data were assessed using uncorrected distance in the principal maximum likelihood tree is shown here (Figure 1). The principal Bayesian inference trees all presented similar topologies, only the statistical support is weak. All the results suggest the monophyly of the Stellifer group, with significant bootstrap and posterior probability values (Figures 1 and 2). However, it was not possible to determine which of the group’s lineages is basal because all three approaches produced a polytomous arrangement (Figures 1 and 2).

The close relationship between Bairdella and Odontoscion was well supported in all of the analyses (Figures 1 and 2). Our results also suggest that the genus Stellifer is not monophyletic because the species S. rastrifer, S. stellifer, and Stellifer sp. B form a clade closely related to Ophioscion, with significant statistical support (Figures 1 and 2), whereas S. micros, S. naso, and S. brasiliensis form a distinct clade, which is also strongly supported by bootstrap and posterior probability values (Figures 1 and 2).

Regarding the interspecific relationships within genus Stellifer, S. naso is a sister group to S. micros, composing a clade along with S. brasiliensis (Figures 1 and 2). In the second clade containing the other species of Stellifer, the low bootstrap and posterior probability values did not allow a reliable definition of the evolutionary relationships among Stellifer sp. B, S. rastrifer and S. stellifer (Figures 1 and 2).

All the analyses supported the separation of the northern (Pará) and southern (São Paulo) lineages of O. punctatissimus, based on high bootstrap and posterior probability values (Figures 1 and 2).

**Discussion**

This is the first molecular phylogeny that includes species representative of all four genera of the Stellifer group, as proposed by Chao [3]. The results of all of the analyses suggest the monophyly of the group (Figures 1 and 2), and are consistent with those of morphological analyses [5] and a molecular study of 17 sciaenid genera, including those of the Stellifer group [17]. However, as the Sciaenidae is a large family that includes some 70

Table 2. Primers and amplification protocols for the mitochondrial and nuclear markers.

| Marker | Primer | Reference | Amplification protocol |
|--------|--------|-----------|------------------------|
| 16S rRNA | L1987: 5’ GCCCTGCGCTTTTACCAAAAAC 3’ | Modified from Palumbi [28] | Initial denaturation at 94°C for 3’; 30 cycles at 94°C for 30’ (denaturation), 50°C for 30’ (annealing), and 72°C for 30’; and final extension at 72°C for 30’ |
|         | H2609: 5’ CCGGTCGAACTGGTACAC 3’ | | Initial denaturation at 94°C for 3’; 30 cycles at 94°C for 40’ (denaturation), 59°C for 30’ (annealing), and 72°C for 30’; and final extension at 72°C for 7’ |
| COI     | FishF1: 5’ TCACCAACCAACAAAGACATTGGC 3’ | [29] | Initial denaturation at 94°C for 3’; 30 cycles at 94°C for 30’ (denaturation), 60°C for 30’ (annealing), and 72°C for 1’; and final extension at 72°C for 7’ |
|         | FishR1: 5’ TAGACCTCTGGGTGGCACAAGATCA 3’ | | |
| TMO-4C4 | F2: 5’ GGGCCTTCTAAAACCTCTCATTAAG 3’ | [30] | Initial denaturation at 95°C for 2’; followed by 35 cycles at 95°C for 30’ (denaturation), 60°C for 30’ (annealing), and 72°C for 1’; and final extension at 72°C for 7’ |
| R2: 5’ GTGCCTCGGTGACAAGATCTACAG 3’ | | |
| Rhodopsin Rod-F2 W: 5’ AGCAATTCGCGCTCCTCATAAG 3’ | [31] | Initial denaturation at 95°C for 7’; 40 cycles at 94°C for 30’ (denaturation), 59°C for 30’ (annealing), and 72°C for 30’; and final extension at 72°C for 7’ |
| Rod-4R: 5’ CTGCTTGTCAATGCAGATGTAGAT 3’ | | |
| RAG-1   | 2510 L: 5’ TGGGCCATCCGGTMAAAC 3’ | [20, 32] | Initial denaturation at 94°C for 3’; followed by 40 cycles at 94°C for 30’ (denaturation), 58°C for 45’ (annealing), and 72°C for 45’; and final extension at 72°C for 10’ |
| RAG1R1: 5’ CTGATCCTGTCAGGCTCCATRAAYTT 3’ | | |
| RAG-1   | RAG1F1: 5’ CGCCGTCGTCAGTACCAATAAGATGT 3’ | [32] | Initial denaturation at 94°C for 3’; followed by 40 cycles at 94°C for 30’ (denaturation), 58°C for 45’ (annealing), and 72°C for 45’; and final extension at 72°C for 10’ |
| RAG1R2: 5’ TGGCCCTCCTAATTTCTGAAGTAYTT 3’ | | |

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genera, further analyses including the Stellifer group and other closely-related sciaenids, will be necessary for a more conclusive evaluation of the group’s monophyletic status.

Bairdiella is a sister group to Odontoscion in all the topologies generated in the present study (Figures 1 and 2), which corroborate Chao’s [5] arrangement, based on morphological traits. By contrast, the findings of Sasaki [1] indicate that Stellifer/Ophioscion/Bairdiella share a common ancestor, whereas Odontoscion would be more closely related to the eastern Pacific Ellatarchus and Corvulla. These results contrast with those obtained in the present study and the phylogenies determined by Chao [5] and Santos et al. [17]. However, Ellatarchus and Corvulla were not included in either the present study or the previous ones [5,17], which means that further phylogenetic analyses will be necessary to resolve these contradictions.

The results of the present study confirm that Stellifer is not monophyletic. The Stellifer sp. B/S. rastrifer/S. stellifer clade shares a common ancestry with O. punctatissimus, whereas S. microps, S. naso, and S. brasiliensis form a distinct clade, in both cases supported by significant bootstrap and posterior probability values (Figures 1 and 2). These results refute the morphology-based hypotheses [1,5] and are consistent with the arrangement proposed by Santos et al. [17], who concluded that Stellifer comprises two distinct lineages, and that Stellifer sp. B/S. stellifer/S. rastrifer would be closer to O. punctatissimus than the second clade. Given these findings, we suggest that either one of the two Stellifer clades should be assigned to a new genus or that the entire group should be subsumed into a single genus. Either way, additional morphological and molecular studies, including more species from the Stellifer group, will be necessary to reach a more conclusive evaluation of the phylogenetic relationship of this group.

Within Stellifer, our results corroborate the close phylogenetic relationship between S. microps and S. naso proposed by Vinson et al. [16], as well as the conclusions of Santos et al. [17] on the S. naso/S. microps/S. brasiliensis clade. However, our findings contrast with those of the latter study [17] with regard to the relationship

Figure 1. Maximum likelihood tree for the Stellifer group, based on mitochondrial (COI and 16S rRNA) and nuclear DNA sequences (rhodopsin, TMO-4C4, and RAG-1). The numbers above the branches represent the bootstrap values for maximum likelihood and maximum parsimony, and posterior Bayesian probabilities, respectively.
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between S. rastrifer, S. stellifer, and Stellifer sp. B. In the earlier study, S. stellifer was identified as a sister group of O. punctatissimus, whereas in the present one, this species is closer to its congeners than Ophioscion (Figures 1 and 2).

One surprising result of this study was the formation of two distinct and statistically well-supported clades of O. punctatissimus from northern (Pará) and southern (São Paulo) coasts of Brazil (Figure 1). In fact, genetic divergence in both mitochondrial and nuclear genes (2.2% for rRNA 16S, 7.3% for COI, 0.8% for TMO-4C4, 0.2% for Rhod, and 0.9% for RAG-1) is similar to or greater than that found between valid sciaenid species [16] and those of other fish families [23,45,46], which leads us to suggest that speciation occurred in the taxa. Ophioscion punctatissimus is the only species of this genus found in Brazil, which eliminates possible errors of identification of the specimens. The northern and southern populations are separated by more than 5000 km of coastline, and inhabitat areas with distinct geomorphological and oceanographic characteristics [47,48], all of which may have contributed to a reduction in the gene flow between the two populations, and the differentiation observed in the present study.

A number of studies have nevertheless pointed out other factors, such as life-history traits, the ecological requirements of the species [49–53], or historic events, such as glaciations, as the primary determinants of genetic differentiation and speciation in fish [45,54–57]. Population differentiation and speciation have been recorded in western Atlantic sciaenids, such as Macrodon [58,59], which has two highly divergent lineages distributed in the western South Atlantic that were recently differentiated as M. ancylodon and M. atricandus by Carvalho-Filho et al. [60]. Mitochondrial and nuclear DNA sequences also indicate that the two distinct lineages of Larimus breviceps from the western South Atlantic may also represent distinct species [17,61]. Given these findings, there is a clear need for more comprehensive data on the populations of O. punctatissimus, including additional molecular markers and specimens from a wider geographical area, in order to determine the exact levels of genetic differentiation and the range of each lineage.

In summary, the morphological similarities of the members of the Stellifer group [5] is reinforced by the molecular data from both the present study and previous analyses [16,17], which have questioned the taxonomic status of the Stellifer group. If, on the one hand, the group is in fact composed of four genera (Stellifer, Ophioscion, Odontoscion, and Bairdiella), one of the two Stellifer clades should be reclassified as a new genus. However, if the close relationship and the reduced genetic diversity (data not shown) found within the group is confirmed in a more extensive study, including representatives of additional taxa, this, together with the morphological evidence, would support downgrading the whole group to a single genus. Obviously, these contradictory findings reinforce the need for a more systematic taxonomic revision of the Stellifer group as a whole.

Conclusions

This study presents the most comprehensive molecular phylogeny yet produced for the genera of the Stellifer weakfish group. The analyses found close relationships among the taxa of the group, as well as two distinct lineages of Stellifer. In addition, marked genetic differentiation was found between the O. punctatissimus populations from northern and southern Brazil, suggesting that speciation occurred in the taxa. All these findings reinforce the need for more comprehensive analyses using both molecular markers and morphological traits for the definition of the phylogenetic relationships within the group.
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Author Contributions

Conceived and designed the experiments: AJBB SS. Performed the experiments: AJBB SS. Analyzed the data: AJBB IS HS SS. Contributed reagents/materials/analysis tools: IS HS SS. Contributed to the writing of the manuscript: AJBB IS HS SS. Obtained permission for sample collection: IS.

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