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Short Communication

# Phylogenetic relationships of *Gymnothorax bacalladoi* Böhlke and Brito (1987) a poorly known moray of the Macaronesian islands

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## 1. Introduction

Moray eels, in spite of their diversity, presence in all tropical and temperate oceans (Nelson, 2006) and their role as major predators in reef ecosystems (Thresher, 1984; Ineich et al., 2007) have been poorly studied from a phylogenetic perspective, with obvious consequences for their taxonomy. Northeastern Atlantic morays belong to several genera namely *Muraena*, *Gymnothorax*, *Enchelycore* and *Anarchias*, which are also present in the Indian and Pacific oceans (Froese and Pauly, 2008) an observation that raises the possibility that the morays of the Lusitanian province may belong to lineages which diverged before the closure of the Tethys Sea.

As recently as 2007, Jiménez et al. used genetic markers to demonstrate that *Muraena helena* and *Muraena augusti* are indeed valid species. The same authors showed that *Muraena robusta*, a species from tropical Africa, was retrieved in groups with species of the genus *Gymnothorax*, casting doubts on the monophyly of both genera.

*Gymnothorax bacalladoi* (Böhlke and Brito, 1987) is a poorly known species described from waters around the Canaries, which likely ranges also to Madeira and Cape Verde (Brito et al., 1999; Brito et al., 2002; Smith and Brito, in press). Only a few specimens have been collected and its phylogenetic relationships are unknown. Böhlke and Brito (1987) noted that it differs in teeth morphology and lateral-line pore system from other Atlantic members of the genus *Gymnothorax*, showing closer affinities with several Indo-Pacific species, a finding which raised the possibility of it being a paleoendemism from the Macaronesian islands related with the Tethys Sea.

In this note, we use three mitochondrial markers and a fragment of the nuclear gene for rhodopsin to investigate the phylogenetic position of *G. bacalladoi*. Samples of other morays present in eastern Atlantic from the genera *Gymnothorax*, *Muraena* and *Enchelycore* were also included in the analysis.

## 2. Material and methods

### 2.1. Morays samples

Total DNA from moray samples belonging to 27 specimens of *Gymnothorax polygonius, Gymnothorax maderensis, Gymnothorax miliaris, Gymnothorax unicolor, Gymnothorax bacalladoi, M. augusti, M. helena* and *Enchelycore anatina* was extracted with a REDExtract-N-Amp kit (Sigma–Aldrich, www.sigma.com). Voucher specimens are deposited in ISPA collections (ethanol preserved tissues). A table with the name of the vouchers and the correspondence with GenBank Accession Numbers, per gene fragment is provided as Supplementary material.

Fragments of 16S, 12S and cytochrome *b* mitochondrial genes and the rhodopsin nuclear gene were amplified and sequenced. Specific moray primers for 16S, 12S and cytochrome *b* fragments were designed (see Table 1) with the software PRIMER 3.0 (http://fokker.wi.mit.edu/primer3/input.htm). PCR amplification

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**Table 1**
Primers and annealing temperatures used for each fragment.

| Primer name | Sequence | Fragment | Source | Melting temperature (°C) |
|---|---|---|---|---|
| 16SM-F | 5′AGCTGGTTGCTCAGAAAATG3′ | 16S | This study | 58 |
| 16SM-R | 5′CAACATCGAGGTCGTAAACC3′ | | | |
| 12SM-F | 5′ACGCCTTGCTTAGCCACAC3′ | 12S | This study | 60 |
| 12SM-R | 5′GCACCTTCCAGTACACTTACCA3′ | | | |
| CytBM-F | 5′TGGCAAACCTACGAAAAACC3′ | Cyt *b* | This study | 50 |
| MMB-R | 5′TTATCAGTCGAGTATTTT(AG)TTTTCT3′ | | Jiménez et al. (2007) | |
| Rod-F2B | 5′GTCTGCAAGCCCATCAGCAACTTCCG3′ | Rhodopsin | Jiménez et al. (2007) | 62 |
| Rod-5R | 5′GGTGGTGATCATGCAGTGGCGGAA3′ | | | |

reactions were performed in a 20 μl total-reaction volume with 10 μl of REDExtract-N-ampl PCR reaction mix (Sigma–Aldrich), 0.8 μl of each primer (10 μM), 4.4 μl of sigma-water and 4 μl of template DNA. An initial denaturation at 94 °C for 7 min was followed by 40 cycles (denaturation at 94 °C for 30 s, annealing at 50°/62° for 30 s, and extension at 72 °C for 35 s, for annealing temperature for each fragment see Table 1) and a final extension at 72 °C for 7 min on a BioRad Mycycler thermal cycler. The same primers were used for the sequencing reaction, and the PCR products were purified and sequenced in STABVIDA (http://www.stabvida.net/).

### 2.2. Phylogenetic analyses

Sequences were edited with CodonCode Aligner v. 2.0 (http://www.codoncode.com/) and aligned with Clustal X (Thompson et al. 1997). All sequences have been deposited in GenBank (available at www.ncbi.nlm.nih.gov/) with the following accession numbers: EU921674 to EU921698 (12S), EU921725 to EU921743 (16S), EU921699 to EU921724 (cytochrome *b*) and EU921744 to EU921756 (rhodopsin).

For phylogenetic analyses we included *Anguilla anguilla* as an outgroup (GenBank Accession numbers AF266495, AB021749, AB021776, EU492236 for 12S, 16S, cytochrome *b* and rhodopsin, respectively). For each of the four fragments, Maximum Parsimony (MP), Minimum Evolution (Neighbor-joining, NJ), and Bayesian Inference (BI) methods were applied separately. In addition, the three mitochondrial makers were combined to form a single dataset.

MP and NJ analysis were performed with PAUP*4.0 (Swofford, 2002). MP analysis was conducted using a heuristic search strategy with random stepwise addition (1000 replicates) and TBR branch swapping. Bootstrap analyses (1000 replicates) were used to assess the relative robustness of branches of the ME and the MP trees (Felsenstein, 1985). For each gene, the saturation of transitions and transversions was checked by plotting the absolute number of changes of each codon position against uncorrected sequence divergence values (*p*).

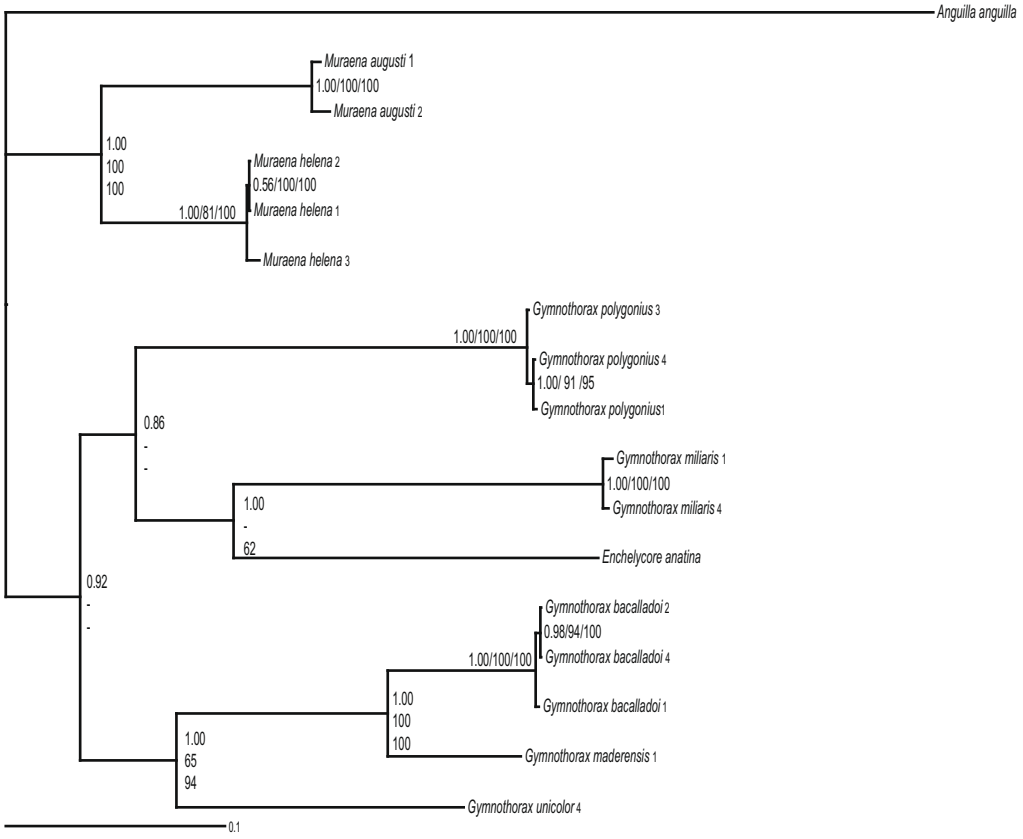


**Fig. 1.** Tree of mtDNA dataset (12s, 16s and cytochrome *b* fragments concatenated) based on BI inference tree. The values presented in the nodes correspond to posterior probabilities, MP and NJ bootstraps. Nodes with no bootstrap support (inferior to 50) in a given method are represented by - .

There was no evidence of saturation in the ingroup (graphs not shown). Thus, we gave equal weights to transitions and transversions when performing the MP trees. Giving transversions 10 times the weight of transitions did not affect the results.

For NJ and IB analyses the best-fit models of nucleotide substitution were selected with the program Modeltest 3.0 (Posada and Crandall, 1998) with the Akaike Information Criterion (AIC). In NJ analysis bootstrap was also used to assess the robustness of the nodes.

For Bayesian Inference (BI) we used MrBayes v3.1.2 (Huelsenbeck and Ronquist, 2001). Values of the evolution models selected were input and runs of two million generations were executed with a sampling frequency of 1000 and a burn-in parameter of 200. When different genes were analyzed together, a partition of the dataset was needed to implement the different models of evolution. Partitioning models should improve the fit of the model to the data and result in a more accurate estimate of phylogeny (Ronquist and Huelsenbeck, 2003). P distances within and between species were computed with the software Arlequin v. 3.01 (Excoffier and Schneider, 2005). Intraspecific distances were corrected by subtracting the mean intra specific pairwise distances and implemented in Arlequin.

## 3. Results

A total of 747, 826, 1006 and 460 bp were amplified corresponding to 12S, 16S, cytochrome *b* and rhodopsin, respectively. The three mitochondrial fragments combined yielded 2581 bp (after alignment).

Concerning parsimony analyses, for the mtDNA dataset, 1635 characters were constant, 203 parsimony-uninformative and 743 were parsimony-informative. For the rhodopsin gene the corresponding values were 336, 60 and 64. The number of retained trees was 4 for the mtDNA dataset and 6 for the rhodopsin gene.

In Figs. 1 and 2 the Bayesian trees for the combined mitochondrial dataset and the rhodopsin are shown respectively, together with bootstrap values from MP and NJ analysis. The corresponding trees for each individual fragment are presented in Supplementary material. In all trees, sequences retrieved from GenBank present their Accession Number between ( ) after the name of the species.

The topology of the parsimony trees did not change when the weight of transversions was changed from 1 to 10 times the weight of transitions.

The tree for the mitochondrial combined dataset is the best resolved. The more salient features of that tree can be summarized as follows:

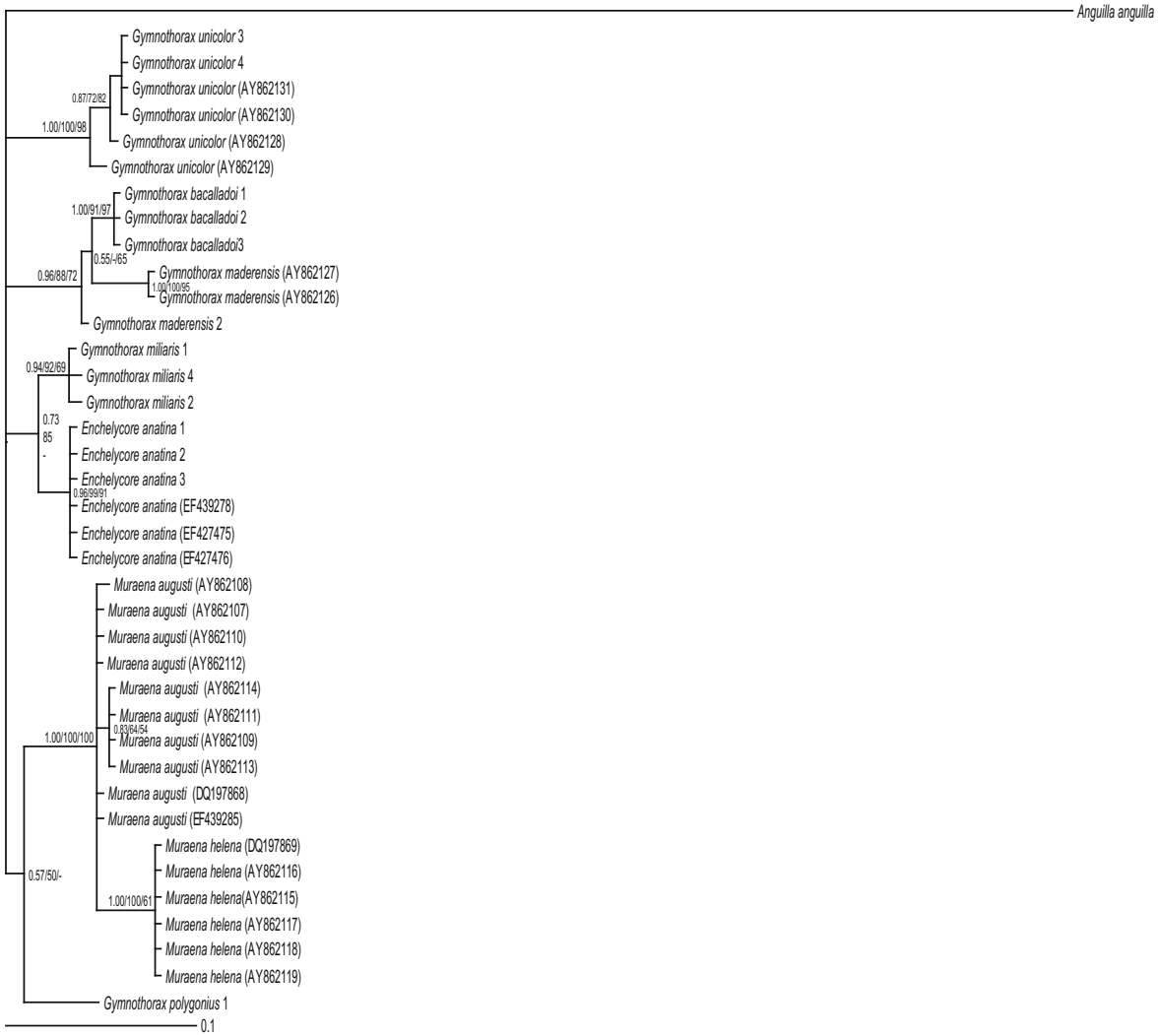


**Fig. 2.** Tree of rhodopsin gene based on BI inference tree. The values presented in the nodes correspond to posterior probabilities, MP and NJ bootstraps. Nodes with no bootstrap support (inferior to 50) in a given method are represented by -. Sequences retrieved from GenBank present their Accession Number between ( ) after the name of the species.

1- *Gymnothorax bacalladoi* is the sister species of *G. maderensis*, a result consistently recovered by all inference methods for all datasets.

2- The clade *G. maderensis*/*G. bacalladoi* is sister to *G. unicolor* although not all inference methods support this conclusion.

3- *Gymnothorax miliaris* seems to belong to a clade which also includes *E. anatina* pointing to the likely lack of monophyly of *Gymnothorax*.

4- The two species of *Muraena* occur in a distinctive clade but the sequences of each species are distinct, both for mitochondrial and nuclear fragments, confirming the finding of Jiménez et al. (2007) that they are two valid species.

Although the rhodopsin tree (Fig. 2) is much less resolved, the clade *G. maderensis*/*G. bacalladoi* was also recovered with high bootstrap and posterior probabilities with *G. bacalladoi* forming a well defined clade nested in a larger group where our samples of *G. maderensis* and those from GenBank occur separately. MP and BI also support the close relationship of *G. miliaris* and *E. anatina* and the distinctiveness and sister relationships of the two species of *Muraena* were also strongly supported. The phylogenetic analyses of the individual mitochondrial fragments all supported with high bootstraps and posterior probabilities the sister relationships of *G. bacalladoi* and *G. maderensis* and those of the two *Muraena* species.

All sequences obtained in this work grouped consistently with the conspecific sequences retrieved from GenBank, a finding that straightens our confidence in the results.

## 4. Discussion

When compared with the distances among the samples of all the species analyzed, those between *G. bacalladoi* and *G. maderensis* are small (8%), for the mitochondrial combined data set, after correcting for intra specific variability. Moreover, all markers consistently recovered *G. bacalladoi* and *G. maderensis* as sister taxa. Interestingly, *G. bacalladoi* and *G. maderensis*, despite their differences, are the only two *Gymnothorax* species in the Eastern Atlantic showing serrate teeth. The species showing the highest morphological similarities with the pair *G. bacalladoi*/*G. maderensis* in the Atlantic Ocean is the small sized littoral *G. hubbsi* moray. This moray not yet studied genetically, is only known from the tropical and subtropical Western Atlantic (Böhlke et al., 1989). The relationships of the Macaronesian morays as a whole are however in need of further investigation.

As already referred in the introduction, the resemblance of some characters of *G. bacalladoi* with several Indo-Pacific species, raised the possibility of it being a paleoendemism related with the Tethys Sea. However, the present data, which support a sister relationship of *G. bacalladoi* and *G. maderensis*, a species with dentition and lateral-line pore system pattern more similar to other Atlantic forms, rejects the hypothesis mentioned above and call into question the taxonomic usefulness of these morphological traits.

As stated in the introduction, Jiménez et al. (2007) had already noted that *M. robusta* was genetically closer to species of *Gymnothorax* than to other species of *Muraena*. In the present study *G. miliaris* seems to be closer to *Enchelychore anatina* than to other *Gymnothorax*. Thus there is a serious possibility that the genera *Muraena*, *Gymnothorax* and *Enchelycore* are not monophyletic.

Only a broader phylogenetic analysis with an adequate coverage of the morays of different oceans will identify the existing clades and which taxa are to be included in a revised taxonomy of this family. Indeed, if the major cladogenic events in the family pre-dated the closure of the Tethys sea and of the Isthmus of Panama (events that isolated the Atlantic from the Indian and Pacific Oceans) any attempts to perform a phylogenetic analysis using only Atlantic species may fail to capture the relevant features of the phylogeny of the group. Anyway, this limitation is unlikely to affect the relationships between *G. maderensis* and *G. bacalladoi*. Applying the frequently used bench mark of 2% divergence per million years for the mtDNA of vertebrates (Brown et al., 1979) we find that the divergence between the combined dataset of these two morays is 8%, 4MYA (after correction) which places their divergence at a much more recent time than the closure of the Tethys Sea, which occurred about 20 MYA (Briggs, 1995). Thus it is unlikely that *G. bacalladoi* is sister to any as yet unsampled taxon from the Indo-Pacific.

The presence of a series of island groups may have played a decisive role in the differentiation of *G. bacalladoi* and *G. maderensis*. If the ancestors of *G. bacalladoi* became temporarily isolated, in one of the Atlantic archipelagos, from the more widespread *G. maderensis* (which occurs in both sides of the Atlantic and Central Islands groups, Böhlke et al., 1989; Smith and Brito, in press), they could have become reproductively isolated. As the circulation in the area favors the spread of fish larvae along the entire island ark (Brito et al., 2007; Domingues et al., 2007, 2008) the two morays could have become sympatric secondarily (namely in Canary Islands, Madeira and Cape Verde) evolving different ecological niches. Indeed *G. bacalladoi* is smaller (44 cm maximum size; Smith and Brito, in press) that *G. maderensis* (130 cm maximum size) and has been mostly collected in shallower waters (5–20 m depth); *G. maderensis* lives exclusively in deep water (120–300 m depth). In any case, a proper test of this hypothesis will require a genetic analysis of the Western Atlantic *G. hubbsi*.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.01.007.

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