

Molecular phylogeny of Clupeiformes and the placement of some Western Atlantic and Amazonian Taxa

Cleonilde da Conceição Silva Queiroz¹, Rosália Furtado Cutrim Souza², Simoni Santos Silva³, Claudio Alberto Gellis de Mattos Dias⁴, Amanda Alves Fecury⁵, Euzébio de Oliveira⁶, Divino Bruno da Cunha⁷, Horacio Schneider³, Iracilda Sampaio³

1. Laboratório de Genética e Biologia Molecular, Centro de Ciências Exatas Naturais e Tecnológicas (CCENT), Universidade Estadual da Região Tocantina do Maranhão (UEMASUL), Rua Godofredo Viana, 1300, Centro Imperatriz- MA- Brazil.

cleo_queiroz@yahoo.com.br

<http://lattes.cnpq.br/4857618533003634>

<http://orcid.org/0000-0001-7906-7379>

2. Laboratório de Populações Pesqueira, Instituto Socioambiental e dos Recursos Hídricos (ISARH), Universidade Federal da Amazônia (UFRA), Belém- PA- Brazil.

rosaliacutrim@superig.com.br

<http://lattes.cnpq.br/7336549637280337>

<http://orcid.org/0000-0002-2843-7760>

3. Laboratório de Genética e Biologia Molecular, Instituto de Estudos Costeiros (IECOS), Universidade Federal do Pará (UFPA), Campus de Bragança. Alameda Leandro Ribeiro, SN, Aldeia Bragança-PA- Brazil.

sssimoni@yahoo.com.br

<http://lattes.cnpq.br/0818422393872186>

<http://orcid.org/0000-0001-7261-6793>

horacio.schneider@gmail.com

<http://lattes.cnpq.br/3621033429800270>

<http://orcid.org/0000-0002-5987-6395>

iracilda@uol.com.br

<http://lattes.cnpq.br/2482763145819602>

<http://orcid.org/0000-0002-2137-4656>

4. Laboratório de Neurociências e Comportamento II (Lab/NeC II), Instituto Federal do Amapá (IFPA), Macapá - AP - Brazil.

claudio.gellis@ifap.edu.br

<http://lattes.cnpq.br/8303202339219096>

<http://orcid.org/0000-0003-0840-6307>

5. Núcleo de Estudos em pesca e Aquicultura (NEPA), Universidade Federal do Amapá (UNIFAP), Macapá - AP - Brazil.

amanda@unifap.br

<http://lattes.cnpq.br/9314252766209613>

<http://orcid.org/0000-0001-5128-8903>

6. Instituto de Ciências Biológicas (ICB), Universidade Federal do Pará (UFPA), Castanhal - PA- Brazil.

euzebio21@yahoo.com.br

<http://lattes.cnpq.br/1807260041420782>

<http://orcid.org/0000-0001-8059-5902>

7. Laboratório de Genética e Biodiversidade, Instituto de Estudos do Xingu (IEX), Universidade Federal do Sul e Sudeste do Pará (Unifesspa), Campus de Universitário de São Félix do Xingu, Travessa Manoel Antônio dos Santos, São Félix - PA- Brazil.

divinobruno@unifesspa.edu.br

<http://lattes.cnpq.br/8700473654101376>

<http://orcid.org/0000-0003-2668-137X>

ABSTRACT

The taxonomy of herrings, sardines and anchovies from morphological and molecular characters has been largely approached, but the phylogenetic relationships among most of the taxa are little resolved. The present study of the mitochondrial ribosomal 16S gene using, adding sequence data for 14 species of Neotropical sardines from both marine and freshwater habitats, 10 of which appear for the first time in molecular phylogenetic analysis. Results from Bayesian, Maximum Likelihood and Parsimony analyses strongly support the monophyly of the families Pristigasteridae and Engraulidae, but were not able to recover the monophyly of the family Clupeidae. In addition, Chirocentridae and Sundasalangidae, appear in polytomic arrangements among some Clupeidae/Clupeiformes lineages. Curiously, *Odontognathus mucronatus* and *Pellona harroweri*, a Western Atlantic species which were included in a molecular phylogenetic analysis for the first time were key species, breaking up the monophyly of both *Pellona* and *Ilisha* inside the Pristigasteridae. Additional examples of paraphyly and/or polyphyly were observed for several traditional genera, such as *Sardinella*, *Anchoa* and *Engraulis*. The present analysis successfully resolved some relevant aspects of its taxonomy and opened several questions that will demand additional sampling effort and new phylogenetic analyses to better clarify the taxonomy of this rich group of fishes.

Keywords: Clupeiformes; molecular phylogeny; Amazonian; sardines; mitochondrial.

Filogenia molecular de Clupeiformes e o posicionamento de alguns táxons das regiões do Atlântico Ocidental e Amazônia

RESUMO

A taxonomia dos arenques, sardinhas e anchovas a partir de caracteres morfológicos e moleculares tem sido amplamente abordada, mas as relações filogenéticas entre a maioria dos táxons são pouco resolvidas. O presente estudo utilizou o gene ribossômico mitocondrial 16S, e adicionando dados de sequência para 14 espécies de sardinha neotropical de habitats marinhos e de água doce, 10 das quais aparecem pela primeira vez em análises filogenéticas moleculares. Os resultados das análises bayesiana, máxima verossimilhança e parcimônia suportam fortemente a monofilia das famílias Pristigasteridae e Engraulidae, mas não foram capazes de recuperar a monofilia da família Clupeidae. Chirocentridae e Sundasalangidae, aparecem em arranjos politômicos entre algumas linhagens de Clupeidae/Clupeiformes. Curiosamente, *Odontognathus mucronatus* e *Pellona harroweri*, espécies do Atlântico Ocidental que foram incluídas em uma análise filogenética molecular pela primeira vez, sendo espécies-chave, quebraram a monofilia de *Pellona* e *Ilisha* dentro de Pristigasteridae. Exemplos adicionais de parafilias e/ou polifilias foram observados para vários gêneros tradicionais, como *Sardinella*, *Anchoa* e *Engraulis*. A presente análise resolveu com sucesso alguns aspectos relevantes de sua taxonomia e abriu várias questões que exigirão um esforço adicional de amostragem e novas análises filogenéticas para melhor esclarecer a taxonomia desse rico grupo de peixes.

Palavras-chaves: Clupeiformes, filogenia molecular, Amazônia, sardinhas, mitocondrial.

Introduction

The order Clupeiformes (Teleostei, Actinopterygii) is currently divided into the suborders Denticiptioidei and Clupeoidei (GRANDE, 1985; WHITEHEAD, 1985; DI DARIO; DE PINNA, 2006; NELSON et al., 2016). Species in the order are commonly known as herrings, sardines and anchovies. The main synapomorphic character for this order is the presence of the *recessus lateralis* – an intercranial space in the optic region of the skull (DI DARIO, 2004). Clupeiformes have flattened or rounded bodies, live in large shoals and feed mainly on plankton which they filter from water using their many branchial

arches (WHITEHEAD, 1985; WHITEHEAD et al., 1988). This order is cosmopolitan, with 364 species in 84 genera (Nelson, 2006). They are primarily pelagic marine species inhabiting coastal areas, though some species live in estuaries and freshwaters. Sardines are globally important as basis for many commercial fisheries (WHITEHEAD, 1985; NELSON et al., 2016).

The wide distribution, molecular and morphological diversity of Clupeiformes has resulted in a considerable debate about its taxonomy in recent years. Currently there are two suborders recognized. The Denticiptioidei (represented by a

single family, Denticiptidae) and the Clupeoidei, with four families: Chirocentridae, Pristigasteridae, Engraulidae and Clupeidae (NELSON, 1970; GRANDE, 1985; WHITEHEAD, 1985; WHITEHEAD et al., 1988; NELSON et al., 2016). However there is a taxonomic revision and proposed 15 family groups (LAVOUÉ et al., 2014): Engraulidae, Engraulinae, Coiliinae, Chirocentridae, Pristigasteridae, Pristigasterinae, Pelloninae, Clupeidae, Clupeinae, Ehiravinae, Dorosomatinae, Alosinae, Dussumieriidae, Dussumieriinae, and Spratelloidinae.

For the Clupeoidei suborder it was proposed that Clupeidae and Chirocentridae to be sister groups based on morphology (GRANDE, 1985), and placed them in the superfamily Clupeoidea. Di Dario (2002) followed Grande (1985) and proposed Engraulidae to be a sister group to this superfamily based on a synapomorphic character: the presence of cartilaginous chevrons at the tips of epicentrals. Di Dario (2002a) described two more characters (orientation of parapophyses of the second vertebra and the complex pattern of interzyga-pophysal articulation) supporting this hypothesis.

On the basis of morphological evidence, Siebert (1997) proposed the inclusion of *Sundasalanx* (previously belonging to the family Sundasalangidae, order Osmeriformes) in the suborder Clupeoidei, family Clupeidae, and as sister group to *Jenkinsia*. *Sundasalanx* inclusion in Clupeiformes was supported in subsequent molecular analyses using mitochondrial genome (ISHIGURO et al., 2005; LAVOUÉ et al., 2007; LAVOUÉ et al., 2013) and mitochondrial genes (12S and 16S) (LI; ORTÍ, 2007), (12S, 16S and Cytochrome *b*) (WILSON et al., 2008), (16S, rag1, rag2 and Cytochrome *b*) (BLOOM; LOVEJOY, 2012), though it was not found to be closely related to *Jenkinsia*.

The order Clupeiformes is accepted as including six families: Denticiptidae, Sundasalangidae, Chirocentridae, Engraulidae, Pristigasteridae and Clupeidae (LAVOUÉ et al., 2007; LI; ORTÍ, 2007; WILSON et al., 2008; LAVOUÉ et al., 2013). Monophyly of the families Pristigasteridae and Engraulidae, and paraphyly of Clupeidae have been suggested based on available molecular data (LAVOUÉ et al., 2007; LI; ORTÍ, 2007; BLOOM; LOVEJOY, 2012; LAVOUÉ et al., 2013; BLOOM; LOVEJOY, 2014). These studies place Chirocentridae within Clupeidae. Lavoué et al. (2007) noted that the high rate of molecular evolution in Chirocentridae made the phylogenetic placement of this family within Clupeiformes difficult.

The analysis of Lavoué et al. (2007) based on complete mitochondrial genomes and Bayesian analysis of 22 taxa of Osteoglossomorpha, Elopomorpha Euteleostei, Alepocephaloidei and Ostariophysii suggested that the Clupeiformes is a monophyletic group (Bayesian posterior probability, BPP = 1.00). Within the order, *Denticeps clupeoides* (suborder Denticiptoidei) is the sister group to the suborder Clupeoidei, which was also monophyletic. In the suborder Clupeoidei, the family Engraulidae (represented by the genera *Coilia* and *Engraulis*) was shown to be monophyletic and most basal (BPP = 0.99), following this there was a polytomic group consisting of Sundasalangidae (*Sundasalanx*), Pristigasteridae (*Ilisha*) and Clupeoidea (Chirocentridae and Clupeidae). Curiously, within Clupeoidea, the family Clupeidae was not found to be monophyletic, with *Chirocentrus dorab* (the only genus of Chirocentridae) appearing within a basal group along with *Jenkinsia* and *Spratelloides*.

Li and Ortí (2007) analyzed the sequence of mitochondrial genes 12S and 16S (1986 bp) and nuclear genes RAG1 and RAG2 (2763 bp) in 37 taxa of Clupeiformes, with seven outgroup taxa. The global arrangement of this analysis revealed monophyly of Pristigasteridae (*Pellona* and *Ilisha*) and Engraulidae (*Setipinna*, *Coilia*, *Engraulis* and *Anchoa*), and

showed again the paraphyly of Clupeidae, with *Chirocentrus* (Chirocentridae) inserted into this group. As with the findings of Lavoué et al. (2007) using mitochondrial genome, the paraphyly of the subfamilies of Clupeidae was again observed in nuclear gene sequences.

The present study approaches the molecular phylogeny of Clupeiformes by including various taxa from the Atlantic coast of South America and Brazilian Amazon. Some of these neotropical species were subjected molecular analyzed for first time in this study.

Materials and Methods

Sampling and DNA procedures

A total of 55 new samples of sardines (order Clupeiformes) from the coast of Brazil (Pará, Bahia and Santa Catarina), and from the Amazon basin (Santarém - Pará and Manaus - Amazonas (Table 1) were analyzed. Taxonomic identifications were done following descriptions in Whitehead (1985) and Whitehead et al. (1988). Ethics Statement, all samples of fishes were obtained directly at local fish markets or markets. According to Instituto Brasileiro de Meio Ambiente (IBAMA), these species are not under a fishing ban legislation.

Table 1. Clupeiformes species of the Brazilian coast and Amazonian basin collected for the present study.

Species	Number	Collecting Site
ENGRAULIDAE		
<i>Anchoa spinifer</i>	5	Bragança (4), Ilhéus (1)
<i>Anchovia clupeoides</i>	4	Bragança, Pará
<i>Anchoviella lepidentostole</i>	3	Bragança, Pará
<i>Cetengraulis edentulous</i>	4	Bragança, Pará
<i>Lycengraulis grossidens</i>	7	Bragança, Pará
<i>Pterengraulis atherinoides</i>	2	Bragança, Pará
PRISTIGASTERIDAE		
<i>Odontognathus mucronatus</i>	4	Bragança (2), Ilheus (2)
<i>Pellona castelnaeana</i>	6	Manaus, Amazonas
<i>Pellona flavipinnis</i>	4	Santaré, Pará
<i>Pellona harroweri</i>	3	Bragança (2), Ilhéus (1)
CLUPEIDAE		
<i>Harengula clupeola</i>	4	Ilhéus, Bahia
<i>Opisthonema oglimum</i>	2	Bragança, Pará
<i>Rhinosardinia Amazonica</i>	3	Bragança, Pará
<i>Sardinella aurita</i>	4	Florianópolis, Santa Catarina

Total DNA was extracted from muscular tissue using the method of Sambrook et al. (2001), which consists of isolating the DNA using ribonuclease, proteases and pheno-chloroform, followed by precipitation with sodium acetate and isopropanol. The 16S mtDNA fragments were amplified by Polymerase Chain Reaction (PCR). Each PCR reaction was carried out in a total volume of 25 µL which contained: Taq polymerase buffer (10X), MgCl₂ (25 mM), Primers (50 ng), genomic DNA (200 ng), dNTPs (1,25 mM), Taq DNA polymerase enzyme (2 U) and sterile water to complete the volume. Primers used were those found in Palumbi et al. (1991): 16S-L (5'-CGCCTGTTTATCAAAAACAT-3') and 16S-H (5'-TTTCCC CGGTGCGCCC-3'). Amplification reactions were adjusted for each stage: denaturation, annealing and extension. An initial denaturation step of 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds (denaturation), 50°C for 1 minute (annealing) and 72°C for 2 minutes (extension), followed by a final extension time at the same temperature for 7 minutes. PCR products were purified using the ExoSAP-IT (Amersham Pharmacia Biotech) and sequenced using Big Dye 3.1 in a ABI 3500 automatic DNA sequencer, following the maker's instructions (Applied Biosystems, Foster, CA, USA).

Molecular Methods

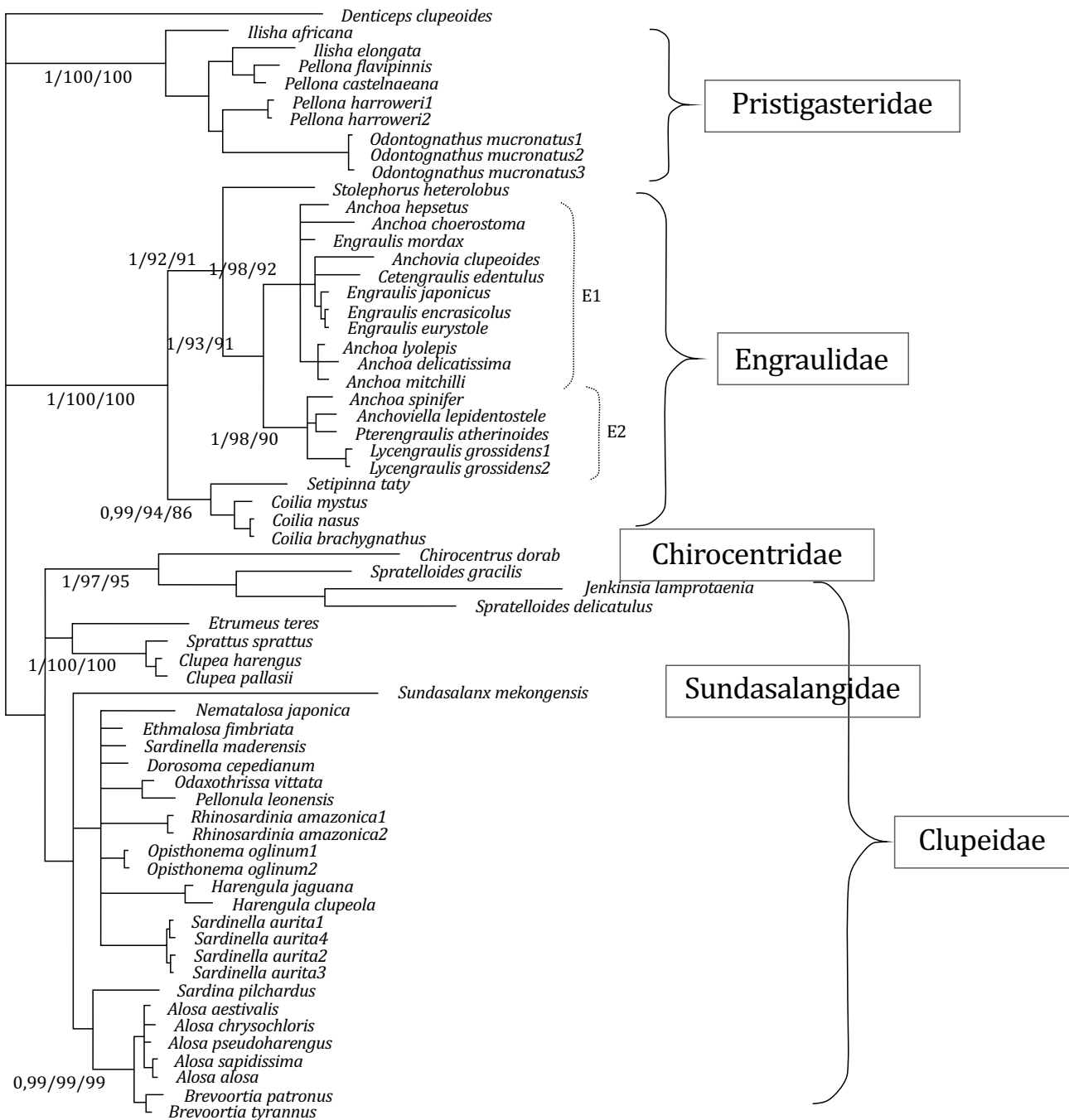
The sequences were aligned using Clustal W (THOMPSON et al., 1994) implemented in the program Bioedit (HALL, 1999). Additional sequences for 38 species already existing in GenBank were added (Supplementary material). The final alignment was 475 base pairs long. Phylogenetic trees were obtained through Bayesian, Maximum Likelihood (ML) and Maximum Parsimony (MP) analyses. MrBayes version 3.1.2 (HUELSENBECK; RONQUIST, 2001) was used for Bayesian analysis using the evolutionary model selected by the script MrAIC version 1.4.3 (NYLANDER, 2014) and the following parameters for Markov Chain Monte Carlo runs: ngen=8000000, nruns=4, nchains=4 and burninfrac=0.5. Remaining parameters were left at default settings. MrBayes provides support for groupings based on posterior probability (BPP) with a range of 0 to 1. For ML analysis, the evolutionary model used was also that determined by the script MrAIC 1.4.3 while PHYML version 2.4.4 (GUINDON; GASCUEL, 2003) was used to obtain the ML tree, with support determined by 1000 bootstrap pseudo-replicates. MP analyses used the program PAUP*

version 4.0b10 (*Phylogenetic Analysis Using Parsimony*) (SWOFFORD, 2002) with default settings. Support was again provided by 1000 bootstrap pseudo-replicates.

Denticeps clupeioides was used as an outgroup in these analyses based on the previous work of Li and Orti (2007) and preliminary analysis using all available sequences.

Results

Bayesian, ML and MP methods produced trees with generally similar topologies, and the species *Denticeps clupeioides* was used as outgroup (Figure 1). The methods showed consensus that the families Engraulidae and Pristigasteridae were monophyletic with BPP=1 and 100% bootstrap support under ML and MP. However, it was not possible to demonstrate monophyly of Clupeidae, as *Chirocentrus dorab* (Chirocentridae) was found within this clade, closely related to *Jenkinsia* and *Spratelloides* (with support of BPP=1, 90% bootstrap support under ML and 85% bootstrap support under MP). Similarly, *Sundasalanx mekongensis* (Sundasalangidae) was also found to occur within Clupeidae.



0.1

Figure 1. Consensus phylogenetic tree with support at nodes based on Bayesian analysis (BPP), Maximum Likelihood (ML) and Maximum Parsimony (MP) in that order. Support values only shown where bootstrap values are over 90%.

Engraulidae

As previously mentioned, the family Engraulidae, represented here by 21 species, was found to be monophyletic with 100% support under all analyses (Figure 1). Within the family, five potentially monophyletic lineages can be seen based on support values.

Among the genera analyzed in the present study, the most basal from the Engraulidae family were *Coilia* (*C. mystus*, *C. nasus* and *C. brachygnathus*) and *Setipinna* (*S. taty*); following this, *Stolephorus heterolobus* is the sister group to the remaining monophyletic clades, the first of which (labeled E1 in Figure 1) comprises *Anchoa*, *Anchovia*, *Cetengraulis* and *Engraulis* species, and the second (labeled E2 in Figure 1) comprises *Anchoa*, *Anchoviella*, *Lycengraulis* and *Pterengraulis* species. The fifth well supported clade is a subgroup of the clade E1 comprising three species of *Anchoa* (*A. delicatissima*, *A. lyolepis* and *A. mitchilli*). Based on these analyses it is therefore not possible to demonstrate monophyly of either *Anchoa* or *Engraulis*.

In relation to the six engraulid species from the Brazilian coast included in the genetic analysis, *Anchovia clupeoides* and *Cetengraulis edentulus* are closely related to the species in E1 clade, while the other four species together form the E2 clade (*Anchoa spinifer*, *Anchoviella lepidentostole*, *Pterengraulis atherinoides* and *Lycengraulis grossidens*).

Pristigasteridae

The family Pristigasteridae, here represented by two *Ilisha*, three *Pellona* and one *Odontognathus* species (Figure 1), is shown to be monophyletic with strong support BPP=1, 100% bootstrap support under MP and 99% bootstrap support under ML. Strangely, a large polytomy results for the six species analyzed here, with monophyly not supported for either *Pellona* or *Ilisha*. However, the grouping of the two freshwater species of the Amazon basin (*Pellona castelnaeana* and *P. flavipinnis*), which are extremely similar morphologically, was supported by Bayesian analysis, though it only had moderate support under ML and MP analyses (bootstrap support ~80%). *Pellona harroweri* and *Odontognathus mucronatus*, both from the Brazilian coast, appear for the first time in a molecular analysis, and in the case of *P. harroweri*, it was found not to be significantly related with the two other species of *Pellona* from freshwater habitats.

Clupeidae-Chirocentridae-Sundasalangidae

The family Clupeidae is represented in this analysis by 25 species of the genera *Alosa*, *Brevoortia*, *Clupea*, *Dorosoma*, *Ethmalosa*, *Etrumeus*, *Harengula*, *Jenkinsia*, *Nematalosa*, *Odaxothrissa*, *Opisthonema*, *Pellonula*, *Rhinosardinia*, *Sardina*, *Sardinella*, *Sprattus* and *Spratelloides*. None of the phylogenetic analyses recovers Clupeidae as a monophyletic group. Representatives of the families Chirocentridae (*Chirocentrus dorab*) and Sundasalangidae (*Sundasalanx mekongensis*) were found inside this traditional group of clupeids.

Only three clades are strongly supported within this group. *Sprattus* is grouped with *Clupea* (BPP=1, 100% bootstrap support under ML and MP), *Alosa* with *Brevoortia* (BPP=0.99, 99% bootstrap support under ML and MP), and *Chirocentrus* (Chirocentridae) with *Spratelloides* and *Jenkinsia* (BPP=1, 97% bootstrap support under ML and 95% bootstrap support under MP).

The four Brazilian species of clupeids (*Harengula clupeola*, *Opisthonema oglinum*, *Rhinosardinia amazonica* and *Sardinella aurita*) end up with most other species grouped in a large polytomy.

Discussion

Family Engraulidae

The family Engraulidae comprises 16 genera and 139 species (NELSON et al., 2016), identifiable mainly by a characteristic prominent snout (WHITEHEAD et al., 1988). They are commonly known as *manjubas* and only a few species have commercial importance (WHITEHEAD et al., 1988). The present study includes representatives of the genera *Stolephorus*, *Setipinna* and *Coilia* (exclusive to the Indo-Pacific). In the analysis of Li and Ortí (2007), *Setipinna* and *Coilia* form a monophyletic group when analyzed using mitochondrial genes or concatenations of mitochondrial and nuclear genes, but were paraphyletic when analyzed using only the nuclear genes. Our analysis with the 16S mitochondrial gene recovers the same arrangement as in Li and Ortí (2007), with *Setipinna* and *Coilia* supported with BPP=0.99, 94% bootstrap support under ML and 86% bootstrap support under MP.

In the present analysis, the species *Stolephorus heterolobus* forms an outgroup to the two clades (E1 and E2) in which predominated species from the New World. Sardines from the New World are here represented by the genera *Anchoa*, *Anchovia*, *Anchoviella*, *Cetengraulis*, *Engraulis*, *Lycengraulis* and *Pterengraulis*. Of these, only *Engraulis* is cosmopolitan (WHITEHEAD et al., 1988; NELSON et al., 2016). Clade monophyly for taxa from the New World was previously recovered by Li and Ortí (2007) using mitochondrial (12 and 16S) and nuclear (RAG1 and RAG2) sequence data, but only including representatives of *Anchoa* (5 species) and *Engraulis* (3 species), both included in clade E1 here. Our analysis added two extra genera to clade E1, *Anchovia* (*A. clupeoides*) and *Cetengraulis* (*C. edentulus*). As in the arrangement of Li and Ortí (2007), the genera *Anchoa* and *Engraulis* do not appear monophyletic and with the addition of *Anchovia* and *Cetengraulis*, an unresolved polytomy is obtained. Our study corroborates the arrangements previously found (LAVOUÉ et al., 2010; BLOOM; LOVEJOY, 2012; LAVOUÉ et al., 2013).

Four species of Engraulidae from the New World (Brazilian coast) included in the present study (*Anchoa spinifer*, *Anchoviella lepidentostole*, *Pterengraulis atherinoides* and *Lycengraulis grossidens*) form a separate monophyletic group identified here as clade E2, and is found to be the sister group to clade E1. Interestingly, *Anchoa spinifer* is found in clade E2 while other members of that genus are in clade E1. Whitehead et al. (1988) proposed that the genus *Anchoa* (which occurs in both the Pacific and Atlantic oceans) be divided into two subgenera: *Anchoa* (short pseudobranch, except *Anchoa marinii*; anal fin origin below or just preceding dorsal fin base), and *Anchoviella* (pseudobranch longer than eye, reaching into inner face of operculum; anal fin origin under or behind the base of the last dorsal fin ray). Following this division, the species, analyzed here, *Anchoa mitchilli*, *A. hepsetus*, *A. choerostoma*, *A. delicatissima* and *A. spinifer* belong to the subgenus *Anchoa* while *Anchoa lyolepis* belongs to the subgenus *Achoviella*.

Our results, similarly to those from Li and Ortí (2007), do not corroborate the division proposed by Whitehead et al. (1988), as *A. mitchilli*, *A. delicatissima* (both *Anchoa*) and *A. lyolepis* (*Anchoviella*) form a monophyletic group with strong support (BPP=0.99 and 90% bootstrap support under ML and MP) within the clade E1, while *A. hepsetus* and *A. choerostoma* occupy an uncertain position within clade E1 and *A. spinifer* is grouped within clade E2 along with the genera *Lycengraulis*, *Pterengraulis* and *Anchoviella* supported with BPP=1, 98% bootstrap support under ML and 90% bootstrap support under MP.

Anchovia clupeioides, *Cetengraulis edentulus*, *Lycengraulis grossidens*, *Pterengraulis atherinoides*, *Anchoviella lepidentostole* and *Anchoa spinifer* are species from the Atlantic coast of Brazil included here for molecular phylogenetic analysis. *Anchovia clupeioides* and *Cetengraulis edentulus* form a monophyletic group (E1, Figure 1), along with four species of *Engraulis* and six species of *Anchoa*. In turn, *Lycengraulis*, *Pterengraulis*, *Anchoviella* and *Anchoa spinifer* form another monophyletic group (E2). This arrangement is in accordance with an observation of Nelson (1970), where *Cetengraulis*, *Anchovia* and *Engraulis* have teeth of the casting plate and indications of secondary reduction with total loss of jaw teeth in *Cetengraulis*. He also noted that the *A. spinifer* teeth are similar to those of *Lycengraulis* and *Pterengraulis*, which supports the position within clade E2 separated from other species of *Anchoa* (NELSON, 1970).

Pristigasteridae was previously classified as a subfamily of Clupeidae (DI DARIO, 2009; NELSON et al., 2016). After a detailed study of morphological and osteological characters of the genera *Pellona*, *Raconda*, *Ilisha*, *Opisthopterus*, *Pristigaster*, *Pliosteostoma*, *Chirocentrodon* and *Odontognathus*, it was concluded that this group presented significantly different characteristics from those of other members from the family Clupeidae and therefore deserved to be recognized as their own distinct family. This was supported by the analyses of Di Dario (2002), in which a greater similarity was found between the Clupeidae and Engraulidae than between either of those groups and the Pristigasteridae. In recent molecular phylogenies (LAVOUÉ et al., 2007; LI; ORTÍ, 2007; WILSON et al., 2008; BLOOM; LOVEJOY, 2012; LAVOUÉ et al., 2014), the relationships between these three main lineages of Clupeiformes were not well resolved.

Pristigasteridae are represented by 9 genera and 34 species, from which three have global distributions, while five are found in the New World and one is found in the Indo-Pacific (WHITEHEAD, 1985; NELSON et al., 2016). Grande (1985) recognized two subfamilies within the Pristigasteridae, Pelloninae (recognized on the basis of having the maxillary-premaxillary gap covered by bone and either a toothed hypomaxilla or an extension of the maxilla) and Pristigasterinae (recognized on the basis of having a bony process on the first pleural rib that articulates with the shoulder girdle). Following this, Pelloninae comprises the genera *Chirocentrodon*, *Ilisha*, *Neopisthopterus*, *Pliosteostoma* and *Pellona*, while Pristigasterinae would comprise the genera *Opisthopterus*, *Raconda*, *Pristigaster* and *Odontognathus*. Molecular data do not clearly support this morphological separation. Alternatively, Grande (1985) suggested that *Ilisha* might not be monophyletic, and indicated the necessity of analyses with more samples before a taxonomic revision could be made. Other authors also noted the possible paraphyly of *Ilisha* and polyphyly of *Pellona*, stating that the evolutionary history of *Pellona* from South America indicated a possible recent invasion of freshwaters (DI DARIO; DE PINNA, 2003). The present phylogenetic arrangement based on the 16S data reveals a complex relationship among species within the Pristigasteridae as the monophyly of neither *Ilisha* nor *Pellona* could be recovered in any of the analyses performed, and leaves open to question the relationships of these species. These relationships are important to understand marine introgressions into the Amazon basin.

Families Clupeidae-Chirocentridae-Sundasalangidae

The family Clupeidae is represented by 188 species in 57 genera (NELSON et al., 2016). Members of this family are found

throughout the world, and are recognized by their small to medium body size, small or absent teeth, and a small terminal or somewhat superior mouth. From an economic perspective, this is the most important family of Clupeiformes, with a high commercial value (WHITEHEAD, 1985; WHITEHEAD et al., 1988; NELSON et al., 2016).

Recently Bloom and Egan (2018) used mitochondrial (Cytb+16S) and nuclear (rag1+rag2) loci, was not recovered as monophyletic the family Clupeidae. Clupeinae being grouped with a lineage of Dussumieriinae, and these positioned as sister to a lineage containing Pristigasteridae+Chirocentridae+Engraulidae+the remaining clupeid lineages.

The present study corroborates the arrangements previously found in molecular analyses (LAVOUÉ et al., 2007; LI; ORTÍ, 2007; WILSON et al., 2008; LAVOUÉ et al., 2013; LAVOUÉ et al., 2014) showing that Clupeidae is not a monophyletic taxon as *Chirocentrus dorab* (Chirocentridae) is included within it, forming a monophyletic group with *Jenkinsia* and *Spratelloides* (Figure 1). Similarly, Sundasalangidae, transferred by Sieberts (1997) to Clupeiformes, appears in an unresolved polytomy along with many traditional clupeids (LAVOUÉ et al., 2014; NELSON et al., 2016). The remaining two arrangements supported in this large group of sardines are those grouping *Alosa* with *Brevortia* and *Sprattus* with *Clupea*.

The genera of clupeids from the Brazilian coast included in this analysis (*Harengula*, *Sardinella*, *Rhinosardinia* and *Opisthonema*), appear in a polytomy and their relationships to other clupeids remain unclear. Even so, Li and Ortí (2007) and Lavoué et al. (2007) attempted to divide the clupeids into subgroups. None of these groups is strongly supported by bootstrap values. Recently Lavoué et al. (2014) proposed 15 level families for Clupeiformes. As such, there are remaining questions regarding to the phylogenetic relationships within the clupeids, especially related to the position of Chirocentridae and Sundasalangidae within Clupeiformes.

Acknowledgements

This study was funded by grants from CNPq-PADCT/MCT 620052/01-5 (Millenium Project), CNPq 308477/2006-5 and 300741/2006-5 (Bolsas Produtividade em Pesquisa, IS and HS) and CNPq 477542/2006-9 (Edital Universal). We would also like to thank Jonathan Ready for assistance in the translation of the manuscript.

References

- BLOOM, D. D.; LOVEJOY, N. R. Molecular phylogenetics reveals a pattern of biome conservatism in New World anchovies (family Engraulidae). **Journal of Evolutionary Biology**, v. 25, p. 701-715, 2012.
- BLOOM, D. D.; LOVEJOY, N. R. The evolutionary origins of diadromy inferred from a time-calibrated phylogeny for Clupeiformes (herring and allies). **Proceedings of the Royal Society B: Biological Sciences**, v. 281, 2014.
- BLOOM, D. D.; EGAN, J. P. Systematics of Clupeiformes and testing for ecological limits on species richness in a trans-marine/freshwater clade. **Neotropical Ichthyology**, v. 16, DOI: 10.1590/1982-0224-20180095, 2018.
- DI DARIO, F. Evidence supporting a sister-group relationship between Clupeoidea and Engrauloidea (Clupeomorpha). **Copeia**, v. 2, p. 496-503, 2002.
- DI DARIO, F. Evidence Supporting a Sister-Group Relationship between Clupeoidea and Engrauloidea (Clupeomorpha). **Copeia**, v. 2, p. 496-503, 2002a.
- DI DARIO, F. Homology between the Recessus lateralis and cephalic sensory canals, with the proposition of additional synapomorphies for the Clupeiformes and the Clupeoidei. **Zoological Journal of the Linnean Society**, v. 141, p. 257-270, 2004.
- DI DARIO, F. Chirocentrids as engrauloids: evidence from suspensorium, branchial arches, and infraorbital bones (Teleostei, Clupeomorpha). **Zoological Journal of the Linnean Society**, v. 156, p. 363-383, 2009.
- DI DARIO, F.; DE PINNA, M. C. C. Family Pristigasteridae (Pristigasterids), in Check list of the freshwater fishes of South and Central America. Porto Alegre RS: EDIPUCRS, 2003.

- DI DARIO, F.; DE PINNA, M. C. C. The supratemporal system and the pattern of ramification of cephalic sensory canals in *Denticeps clupeoides* (Denticipitoidei, Teleostei): additional evidence for monophyly of Clupeiformes and Clupeoidei. **Papéis Avulsos de Zoologia**, v. 46, p. 107-123, 2006.
- GRANDE, L. Recent and fossil Clupeomorph fishes with materials for revision of the subgroups of Clupeoids. **Bulletin of the American Museum of Natural History**, v. 181, p. 231-372, 1985.
- GUINDON, S.; GASCUEL, O. A. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. **Systematic Biology**, v. 52, p. 696-704, 2003.
- HALL, T. A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. **Nucleic Acids Symposium Series**, v. 41, p. 95-98, 1999.
- HUELSENBECK, J. P.; RONQUIST, F. MrBayes: Bayesian inference of phylogeny. **Bioinformatics**, v. 17, p. 754-755, 2001.
- HUELSENBECK, J. P.; RONQUIST, F. The evolutionary origins of diadromy inferred from a time-calibrated phylogeny for Clupeiformes (herring and allies). **Proceedings of the Royal Society B: Biological Sciences**, v. 281, 2014.
- ISHIGURO, N. B.; MIYA, M.; INOUE, J. G.; NISHIDA, M. Sundasalanx (Sundasalanxidae) is a progenetic Clupeiform, not a closely-related group of salangids (Osmeriformes): mitogenomic evidence. **Journal Fish of Biology**, v. 67, p. 561-569, 2005.
- LAVOUÉ, S.; MIYA, M.; SAITOH, K.; ISHIGURO, N. B.; NISHIDA, M. Phylogenetic relationships among anchovies, sardines, herrings and their relatives (Clupeiformes), inferred from whole mitogenome sequences. **Molecular Phylogenetics and Evolution**, v. 43, p. 1096-1105, 2007.
- LAVOUÉ, S.; MASAKI, M.; MUTSUMI, N. Mitochondrial phylogenomics of anchovies (family Engraulidae) and recurrent origins of pronounced miniaturization in the order Clupeiformes. **Molecular Phylogenetics and Evolution**, v. 56, p. 480-485, 2010.
- LAVOUÉ, S.; MIYA, M.; MUSIKASINTHORN, P.; CHEN, W. J.; NISHIDA, M.; Mitogenomic evidence for an Indo-West Pacific origin of the Clupeoidei (Teleostei: Clupeiformes). **Plos One**, v. 8, p. 1-15, 2013.
- LAVOUÉ, S.; KONSTANTINIDIS, P.; CHEN, W. J. Progress in Clupeiform systematics. In: GANIAS, K. (Ed.). **Biology and Ecology of Sardines and Anchovies**. Boca Raton FL: CRC Press, 2014.
- LI, C.; ORTÍ, G. Molecular phylogeny of Clupeiformes (Actinopterygii) inferred from nuclear and mitochondrial DNA sequences. **Molecular Phylogenetics and Evolution**, v. 44, p. 386-398, 2007.
- NELSON, G. J. The hyobranchial apparatus of teleostean fishes of the families Engraulidae and Chirocentridae. **American Museum Novitates**, v. 24, n. 10, p. 1-30, 1970.
- NELSON, J. S. **Fishes of the world**. Hoboken, New Jersey: John Wiley & Sons, 2006. 622p.
- NELSON, J. S.; GRANDE, T.; WILSON, M. V. H. **Fishes of the world**. Hoboken, New Jersey: John Wiley & Sons, 2016. 624p.
- NELSON, J. S.; GRANDE, T.; WILSON, M. V. H. The evolutionary origins of diadromy inferred from a time-calibrated phylogeny for Clupeiformes (herring and allies). **Proceedings Royal Society B: Biological Sciences**, v. 281, 2014.
- NYLANDER, J. A. A Perl script for selecting DNA substitution models using PhyML. Evolutionary Biology Centre, 2014. Disponível em: <<https://github.com/nylander/MrAIC>>. Acesso em: 19 FEB 2019.
- SAMBROOK, J.; MACCALLUM, P.; RUSSEL, D. **Molecular cloning: A laboratory manual**. Cold Springs Harbour Press, NY, 2001. 653p.
- SIEBERT, D. J. Notes on the anatomy and relationships of Sundasalanx Roberts (Teleostei, Clupeidae), with descriptions of four new species from Borneo. **Bulletin of the American Museum of Natural History**, v. 63, p. 13-26, 1997.
- SWOFFORD, D. L. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.0b10. 2002. Disponível em: <https://www.researchgate.net/publication/271205405_PAUP_Phylogenetic_Analysis_Using_Parsimony_and_Other_Methods_Version_40b10>. Acesso em: 19 FEB 2019.
- THOMPSON, J. D.; HIGGINS, D. G.; GIBSON, T. J. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Research**, v. 22, p. 4673-4680, 1994.
- WHITEHEAD, P. J. P. **Vol.7. Clupeoid fishes of the world (Suborder Clupeoidei): An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, shads, anchovies and wolf-herrings Part 1. Chirocentridae, Clupeidae and Pristigasteridae**. FAO SPECIES CATALOGUE. Rome IT: Food and Agriculture Organization of the United Nations 1985.
- WHITEHEAD, P. J. P.; NELSON, G. J.; WONGRATANA, T. **Vol.7. Clupeoid fishes of the world (Suborder Clupeoidei): An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, shads, anchovies and wolf-herrings Part 2 Engraulidae (suborder Clupeoidei)**. FAO SPECIES CATALOGUE. Rome IT: Food and Agriculture Organization of the United Nations 1988.
- WILSON, A. B.; TEUGELS, G. G.; MEYER, A. Marine incursion: the freshwater herring of Lake Tanganyika are the product of a marine invasion into West Africa. **PLoS ONE**, v. 3, p. 4-19, 2008