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Multidisciplinary recognizable proof of clupeiform fishes from the Southwestern Atlantic Ocean

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Abstract

In the Southwestern Atlantic Ocean, several described species of Clupeiformes overlap their geographical distribution in the coastal areas from Rio Grande do Sul (Southern Brazil) to Bahía Blanca (Argentina) and the Río de la Plata estuary. Larvae and juveniles of the SW Atlantic menhaden *Brevoortia aurea* are very difficult to discriminate from those of other clupeids belonging to the genera *Platanichthys* and *Ramnogaster* inhabiting the same environments. Here we implemented phylogenetic analyses based on mitochondrial *cytochrome b* sequences and morphometric and osteological studies to achieve unambiguous species recognition in different ontogenetic stages of six endemic species of Clupeiformes from the Southwestern Atlantic Ocean. All phylogenetic analyses based on the *cytochrome b* gene yielded a robust support to the existence of highly structured and monophyletic groups conforming clupeiforms taxa. These monophyletic entities were consistent with major groups accessed through the first two principal components (PCs) from morphometric variation among taxa. Both approaches resulted in accurated and complementary tools for the individual assignment in clades and groups within Clupeiformes during different ontogenetic stages in their life cycle. The detection of species-specific spawning and nursery areas through accurated methodological approaches of identification constitute a prerequisite for a sustainable management in pelagic fisheries.

Keywords: Estuarine, clupeiformes, identification, morphology, cytochrome b.

INTRODUCTION

The order Clupeiformes represented by herrings, sardines, anchovies, menhadens, shads and relatives, constitute a crucial group in world fisheries covering about 26% of the total annual catch of all marine pelagic fishes (Csirke, 2005). Even if to their economical importance, the relationships among and within the main lineages of the order Clupeiformes have been explored in few morphological studies and still remain poorly understood. In fact, a well-supported taxonomical hypothesis among the major groups of clupeiforms is still lacking (Lavoué et al., 2007; Li and Ortí, 2007).

The Clupeiformes constitute a well-accepted group, which is supported by several complex anatomical characters (Grande, 1985; Nelson, 1973). Recently, Nelson (2006) subdivided the order in two suborders, the monospecific Denticipitoidei and the Clupeoidei. Grande (1985) and Nelson (2006) subdivided the last suborder into four families: Engraulidae (two subfamilies, 16 genera, 139 species), Pristigasteridae (two subfamilies, 9 genera, 37 species), Chirocentridae (one genus, two species) and Clupeidae (five families, 66 genera, 216 species). The

relationships among the major lineages of Clupeoidei and, in particular, among the clupeid subfamilies (Dorosomatinae, Alosinae, Pellonulinae, Clupeinae and Dussumieriinae) were mostly unresolved (Lavoué et al., 2007).

Only in the past years, molecular systematic approaches based on mitochondrial genome (that is, the mitogenome) provided new insights into the relationships among teleost fishes (Inoue et al., 2001, 2004; Saitoh et al., 2003). More recently, these approaches were applied into the aforementioned systematic problems among Clupeiformes (Lavoué et al., 2007).

Most species of Clupeiformes inhabit marine tropical and sub-tropical coastal areas, and several groups are euryhaline and anadromous (Whitehead, 1985). These facts have great interest due to the physiological adaptations developed during different ontogenetic stages of their complex life cycle. According to Sinclair and Iles (1989), in complex life histories, eggs, larvae and juveniles exhibit geographic or spatial distributions that are usually different from those characteristics of the adult phase. Many pelagic clupeiforms form dense schools (Whitehead, 1985) which represent random assemblages of similar sized fish (Hauser et al., 1998).

In addition to the aforementioned systematic controversies among clupeiform families and subfamilies, it is being necessary an accurate and unambiguous identification of the described species of menhaden (Alosinae), sardines (Clupeinae) and anchovies (Engraulididae) overlapping their geographical distribution in the South western (SW) Atlantic Ocean coastal areas from Rio Grande do Sul (Southern Brazil) to Bahía Blanca (Argentina) Several spawning and nursery areas shared by several of these clupeiforms species (*Brevoortia aurea*, *Platanichthys platana*, *Ramnogaster* sp. and *Lycengraulis grossidens*) were detected in rivers, in the coastal lagoons system and in the nearshore environments of the Río de la Plata estuary. In this sense, larvae and juveniles (less than 30 mm in length) of the menhaden *B. aurea* are difficult to discriminate from those of other clupeids belonging to the genera *Platanichthys* and *Ramnogaster* inhabiting the same environments. Another marine and coastal small pelagic fish stock particularly abundant in the SW Atlantic Ocean is the anchovy (*Engraulis anchoita*) that usually is found off shore in Southern Brazil, Uruguay and Northern Argentina (Csirke, 2005). However, this species develops its life cycle to about 800 or more kilometers from the shore separated from the other SW Atlantic Clupeiformes.

Most of all these SW Atlantic clupeiform species are target by the commercial and artisanal fisheries and therefore a robust multidisciplinary approach to discrimination among species-specific fish and fish products, from eggs to adults is important to assist in managing fisheries for their long-term sustainability (Ward et al., 2005). Morphological approach in Clupeiformes have showed high performance to differentiate between close populations in the genus *Colia* (Cheng et al., 2005) or between populations of the anchovies genus *Engraulis* (Turan et al., 2004) among other studies. In the other hand, molecular systematic have proved accuracy to validate the existence from populations of the SW Atlantic Ocean of one species in the genus *Brevoortia* in which the taxonomy has been historically controversial (García et al., 2008).

In present work, we implemented phylogenetic analyses based on mitochondrial *cytochrome b* (*cyt-b*) sequences combined with morphometry to achieve species recognition in different ontogenetic stages of endemic species of Clupeiformes from SW Atlantic Ocean. Additionally, osteological study was performed in juvenile clupeids that inhabit in the same environments.

MATERIALS AND METHODS

Sampling

A total of 141 individuals from 29 collecting sites (Appendix 1) covering a widespread range of the geographic distribution of six clupeiforms species (*B. aurea*, *P. platana*, *Ramnogaster melanostoma, Ramnogaster arcuata, Lycengraulis grossidens* and *E. anchoita*) from Río de la Plata to Southern Brazil were used in the molecular phylogenetic and morphometric analyses. All adults individuals were at first place identify by exomorphological diagnosis. Additionally a comparative osteological study of a limited subset of juveniles from four clupeids taxa was implemented (Figure 1). The collecting localities were selected among different estuarine environments in the Río de la Plata, SW Atlantic Ocean and their associated system of coastal lagoons and rivers. Two set of samples were included: Adult individuals of large-sizes (>150 mm) obtained from landings of artisanal fisheries using purse seines or drift nets and juveniles ones (< 80 mm) recruited using purse seines from sea coast beaches and coastal lagoons. Individuals of *E. anchoita* were kindly provided onboard fishing vessels by scientific observers from DINARA (Dirección Nacional de Recursos Acuáticos) on board of the commercial fleet.

Morphometric analysis

Shape variation was analysed through a geometric morphometry using the thin plate spline analysis (Bookstein, 1991) which provides shape parameters for subsequent multivariate analyses. Present analysis was based on the measurement of two-dimensional coordinates of 14 landmarks determined on the external morphology of the fish (Figure 2). The data were extracted from digital pictures of samples preserved in 95% ethanol. Due to taxonomic controversies and in order to increase the accuracy of morphometry in the discrimination among clupeiforms taxa, we have included in comparative analyses other species of clupeids not found in the present sampling (*Harengula jaguana*, *Sardinella aurita* and *Opisthonema oglinum)*. In this case, descriptive drawings of Whitehead (1985) were used as “passive” samples, without any weight in other steps of the analyses. The coordinates of the landmarks were obtained with the software tpsDig version 2.04 (Rohlf, 2005a), and the partial-warps were calculated with tpsRelw version 1.41 (Rohlf, 2005b). To examine the extent to which

Appendix 1. Localities, geographic coordinates and GenBank accession numbers of all specimens sampled in present study.

Species	Locality	Latitude/ Longitude	GenBank accession number	Analyses (n)
B. aurea	Aguas Dulces			M (7-a)
B. aurea	Barra del Chuy			M (6-a)
B. aurea	Buceo beach			M (5-j)
B. aurea	Cerro beach			M (5-a)
B. aurea	Ensenada de Valizas		EF564688	S (7-j) M (5-j)
B. aurea	José Ignacio lagoon			M (5-j)
B. aurea	La Paloma		EF564672	S (4-a) M (4-a)
B. aurea	Maldonado stream		EF564665-EF564672-EF564684	S (20-j,1-a) M (7-j) O (2-j)
B. aurea	Malvín beach		EF564673-EF564675	S (1-j,1-a) M (5-j) O (1-j)
B. aurea	Pando stream			M (3-j)
B. aurea	Pascual beach			M (3-j) O (2-j)
B. aurea	Puerto de los Botes			M (3-a)
B. aurea	Puerto del Buceo		EF564683-EF564667-EF564670	S (2-j,1-a) M (3-j, 2-a)
B. aurea	Punta Carretas		EF564672-EF564679	S (2-a) M (4-a)
B. aurea	Punta del Este			M (6-j)
B. aurea	Rocha lagoon		EF564693-EF564686	S (2-j) M (5-j)
B. aurea	Valizas stream		EF564665-EF564687	S (2-a) M (4-a)
B. aurea	Verde beach			M (2-j)
E. anchoita	24 m in depth	35º 16´ S / 53º 49´ W	GQ890158-GQ890168	S (2-a)
E. anchoita	24.5 m in depth	35º 15´ S / 53º 49´ W	GQ890159	S (1-a)
E. anchoita	25 m in depth	35º 17´ S / 54º 65´ W	GQ890162	S (1-a)
E. anchoita	60 m in depth	35º 34´ S / 53º 36' W	GQ890161	S (1-a)
E. anchoita		35º 24´ S / 53º 55´ W	GQ890164- GQ890165	S (2-a)
E. anchoita		35º 35´ S / 54º 39´ W	GQ890160- GQ890163	S (2-a)
E. anchoita		36º 48´ S / 55º 18´ W	GQ890157- GQ890166	S (2-a)
E. anchoita		36º 54´ S / 55º 29´ W	GQ890167	S (1-a) M (1-a)
L. grossidens	EG766	34º 50´ S / 57º 34´ W	GQ890171- GQ890178	S (2-a) M (3-a)

Appendix 1. Contd

L. grossidens	EG785	34º 52´ S / 55º 31´ W	GQ890169- GQ890172	S (2-a)
L. grossidens	EG779	35º 18´ S / 56º 44´ W	GQ890173-GQ890174-GQ890175	S (3-a)
L. grossidens	Valizas stream		GQ890176- GQ890177	S (2-a)
P. platana	José Ignacio lagoon		GQ890194- GQ890195	S (2-a) M (1-a)
P. platana	Rocha lagoon		GQ890207	S (1-j) M (1-j) O (2-j)
P. platana	Valizas stream		GQ890193- GQ890196 GQ890205- GQ890206 GQ890208- GQ890209	S (6-a) M (2-a)
R. arcuata	Pajas Blancas beach			M (1-j) O (2-j)
R. melanostoma	Cufré beach		GQ890211- GQ890212 GQ890213	S (3-a) M (1-a)
R. melanostoma				S (1-j) O (2-j)

When available latitude and longitude coordinates were included for marine and estuarine localities. Type of analyses: S = Sequence analyses; M = morphometric analyses; O = osteology; (n) = number of individuals analyzed (j=juveniles; a=adults).

landmarks reveal phenetic clusters of individuals of different shape of those partial-warps were used as input variables for a principal components analysis (PCA) using the STATISTICA package, version 8.0 (Statsoft, 2007).

Osteological methodology

Only four clupeid species (*B. aurea*, *R. arcuata*, *R. melanostoma* and *P. platana*) in an ontogenetic stage where is very difficult to discriminate them were included in osteological comparative studies. Fourteen characters were analyzed as follows: af, anterior fontanelle; ep, epiotic; epf, epiotic fenestra; f, frontal; let, lateral ethmoid; mes, mesethmoid; n, nasal; pa, parietal; pf, posterior fontanelle; pto, pterotic; so, supraorbital; soc, supraoccipital; sp, sphenotic; tf, temporal fenestra. *R. arcuata*: Specimens between 23.0 to 28.0 mm in length; *R. melanostoma*: Specimens between 30.0 to 31.6 mm in length; *P. platana*: Specimens of 37.2 and 38.4 mm, respectively and *B. aurea*: Specimens between 23.0 to 35.3 mm in length. They were cleared and counterstained (C&S) following Taylor and Van Dyke (1985). The standard length (SL) was taken to posterior margin of hypurals.

Genomic DNA extraction

Tissues of the voucher specimens are deposited in the Evolutionary Genetics collection in the Faculty of Sciences, University of the Republic, Montevideo, Uruguay. Genomic DNA was isolated from liver tissue of freshly sacrificed animals (fixed in 95% ethanol) using sodium chloride protein precipitation, followed by ethanol precipitation (modified from Medrano et al., 1990).

Mitochondrial cytochrome b sequences

A fragment of the *cyt-b* gene of approximately 800 bp was amplified using the CB3-H and Gludg-L primers (Palumbi et al., 1991) following the PCR cycle profile: 94ºC for 1 min, 45ºC for 1 min, 72ºC for 1 min; 30 cycles. PCR products were cleaned with the CONCERT Kit rapid PCR purification System (Life Technology) and subjected to sequencing using the amplification primers in a Perkin-Elmer ABI Prism 377 automated sequencer. The final sequences for analysis were obtained by reconciling chromatograms for the light and heavy DNA strands. Sequence alignment was performed using the CLUSTAL X version 1.8 program (Thompson et al., 1997).

Statistical analyses of mitochondrial cytochrome b sequences

Nucleotide composition and substitution patterns were calculated using the MEGA version 2.0 (Kumar et al., 2001) and DnaSP version 4.0 (Rozas et al., 2003) programs. The corrected estimates of pairwise sequence divergence were obtained using the Kimura (1980) two-parameter algorithm (K2P) implemented in MEGA. Within a population, DNA polymorphism was measured calculating

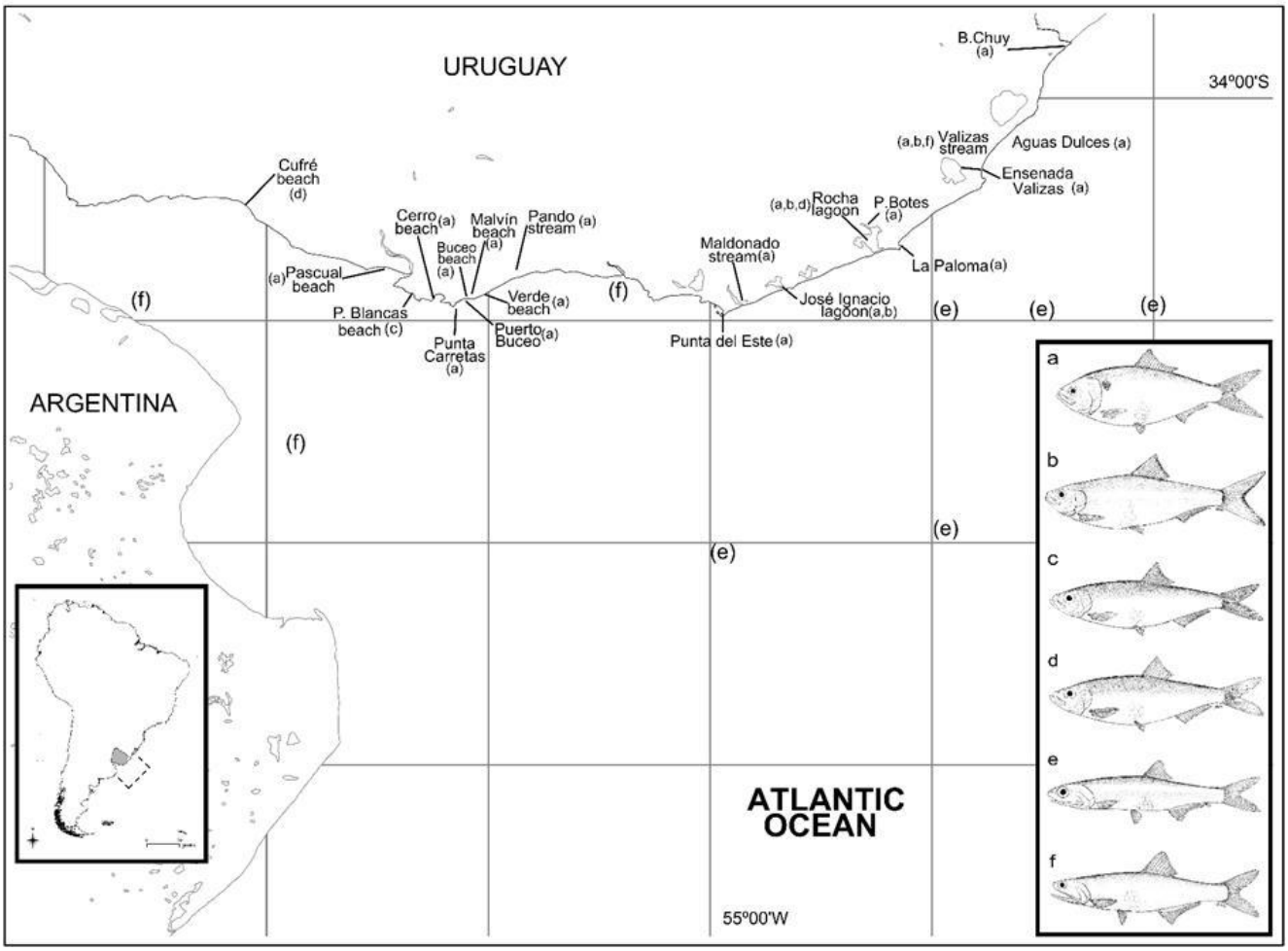

Figure 1. Collecting sites of juveniles and adults individuals of six Southwestern Atlantic Ocean clupeiform species inhabiting coastal lagoons and river systems associated to the Río de la Plata estuary: (a) *B. aurea*; (b) *P. platana*; (c) *R. arcuata*; (d) *R. melanostoma*; (e) *E. anchoita*; (f) *L. grossidens*. (Fish pictures modified from Whitehead, 1985). The dotted line in the South American map represents the area under study.

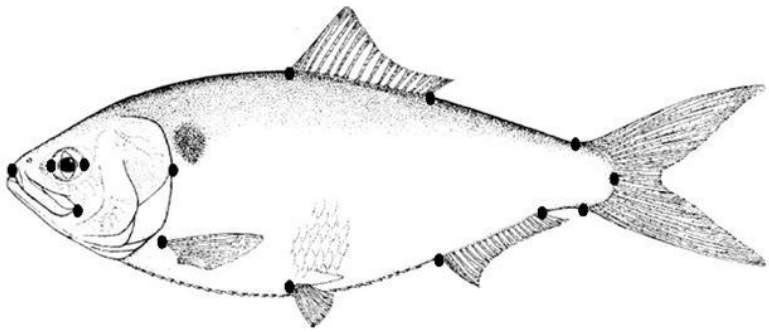

Figure 2. Landmarks generated over the schematic representation of *B. aurea* (Modified from Whitehead, 1985).

the proportion of segregating sites (S), the haplotype diversity (h) (Nei, 1987: 179) and the nucleotide diversity (π) (Nei, 1987: 257) using the ARLEQUIN version 2.0 (Schneider et al., 2000) and DnaSP version 4.0 (Rozas et al., 2003) software packages.

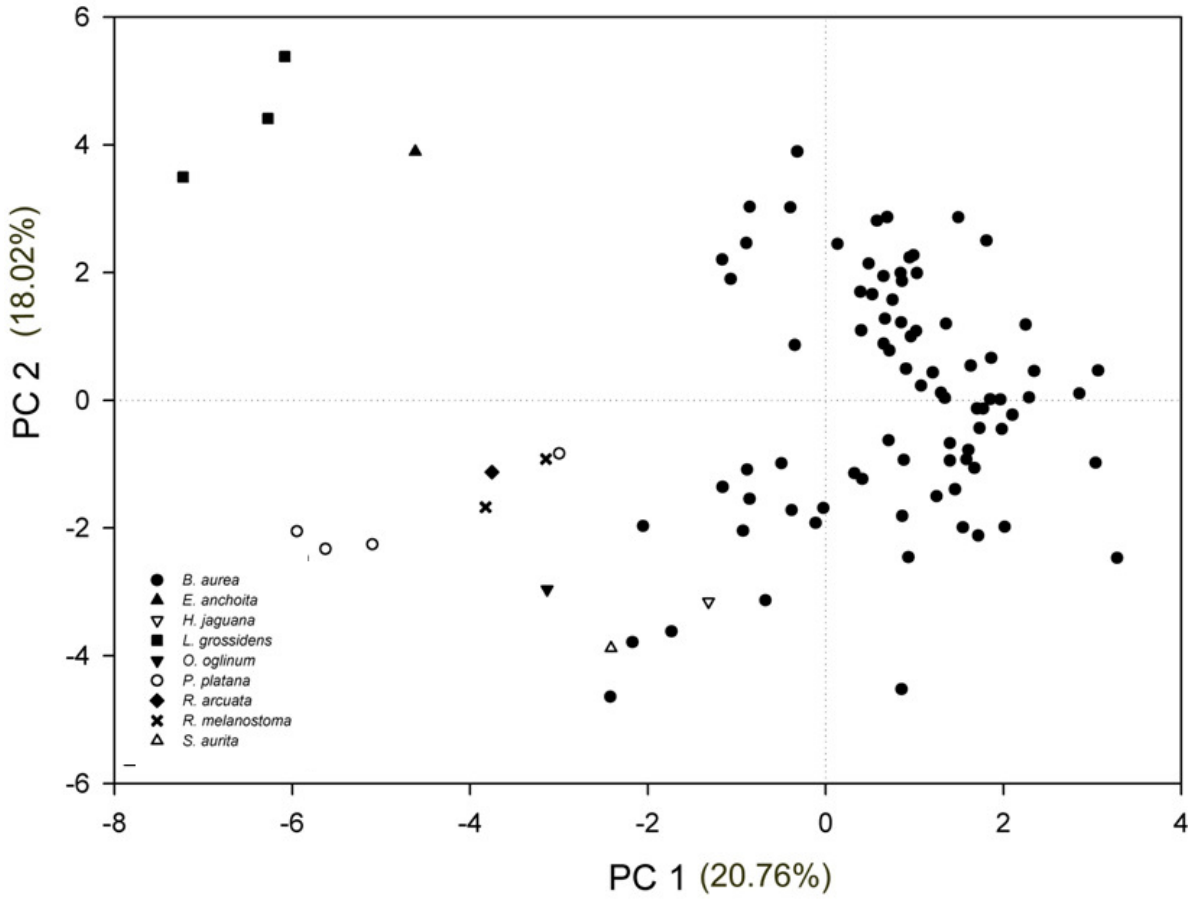

Figure 3. Morphometric analysis of six Southwestern Atlantic clupeiform species present in the samples and other clupeids taxa. Scatterplot of the first two principal components (PC) of partial-warps PCA. Other species of clupeids in the box (*H. jaguana*, *S. aurita* and *O. oglinum)*, not found in the samples were included in comparative analyses.

Tajima´s (1989) test was performed to test for mutation/drift equilibrium and the significant excess of low-frequency haplotypes in order to evaluate the hypothesis of population expansion using the DnaSP version 4.0 (Rozas et al., 2003) program.

Phylogenetic analyses

To resolve the phylogenetic association among mitochondrial sequences from Clupeiformes taxa, four different methods of phylogenetic reconstruction were used: Maximum-parsimony (MP), neighbour-joining (NJ), maximum-likelihood (ML) and Bayesian Inference (BI). They were performed in PAUP*4.0b8 (Swofford, 1998) and MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) programs.

An equally weighted MP analysis was performed through heuristic search (MULPARS option, stepwise addition, tree-bisection-reconnection TBR branch swapping, 100 replications). A strict consensus between rival trees was computed to reconcile equally parsimonious topologies. Distance trees were generated on the basis of the corrected K2P distances implemented in MEGA and subjected to the NJ method of tree reconstruction (Saitou and Nei, 1987). For both methods (MP and NJ), the degree of confidence assigned to nodes in trees was assessed by bootstrapping with 500 replicates.

To identify the best-fitting model for ML and ML-based distance analyses of the Clupeiformes here analyzed, the GTR + G (Rodríguez et al., 1990) substitution model was employed. This model allows us to consider equal base frequencies and among-site rate variation drawn from a gamma distribution (G). It was selected from a comparison among 56 0models using the Akaike information criterion (Akaike, 1974) as implemented by the Modeltest version 3.06 (Posada and Crandall, 1998) software package. A heuristic ML search (again with 100 replications of stepwise addition and TBR branch swapping) was implemented in the PAUP*4.0b8 (Swofford, 1998) software package. Other Clupeiformes sequences retrieved from the GenBank were included in the ingroup: *S. aurita* (AF472584), *Engraulis encrasicolus* (AY923823), *Sardinops sagax* (AF472586), *Sardinops caeruleus* (AF472585). Since the clupeiforms taxonomy remains highly controversial and unresolved (Lavoué et al., 2007; Li and Ortí, 2007) all trees were rooted by means of the outgroup criterion using *Anguilla marmorata* (DQ093417) according to Saitoh et al. (2003).

To access an additional measure of the posterior probability for nodes in the tree, the Bayesian method of phylogenetic inference (Rannala and Yang, 1996) based on the aforementioned GTR + G tree was implemented using the program MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). Searches were run with four simultaneous Markov Chain Monte Carlo chains (MCMC) for 1,000,000 generations, sampling trees every 100 generations and

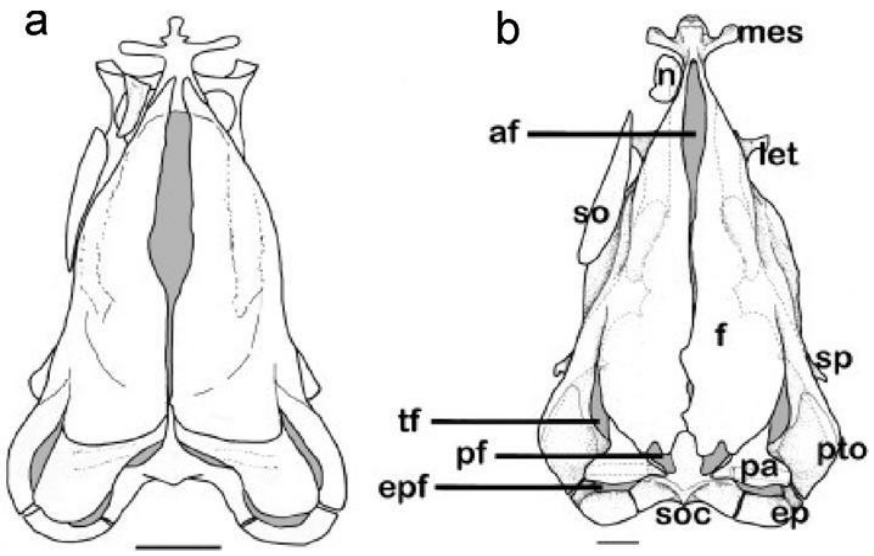

Figure 4. Fourteen osteological characters included in the comparative analysis of clupeid species. (a) *R. arcuata*, 27.8 mm LT showing juvenile character state. (b) *P. platana*, 81.5 mm SL adult as characters reference: af, anterior fontanelle; ep, epiotic; epf, epiotic fenestra; f, frontal; let, lateral ethmoid; mes, mesethmoid; n, nasal; pa, parietal; pf, posterior fontanelle; pto, pterotic; so, supraorbital; soc, supraoccipital; sp, sphenotic; tf, temporal fenestra.

applying temperatures of 0.2. Because the chains appear to reach apparent stationary by about the 9,200th generation, the first 9,199 trees were discarded. The remaining 990,800 were used to compute a 50% majority-rule consensus tree. The percent of times that a clade occurred among the sampled trees has been interpreted as the probability of the existence of that clade (Huelsenbeck et al., 2002).

RESULT

Morphometric analysis

Morphometric variation among Clupeiformes taxa was visualized via scatter plot of the scores of the first two principal components (PCs) (Figure 3). The PC1 accountfor the 20.76 % of the total variance, whereas the PC2 explains the 18.02%. Although these two first principal components show low values, they allow us to discriminate three major groups of taxa: Species of the family Engraulidae (*L. grossidens* and *E. anchoita*) clustered separately from the family Clupeidae, an intermediate group integrated by *P. platana, R. arcuata* and *R. melanostoma* and the last major group including all *B. aurea* samples, *H. jaguana*, *S. aurita* and *O. oglinum.* Remarkably from both PCs, analysis *B. aurea* shows high dispersion of its morphometric pattern overlapping with the remaining taxa in this group. In spite of the low number of individuals analyzed, *P. platana* overlaps its morphometric variation between individuals from both *Ramnogaster* species.

Osteology

Fourteen osteological characters were analyzed in a comparative approach of juvenile specimens belonging to four clupeid species (Figure 4a and b). At a SL ≤30 mm, the four species have the head pointed anteriorly, with very large cranial fontanelles occupying most of the anterior and median regions of skull. The shape of the bones is not well defined because there are some areas with incipient ossifications. Upper and lower jaws are ossified. None of the bones of the specimens examined have teeth. In *R*. *arcuata,* the premaxillary notch is scarcely developed. In *Ramnogaster*, the supramaxilla is well developed and larger than that of *Brevoortia*. A small bone, probably a supramaxilla, appears in some specimens of *P*. *platana*. Abdominal scutes are ossified although their number is lower than in adults: *R*. *arcuata* has 23 to 26; *R*. *melanostoma* 25 to 26; *P*. *platana* 24 to 26 and *B*. *aurea* with 25 to 27. The supraneural bones still have unossified margins. The surface of the gill rakers is smooth.

Mitochondrial cyt-b variation in the Clupeiformes data set

The present study includes 54 mitochondrial *cyt-b* sequences of juveniles and adults belonging to Clupeiformes taxa. In the total data set including the outgroup taxon, among 720 bp analyzed, 395 were

Table 1. Estimates of DNA polymorphism in Southwestern Atlantic Clupeiformes.

Species	Number of sequences	Variable sites	Phylogenetic informative sites	Number of haplotypes	Haplotype diversity (SD)	π (SD)	Kimura 2P distance (Tv+Ts) (SD)	D
B. aurea	17	37	9	13	0.967(0.036)	0.017(0.004)	0.016 (0.005)	-2.025(P<0.05)
E. anchoita	12	55	21	10	0.978(0.003)	0.060(0.030)	0.071(0.011)	-1.121(P>0.10)
L. grossidens	9	30	3	5	0.786(0.151)	0.035(0.015)	0.036(0.007)	-1.652(P>0.05)
P. platana	9	20	8	8	0.973(0.064)	0.023(0.005)	0.071(0.011)	-0.729(P>0.10)
Ramnogaster sp.	4	23	4	4	1.000(0.177)	0.027(0.006)	0.037(0.011)	0.372(P>0.10)

Variables and phylogenetic informative sites among 720 bp in the total data set; Haplotype diversity (h = gene) (Nei, 1987); π = Nucleotide diversity (Nei, 1987). Corrected Kimura 2P distances (1980). D = Neutrality test (Tajima, 1989).

variables sites and 331 were phylogenetically informative ones. Only considering the Clupeiformes data set, 379 were variables and 320 phylogenetically informative sites were found. Among 240 amino acids, 108 represented variables sites and 74 phylogenetic informative sites.

Table 1 show variable and phylogenetically informative sites as well as the haplotypes number (GenBank accession numbers in Appendix 1) for each SW clupeiforms analyzed. Insertions and deletions were notobserved. High values of haplotype diversity (h) were found in four clupeiforms taxa. *E. anchoita* showed the higher value of the nucleotide diversity (π=0.060, SD = 0.015) (Table 1). The gamma distribution shape parameter was 1.637, indicating that more sites evolve at a moderate mutation rate and fewer sites have extremely high or low rates. Table 1 includes the average of the corrected K2P sequence divergence within each taxon of the SW Atlantic clupeiforms analyzed. Remarkably, *P. platana* and *E. anchoita* (7.1%, SD= 0.011) showed the highest value, whereas *B. aurea* (1.6%, SD = 0.005) the lower. Table 2 shows the pairwise corrected genetic distances among taxa under the K2P model of nucleotide substitution. Interestingly, *E. anchoita* appears as the most divergent taxa within the clupeiforms (in average 20%). The maximum divergence by including the outgroup taxon was 32% (SD = 0.047).

Phylogenetic analyses

All phylogenetic analyses showed the same tree topology (Figure 5). Maximum likelihood analysis generated under the GTR + G model of molecular evolution have produced a well-supported phylogeny (-ln likelihood score = 7018.95). Figure 5 resumes the ML and the BI due to the similarity of both tree topologies. Bayesian values over the ML branches showed a high degree of confidence to the posterior probability of monophyly for the major and minor Clupeiformes clades. The two major clades correspond to the families Clupeidae and Engraulidae. The family Clupeidae includes two minor clades integrated by members of the subfamilies Clupeinae and Alosinae. *S. aurita* collapsed basal to the major Clupeidae clade. Remarkably, Alosinae appears derived from the subfamily Clupeinae, including all taxa of *B. aurea*. Species from the genus *Sardinops* (*S. caeruleus* and *S. sagax*) were sister taxa of the *B. aurea* clade. Minor monophyletic clades in *B. aurea* were integrated by mixed populations from the estuarine mouth of rivers and coastal lagoons associated to the Río de la Plata estuary and Southwestern Atlantic Ocean. *P. platana* and *Ramnogaster* sp. constituted well supported monophyletic sister clades, each of one integrated by other minor monophyletic intraspecific group of samples which received considerable Bayesian posterior probability of occurrence (Figure 5). In *P. platana,* two major different and highly diverse structured lineages were detected: One of them including samples from Valizas stream and Rocha coastal lagoon and the other one integrated only by individuals from the José Ignacio coastal lagoon. *Ramnogaster* sp. presented two monophyletic entities showing high probability of occurrence. One group conformed by haplotypes of *R. melanostoma* from Cufré beach in the Río de la Plata estuary. Remarkably, the other one included *R. melanostoma* individuals from the

Table 2. Corrected genetic distances among groups under Kimura 2P model (below diagonal),

	1	2	3	4	5	6	7	8	9
1		0.040	0.047	0.039	0.035	0.037	0.044	0.037	0.040
2	0.253		0.035	0.038	0.035	0.042	0.039	0.036	0.039
3	0.320	0.209		0.036	0.043	0.041	0.036	0.042	0.039
4	0.255	0.219	0.201		0.032	0.042	0.042	0.037	0.038
5	0.222	0.221	0.300	0.208		0.035	0.041	0.037	0.042
6	0.237	0.277	0.257	0.282	0.248		0.041	0.041	0.045
7	0.268	0.247	0.226	0.261	0.282	0.281		0.035	0.041
8	0.250	0.217	0.290	0.247	0.260	0.291	0.217		0.039
9	0.283	0.273	0.274	0.262	0.305	0.329	0.281	0.280	

Standard deviation (S.D) estimated by bootstrap method (above diagonal): 1. Outgroup (*Anguilla marmorata*), 2. *Sardinella aurita*, 3. *Sardinops*, 4. *B. aurea*, 5. *P. platana*, 6. *Ramnogaster*, 7. *E. encrasicholus*, 8. *L.grossidens*, 9. *E. anchoita*.

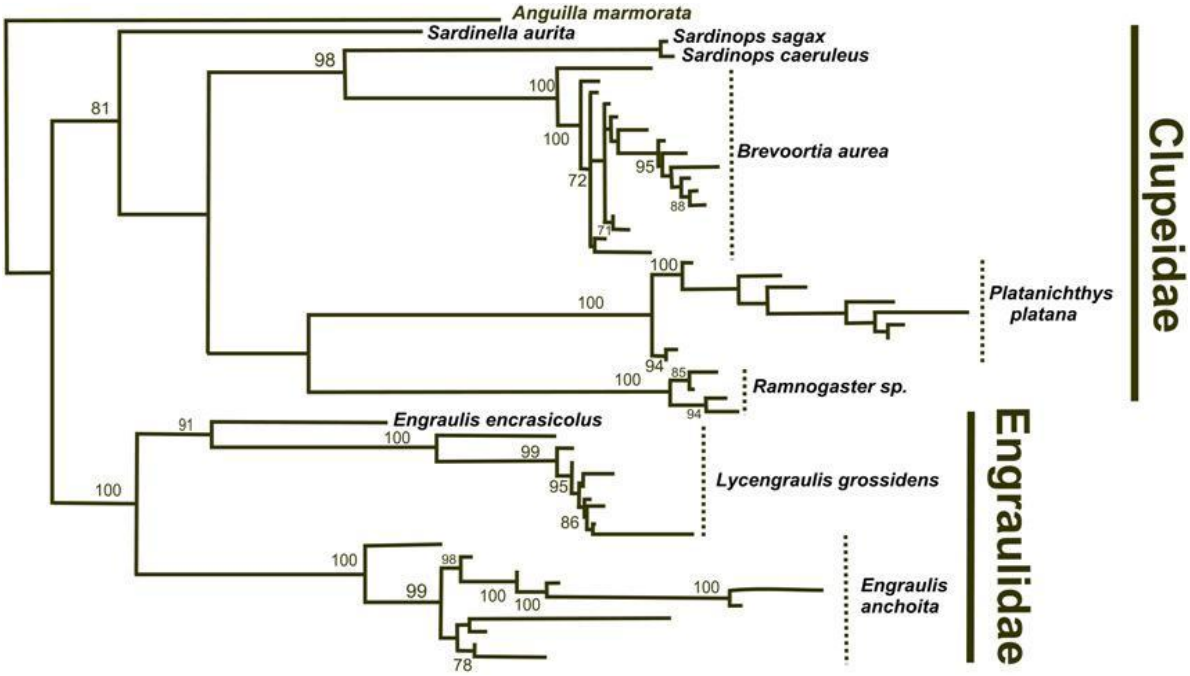

Figure 5. Maximum likelihood analysis under general time-reversible model with gamma correction (GTR+G) and Bayesian inference, based on 54 *cyt-b* sequences of Clupeiformes. Numbers in the branches correspond to the Bayesian posterior probability obtained with the MrBayes program

aforementioned collecting site and other specimen first morphologically identified as *R. arcuata* from the Rocha coastal lagoon, an estuarine environment associated to the Southwestern Atlantic Ocean. The other major monophyletic group includes the family Engraulidae, subfamily Engraulinae, and it was integrated by *E. anchoita* and *L. grossidens* taxa. Interestingly, *E. encrasicolus* appears as a sister taxon of *L. grossidens* and more distantly related to *E. anchoita*. These two last taxa presented minor monophyletic intraspecific groups showing considerable genetic structuring. In *E. anchoita* these monophyletic entities present different geographic locations: One fish stock situated between 35° S and 5° to 53° W; a second one between 36° S and 53 to 52° W and finally the most basal haplotype included samples from 36° S and 55° W in front of the Río de la Plata mouth. *L. grossidens* presented well supported intraspecific clades which were constituted by mixed populations from the Río de la Plata and SW Atlantic Ocean as well as their rivers and coastal lagoons estuarine associated environments.

DISCUSSION

Molecular systematic analysis of the Southwestern Atlantic clupeiforms

All phylogenetic analyses (Figure 5) yielded robust support to the existence of highly structured and monophyletic group conforming clupeiforms taxa from the SW Atlantic Ocean. Present analyses including a limited number of taxa were congruent with previous morphological and molecular phylogenetic hypothesis of relationships among Clupeiformes. Present results were concordant with studies supporting the monophyly of the suborder Clupeoidei, as well as with the monophyly of two, among five, families belonging to the suborder: Engraulidae and Clupeidae (Di Dario, 2004; Grande, 1985; Nelson, 1973). The family Clupeidae (Nelson, 1970) the most specious group within the Clupeiformes (approximately 208 species, Fishbase, September 2008:http://www.fishbase.org/search.php) includes five subfamilies of the marine coastal, some freshwater and anadromous schooling fishes (Grande, 1985; Nelson, 1970; Whitehead, 1985; Whitehead et al., 1988). Several authors have noted that two of these subfamilies, Alosinae and Clupeinae, are certainly not monophyletic (Grande, 1985; Nelson, 1970) but they left unchanged their classification pending clarification from additional studies (Lavoué et al., 2007). In this sense, instead of the conventional subfamilial arrangement, present data showed that the subfamily Clupeinae presented two monophyletic entities. One clade constituted by *B. aurea* belonging to Alosinae subfamily nested with its sister taxon, the genus *Sardinops,* belonging to the Clupeinae subfamily. Similar results were found by Lavoué et al. (2007) in which *Sardinops* and *Alosa* resulted in a well supported monophyletic entity within the Clupeinae group. The other Clupeinae clade included the genera *Ramnogaster* and *Platanichthys* analyzed here. Remarkably, present molecular analysis does not support the existence of the two described *Ramnogaster* species: *R. arcuata* and *R. melanostoma.* This taxonomic incongruence with previous morphological analysis (Cione et al., 1998) will be clarified in further studies including additional number of samples from both taxa. Interestingly, *S. aurita* represented the most distantly taxon which collapsed basal to the family Clupeidae.

On the other hand, our present results corroborated the monophyly of the family Engraulidae supported by morphological characters (Nelson, 1967, 1970; Grande, 1985). Two minor clades, which presented high posterior probability of occurrence, emerged from present analyses. One of them integrated by the sister taxa *E. encrasicolus* and *L. grossidens* and the other one grouping all taxa belonging to *E. anchoita*. Even though the taxonomic sampling was incomplete, the genus *Engraulis* appears as a paraphyletic entity in the present analysis.

Molecular vs. morphological data in the Southwestern Atlantic clupeiforms discrimination

Like the genetic analysis (Figure 5), morphometric approach reveals similar major groups of species (Figure 3). A well discriminated group is composed by *E. anchoita* and *L. grossidens.* Another group comprises the genus *Ramnogaster* and *P. platana* which is closer, but separated to the major cluster integrated mainly by *B. aurea* samples. Remarkably, present analyses based on morphometric data showed high level of intraspecific variability in *B. aurea.* The partial overlapping detected in *B. aurea* in relation to other clupeids taxa (Figure 3) may be due to the inclusion of different ontogenetic stages (juveniles, adults) in the analysis.

However, *S. aurita* displays different patterns of taxa relationship in genetic and morphometric analyses. Whereas in the *cyt-b* phylogenetic analysis, it appears as a basal taxon in the Clupeidae clade (Figure 5); in the PCs analysis (Figure 3) it remains next to other Clupeinae and the most distal Alosinae samples belonging to *B. aurea*. This last *S. aurita* location is concordant with previous molecular phylogenetic analyses of Clupeiformes using nuclear and mitochondrial DNA sequences (Li and Ortí, 2007). The discordant molecular phylogenetic location of *S. aurita* from both analyses would be due to different sample design as well as the lower number of Clupeiformes taxa included in present study.

In contrast to morphometric and molecular results, the osteological characters found in small specimens of *R. arcuata*, *R. melanostoma*, *P. platana* and *B. aurea* do not help to differentiate these four species. Giangiobbe and Sánchez (1993) found teeth in the jaws of *B. aurea* but they were not observed in the specimens analyzed in this study. Nevertheless, adult specimens are distinguished by different morphological and osteological characters (Cione et al., 1998; Segura and Díaz de Astarloa, 2004). Among others, both *B. aurea* and *P. platana* have large cranial fontanelles, smooth frontals and supraoccipital process. The shape of frontals differentiates *B. aurea* from *P. platana*. In *B. aurea,* it is markedly broader anteriorly than posteriorly. Also, *B. aurea* is easily distinguished by the black and round humeral spot that appear well defined at about 30 mm SL. *Platanichthys* and *Ramnogaster* have teeth on jaws, whereas teeth are absent in adult specimens of *B. aurea*. In the skull surface of *Ramnogaster* adult specimens, only the anterior cranial fontanelle is still open but reduced, the frontals have a long ridge and the supraoccipital does not have a conspicuous posterior process. The two species of *Ramnogaster* may be differentiated by the extent of the anterior cranial fontanelle, the separation between both

frontals in the posterior region of the skull in *R*. *arcuata*, the low ridge in the frontal of *R*. *melanostoma*, the larger teeth in premaxilla, maxilla and dentary of *R*. *arcuata* and the large tooth-like structures on the surface of gill rakers. Also, the number of dorsal-fin rays (*R*. *arcuata* 17 to 21, *R*. *melanostoma* 14 to 16), and other detailed characters as the presence of a tooth plate on the pharyngobranchial 2 of *R*. *arcuata* differentiate both species (Cione et al., 1998).

Morphological and genetic data: Accurate tools in sustainable management of pelagic fisheries

According to their actual taxonomic status present, morphometric and molecular data resulted in accurated and complementary tools for the individual assignment in clades and groups within SW Atlantic Ocean Clupeiformes during different ontogenetic stages of their life cycle. However, the osteological characters analyzed here represented an additional useful identification approach only for adult specimens but do not help to differentiate among juveniles clupeids species inhabiting the same environments.

Following Blaber and Blaber (1980), temperate regions nearshore marine waters and estuaries act like important nursery areas for several marine teleost species, before they mature and migrate out to deeper waters. In the present study, several common nurseries areas for clupeids and engraulid species (*Lycengraulis*) were detected in rivers and coastal lagoon system associated to the Río de la Plata and Atlantic Ocean nearshore environments (Figure 1). The main difficulty in discriminating and identifying clupeiforms life stages relates to their similar external morphology and the lack of appropriate descriptions of larval development and identification keys for congeners or closely related taxa (Ditty et al., 2006). Nevertheless, present paper have corroborated the high performance of morphometric and molecular tools to accurated species identification in spite of the limited number and size of individuals sampled in some taxa. On the other hand, in fish assemblage, morphological similarity detected among clupeiform juveniles may be a common strategy shaped by similar environmental pressures according to forage availability and spatial predation refuge. Carlson and Stenseth (2008) stressed that in spatially structured populations in which some component of the population used the nearshore environment relatively more than other components (e.g. juveniles, breeders), the individuals would have differed in their vulnerability to be captured based on their phenotype traits (e.g. size).These issues remain opens to further approaches including a more extensive sample strategy and analyses. In conclusion, the detection of species-specific spawning and nursery areas through multidisciplinary methodological approaches of species identification, constitute a robust contribution to a sustainable management in pelagic fisheries.

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REFERENCES

Akaike H (1974). A new look at the statistical model identification. IEEE Trans. Automat. Contr., 19: 716–723.

Blaber SJM, Blaber TG (1980). Factors aVecting the distribution of juvenile estuarine and inshore Wsh. J. Fish. Biol., 17: 143–162.

Bookstein FL (1991). Morphometric tools for landmark data: geometry and biology. New York: Cambridge University Press.

Carlson SM, Stenseth NC (2008). Commentary: Fishery selection across the millennia. Proc. R. Soc. Lond. B Biol. Sci. 275: 2657-265.

Cheng QQ, Lu DR, Ma L (2005). Morphological differences between close populations discernible by multivariate analysis: A case study of genus Coilia (Teleostei: Clupeiforms). Aquat. Living Resour., 18: 187–192.

Cione AL, Azpelicueta MM, Casciotta JR (1998). Revision of the clupeid genera *Ramnogaster*, *Platanichthys* and *Austroclupea* (Teleostei: Clupeiformes). Ichthyol. Explor. Freshwat. 8: 335-348.

Csirke J (2005). Review of the state of world marine fishery resources. FAO fisheries Technical Paper 457. Publishing Management Service Rome, Italy ISBN 92-5-105267-0

Di Dario F (2004). Homology between the recessus lateralis and cephalic sensory canal, with the proposition of additional synapomorphies for the Clupeiformes and the Clupeoidei. Zool. J. Linnean Soc., 141: 257–270.

Ditty JG, Farooqi T, Shaw RF (2006). Clupeidae: sardines & herrings. In: Richards, W.J. (Ed.) Early life stages of Atlantic fishes: an identification guide for western central North Atlantic. CRC Press, Boca Raton, pp. 73–99.

García G, Vergara J, Gutiérrez V (2008). Phylogeography of the Southern Atlantic menhaden *Brevoortia aurea* inferred from mitochondrial cytochrome b gene. Mar. Biol., 155: 325–336.

Grande L (1985). Recent and fossil clupeomorph fishes with materials for revision of the subgroups of clupeoids. Bull. Am. Mus. Nat. Hist., 181: 235–372.

Giangiobbe A, Sánchez S (1993). Feeding in the menhaden B. aurea. Maritime Front, 14: 71-80.

Hauser L, Carvalho GR, Pitcher TJ (1998). Genetic structure in the Lake Tanganyika sardine *Limnothrissa miodon*. J. Fish. Biol., 53: 413–429.

Huelsenbeck JP, Hall B (2001). Manual for MrBayes: Bayesian inference of phylogeny.Distributed by the authors, Department of Biology, University of Rochester, New York.

Huelsenbeck JP, Larget B, Miller RE, Ronquist F (2002). Potential

applications and pitfalls of Bayesian inference of phylogeny. Syst. Biol., 51: 673–688.

Inoue JG, Miya M, Tsukamoto K, Nishida M (2001). Complete mitochondrial DNA sequence of the Japanese anchovy Engraulis japonicus. Fish. Sci., 67: 828–835.

Inoue JG, Miya M, Tsukamoto K, Nishida M (2004). Mitogenomic evidence for the monophyly of elopomorph fishes (Teleostei) and the evolutionary origin of the leptocephalus larva. Mol. Phylo. Evol., 32: 274-286.

Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol., 16: 111–120.

Kumar S, Tamura K, Jakobsen IB, Nei M (2001). MEGA 2. Version 2.0. Molecular evolutionary genetics analysis software. Bioinformatics, 17: 1244–1245.

Li CH, Orti G (2007). Molecular phylogeny of Clupeiformes (Actinopterygii) inferred from nuclear and mitochondrial DNA sequences. Mol. Phylo. Evol., 44: 386–398.

Lavoué S, Miya M, Saitoh K, Ishiguro NB, Nishida M (2007). Phylogenetic relationships among anchovies, sardines, herrings and their relatives (Clupeiformes),inferred from whole mitogenome sequences. Mol. Phylo. Evol., 43: 1096–1105.

Medrano JF, Aasen E, Sharrow L (1990). DNA extraction from nucleated red blood cells. Biotechniques, 8:43.

Nei M (1987). Molecular evolutionary genetics. Columbia University Press, New York. pp. 505.

Nelson GJ (1967). Gill arches of teleostean fishes of the family Clupeidae. Copeia 2: 389–399.

Nelson GJ (1970). The hyobranchial apparatus of teleostean fishes of the families Engraulidae and Chirocentridae. Am. Mus. Novitates, 2410: 1–30

Nelson GJ (1973). Relationships of clupeomorphs, with remarks on the structure of the lower jaw in fishes. In: Greenwood, P.H., Miles, R.S., Patterson, C. (Eds.), Interrelationships of Fishes. Academic press, London, pp. 333–349.

Nelson JS (2006). Fishes of the World. Wiley-Interscience, New York p. 416.

Palumbi S, Martin A, Romano S, McMillan WO, Stice L, Grabowski G (1991).The simple fool's guide to PCR. Department of Zoologyand Kewalo Marine Laboratory, University of Hawaii, Honolulu. pp. 44.

Posada D, Crandall A (1998). Modeltest: testing the model of DNA substitution. Bioinformatics, 14: 817–818.

Rannala B, Yang Z (1996). Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. J. Mol. Evol., 43: 304–311.

Rodríguez F, Oliver JL, Marín A, Medina JR (1990).The general stochastic model of nucleotide substitution. J. Theor. Biol., 142: 485–501.

Rohlf FJ (2005). TpsDig program, version 2.04, Ecology and Evolution, SUNY at Stony Brook. State University of New York, New York (http://life.bio.sunysb.edu/morph/)

Rohlf FJ (2005). TpsRelw: Thin Plate Spline Relative Warp Analysis (Version 1.41). State University of New York at Stony Brook, Stony Brook, New York. (http://life.bio.sunysb.edu/morph/)

Rozas J, Sánchez-Delbarrio JC, Messeguer X, Rozas R (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics, 19: 2496–2497.

Saitoh K, Miya M, Inoue JG, Ishiguro NB, Nishida M (2003). Mitochondrial genomics of ostariophysan fishes: Perspectives on phylogeny and biogeography. J. Mol. Evol., 56: 464–472.

Saitou N, Nei M (1987). The neighbor-joining method: a new method for Reconstructing phylogenetic trees. Mol. Biol. Evol., 4: 406-425.

Schneider S, Roessli D, ExcoYer L (2000). Arlequin: a software for population genetics data analysis. University of Geneva, Geneva.

Segura V, Díaz de Astarloa J (2004). Osteological analysis of the menhaden Brevoortia aurea (Spix) (Actinopterygii: Clupeidae) in the southern Atlantic Ocean. J. Marine Biol. Oceanogr. 39: 37-52.

Sinclair M, Iles TD (1989). Population regulation and speciation in the oceans. J. Cons. Int. Explor. Mer., 45: 165–175.

StatSoft Inc. (2007). Statistica (data analysis software system). Version 8.0. http://www.statsoft.com.

Swofford DL (1998). PAUP*: phylogenetic analysis using parsimony (*and other methods). Version 4.0b8. Sinauer Associates, Sunderland.

Tajima F (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123: 585–595.

Taylor WR, Van Dyke GC (1985). Revised procedures for staining and clearing small fishes and other vertebrates for bone and cartilage study. Cybium, 9: 107–119.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic. Acids. Res., 25: 4876–4882.

Turan C, Erguden D, Gurlek M, Basusta N, Turan F (2004). Morphometric structuring of Anchovy (*Engraulis encrasicolus* L.) in the Black, Aegean and Northeastern Mediterranean Seas. Turk. J. Vet. Anim. Sci., 28: 865-871.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005). DNA barcoding Australia's fish species Phil. Trans. R. Soc. B, 360: 1847-1857.

Whitehead PJP (1985). FAO species catalogue. Clupeoid Fishes of the world (suborder Clupeoidei). An annotated and illustrated catalogue of the herrings, sardines, pilchards, sprats, anchovies and wolf-herrings. Part 1 Chirocentridae, Clupeidae and Pristigasteridae. FAO Fisheries Synopsis 125, FAO, Rome, pp. 1–303.

Whitehead PJP, Nelson GJ, Wongratana T (1988). FAO species catalogue. Vol. 7. Clupeoid fishes of the world (suborder Clupeoidei). Part 2. Engraulididae. FAO Fisheries Synopsis 125, FAO, Rome, pp. 305-579.