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Genetic diversity of the Southern Black Drum *Pogonias courbina* (Teleostei: Sciaenidae) from Río de la Plata and Atlantic Ocean coasts

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Abstract: Black drum *Pogonias cromis* and *P. courbina* are the only two valid species at present within the genus. These species support extensive commercial and recreational fisheries along the Western Atlantic coasts. Recent studies qualify the species *P. courbina* as vulnerable. We used two mitochondrial markers, partial sequences of cytochrome oxidase I and cytochrome b genes, to study genetic variation and demographic history of *P. courbina* from the Río de la Plata and the adjoining Southwestern Atlantic Ocean. Additionally, for the first time, we used both markers to estimate the divergence time of the species in the genus *Pogonias*. Regarding the study of the species *P. courbina*, the results obtained indicate relatively moderate to low levels of genetic variation. Besides, we found evidence that *P. courbina* has experienced a recent population expansion during the Pleistocene in the Río de la Plata and adjacent waters of the Southwestern Atlantic Ocean. Our analysis has shown that *P. cromis* and *P. courbina* diverged 1,154,800 years ago. The present work suggests a certain vulnerability to overexploitation and therefore conservation measures should be implemented to avoid population decline of *P. courbina* in the Río de la Plata.

Key words: Genetic variation; Molecular dating; Mitochondrial markers; Western Atlantic.

Diversidad genética de la corvina negra del sur *Pogonias courbina* (Teleostei: Sciaenidae) en las costas del Río de la Plata y del Océano Atlántico. Resumen: Las corvinas negras *Pogonias cromis* y *P. courbina* son las únicas dos especies válidas hasta el presente dentro del género. Son especies ampliamente explotadas por la pesca comercial y recreativa a lo largo de las costas del Océano Atlántico Occidental, y particularmente *P. courbina* ha sido calificada como vulnerable. En este trabajo utilizamos secuencias parciales de dos genes mitocondriales, citocromo oxidasa I y citocromo b, para estudiar la variación genética y la historia demográfica de *P. courbina* en el Río de la Plata y el Océano Atlántico Sudoccidental adyacente al mismo. Adicionalmente, utilizamos ambos marcadores para estimar por primera vez el tiempo de divergencia entre estas especies del género *Pogonias*. Los resultados obtenidos indican niveles de variación genética relativamente moderados a bajos. Nuestro análisis muestra que *P. cromis* y *P. courbina* divergieron hace 1,154,800 años (Pleistoceno), y encontramos evidencia de que *P. courbina* ha experimentado una reciente expansión poblacional durante esta época geológica en el Río de la Plata y aguas adyacentes del Océano Atlántico Sudoccidental. El presente trabajo sugiere una cierta vulnerabilidad a la sobreexplotación y, por lo tanto, deberían ser implementadas medidas de conservación para evitar una declinación poblacional de *P. courbina* en el Río de la Plata.

Palabras clave: Diversidad genética; Datación Molecular; Marcadores mitocondriales; Atlántico Occidental.

Introduction

Sciaenidae, that includes the fishes called croakers or drums, is one of the largest families within the Osteichthyes fish group, with approximately 291 species all around the world (Lo *et al*. 2015). The sciaenids are distributed from tropical to temperate areas, fundamentally on sea coasts, although some species inhabit in rivers and estuaries (Chao *et al*. 2015). Most marine species use estuarine environments such as breeding areas, or move along the shore and river margins seasonally for reproduction (Chao *et al*. 2015).

Lo *et al*. (2015) established a comprehensive molecular phylogeny of the family that allowed identify 15 main lineages within Sciaenidae and estimate time to the most recent common ancestor 27.3 million years ago (Mya). Furthermore, based on ancestral area reconstruction and the fossil record, the authors propose that the family originated in tropical America and subsequently diversified during the Oligocene to Early Miocene (Lo *et al*. 2015). The contribution of this work has been essential to the study of particular genera within the Sciaenidae (e.g., Marceniuk *et al*. 2019, Lim *et al*. 2021).

Species of black drum genus *Pogonias* are estuarine-dependent marine fishes and are the largest of the Sciaenidae family (Machado *et al*. 2020). Black drums are distributed along the Western Atlantic coasts, except in tropical areas (Azpelicueta *et al*. 2019). Thus, this tropical gap of about 8000 km defines an antitropical distribution between fishes from northern and southern Western Atlantic Ocean (Azpelicueta *et al*. 2019). In addition, tropical America was suggested as the center of origin of the genus or its ancestor during the mid-Tertiary, based on its distribution and the limited fossil record (Takeuchi & Huddleston 2008). In reference to *Pogonias* fossils, a significant discovery was carried out by Takeuchi & Huddleston (2008), because they described a species, *Pogonias stringeri*, inhabiting the eastern Pacific Ocean from late early Miocene. Its presence has been explained by an expansion of the distribution range of the genus *Pogonias*, currently restricted to Western Atlantic coast, and was related to the Panama seaway (Takeuchi & Huddleston 2008).

At the present time, only two valid species make up this genus: *Pogonias cromis* (Linnaeus 1776) and *Pogonias courbina* (Lacepède 1803) (Azpelicueta *et al*. 2019). Both species support extensive commercial and recreational fisheries (Olsen *et al*. 2018). The former is distributed from Massachusetts (United States) to the Gulf of Mexico (Macchi *et al*. 2002), and the latter is distributed from Rio de Janeiro (Brazil) to south of Buenos Aires Province (Argentina) (Azpelicueta *et al*. 2019). *Pogonias courbina* was recently reappraisal based on molecular and morphological evidence by Azpelicueta *et al*. (2019). For instance, the authors evidenced several features as the characteristic hyperostoses of the dorsal spines, thinner pterygiophores in the dorsal and anal-fin, differences in morphology of the gas bladder and in the duration of advertisement calls (see Azpelicueta *et al*. 2019 for more information) that indicated differentiation between northern and southern individuals. Furthermore, genetic distance between both species of 1% was calculated, based on mitochondrial cytochrome oxidase I (COI) gene, by Azpelicueta *et al*. (2019).

Specimens of *P. courbina* are long-lived and have slow growing, indicating a low natural mortality rate, a low relative fertility (Urteaga & Perrotta 2001). All of this implies a lower biomass replenishment capacity and making them particularly susceptible to fishing (Urteaga & Perrotta 2001). Previous studies of this taxon in Brazil qualify the regional subpopulation of this species as highly vulnerable to overfishing and degradation of coastal habitat (Chao *et al*. 2015). Currently, *P. courbina* is considered as endangered in Brazil (Haimovici *et al*. 2020), due to large catches in the 1970s that led to population declines (Dos Santos *et al.* 2019, Machado *et al*. 2020). Differing from *P. cromis* that is listed globally as Least Concern by IUCN, due to no present indication of significant population declines in U.S. waters, although there is some evidence of local declines off in Mexico (Chao *et al*. 2020).

At regional level, *P. courbina* has great importance in sport or recreational fishing (Nion *et al*. 2013). According to data presented for DINARA (*Dirección Nacional de Recursos Acuáticos*, Uruguay, 2014) in Uruguay, fishing landing of this taxon carried out by industrial and artisanal fishing is irregular, being approximately 2% of the total. In 2015, an increase of this fishing activity was observed, reaching a 3% of total (DINARA 2015). From 2016 to 2017, a slight decrease of landing was observed of the southern black drum, being around

2% of the total, whereas that in 2018, a substantial decrease was evidenced, being only 0.9% of the total fishing landing (DINARA 2019).

The knowledge of population genetic structure is particularly important for management and conservation of fishing resources. According to Hauser & Ward (1998), if different stocks are identified, each of these populations could react independently to exploitation, representing different management units. Differences in habitat, migration, geographic segregation or human activities are considered as contributing factors for the genetic population structuring (Gonzalez & Zardoya 2007, Gao *et al.* 2014, Eschbach *et al.* 2021). Because *P. courbina* is a shared fishing resource in the Southwestern (SW) Atlantic Ocean (Machado *et al*. 2020), it is very important to define whether it constitutes a single population or if this taxon is made up of different stocks in this region. A recent study analyzing sequences of the mitochondrial DNA control region (mtDNA CR) of *P. courbina*, from South of Brazil, Uruguayan coast and Samborombón Bay in Argentina, revealed a high genetic diversity within populations but an absence of inter-population differentiation (Machado *et al*. 2020). Also, they found a high number of migrants from Brazil to Uruguay and Argentina, defining these localities as a single connected population (Machado *et al*. 2020).

In the present paper, we used two mitochondrial markers, COI and cytochrome b (*Cyt b*) genes, to study genetic variation and to describe the spatial differentiation of *P. courbina* distributed in different sampling sites from Uruguayan coast and nearby areas. This is in order to perform a preliminary fine-scale analysis of the population genetic structure in Uruguay. Both markers COI and *Cyt b* have been used to resolve phylogenetic relationships (e.g., Marceniuk *et al*. 2019, Tominaga *et al*. 2020) as well as in studies of population genetics of several species of fish (e.g., García *et al*. 2014, Ríos *et al*. 2017). Also, we used these markers to estimate divergence time of the species in the genus *Pogonias*. We assessed the genetic diversity of this species in the Río de la Plata and Atlantic Ocean in order to provide useful information which would contribute to construct adequate conservation strategies for this endemic taxon in the SW Atlantic Ocean.

Materials and Methods

Sample collection and DNA extraction: A sampling of 38 specimens of the species *Pogonias courbina* belonging to three collecting sites in the coastal area of Río de la Plata and the Atlantic Ocean of Uruguay were included in present work (Fig. 1 and Table I). We chose three principal fishing points in Uruguay for sampling in order to perform a fine-scale investigation. These specimens were obtained from artisanal and industrial fisheries belonging to the sampled area in the Rio de la Plata and Atlantic coast. The samples of different tissues (muscle and/or fin) were preserved in ethanol 95° at the *Sección Genética Evolutiva* of *Facultad de Ciencias*, Montevideo, Uruguay or at the *Unidad de Gestión Pesquera Atlántica* - DINARA, Rocha, Uruguay. The total genomic DNA was extracted using proteinase K digestion, followed by sodium chloride extraction and ethanol precipitation (modified from Medrano *et al*. 1990).

PCR amplifications and sequencing of Cyt b and COI mitochondrial genes: A fragment of approximately 650 bp from the COI gene was amplified using the LCO1490 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HC02198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers (Folmer *et al*. 1994). For the amplification of approximately 800 bp from the *Cyt b* gene, we use the CB3-H (5'-GGCAAATAGGAARTATCATTC-3') and Gludg-L (5'-TGACTTGAARAACCAYCGTTG-3') primers (Palumbi *et al*. 1991). The total volume for PCR amplifications was 20 µL, containing 30 ng of DNA, 1× Taq buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U of Taq DNA polymerase (Invitrogen), and 0.4 µM of each primer. COI gene was amplified using the following cycling profile: an initial denaturation of five min at 94°C; followed by 35 cycles of 94°C for one min, 45°C for one min, and 72°C for one min; and a final extension of seven min at 72°C. To amplified *Cyt b* gene we used the following cycling conditions: an initial denaturation of five min at 94°C; followed by 35 cycles of 94°C for 30 sec, 46°C for 45 sec, and 72°C for one min; and a final extension of seven min at 72°C. PCR products were sequenced directly using the same amplification primers with an ABI Prism 377 Automated Sequencer (MACROGEN, Seoul, Korea).

Sequence analyses: The new DNA sequences were edited in FinchTV (Finch TV 1.4.0, Geospiza, http://www.geospiza.com/finchTV). Sequence alignments were performed using the CLUSTAL X (Thompson *et al*. 1997) under default parameters and implemented in MEGA version X (Kumar *et al*. 2018). Additionally, we included in the total COI

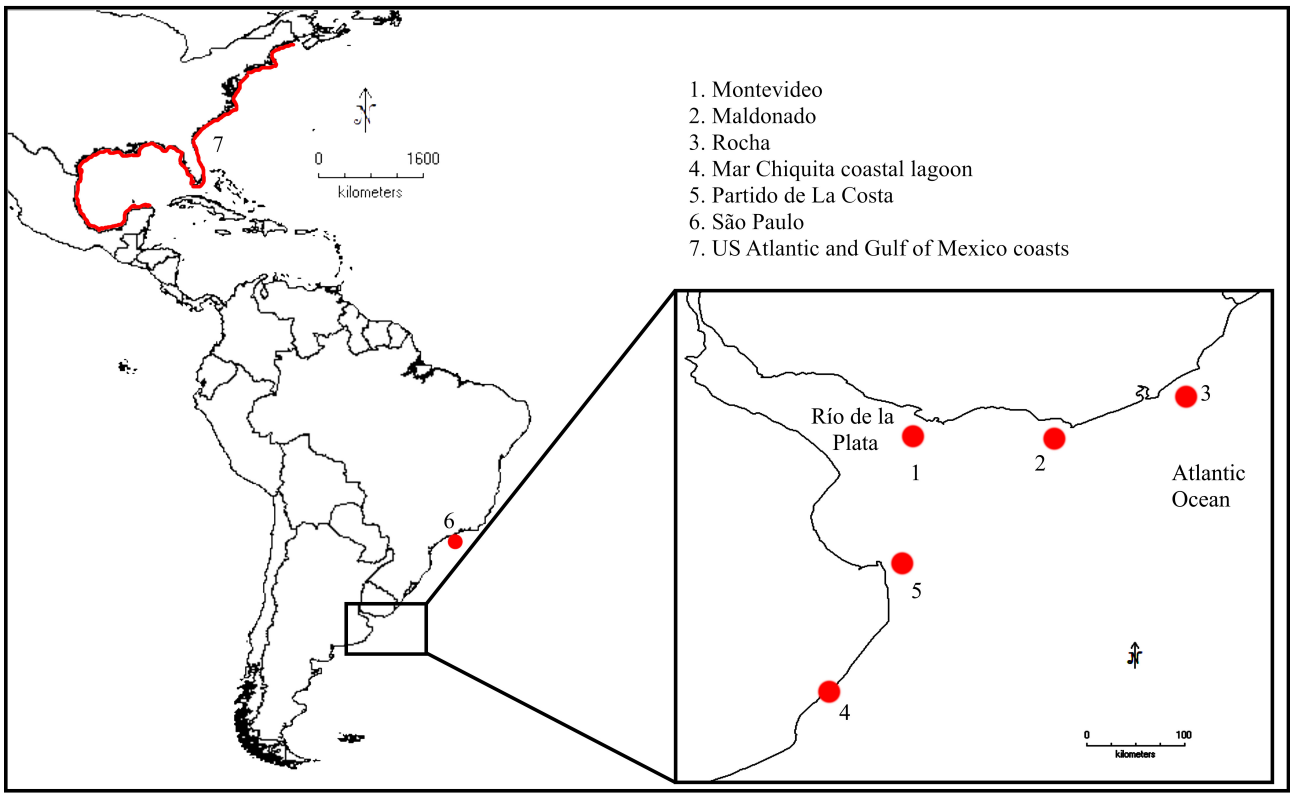

Figure 1. Collecting sites of all *Pogonias* samples analyzed: 1-6 - *Pogonias courbina*, 7 - *Pogonias cromis*. More details of the collecting site are provided in Table I.

Table I. Geographic distribution of *Pogonias* specimens studied: Species, Basin, Collecting site, Catalog sample number, COI haplotype, GenBank accession number for each marker and their corresponding references.

	Basin	Collecting site	Sample number	COI haplotype	Accession Number		References
					COI	*Cyt b*	
Pogonias courbuna	Río de la Plata - SW Atlantic Ocean	Unknown	P2326*	H_1	MZ065214		Present study
			P2327*	H_2	MZ065215		
	Río de la Plata	Montevideo (1) N = 9	P2333*	H_2	MZ065239	MZ065201	
			P2334*	H_2	MZ065240	MZ065202	
			P2350*	H_2	MZ065241		
			P2351*	H_2	MZ065242		
			P2353*	H_2	MZ065244		
			P2332*	H_10	MZ065238	MZ065200	
			P2299*	H_14	MZ065236	MZ065198	
			P2300*	H_15	MZ065237	MZ065199	
			P2352*	H_16	MZ065243		
		Maldonado (2) N = 10	P2345*	H_2	MZ065231	MZ065208	
			P2346*	H_2	MZ065232	MZ065209	
			P2347*	H_2	MZ065233	MZ065210	
			P2348*	H_2	MZ065234	MZ065211	
			P2349*	H_2	MZ065235		
			P2356*	H_2	MZ065245		
			P2357*	H_2	MZ065246		
			P2358*	H_15	MZ065247		
			P2359*	H_2	MZ065248		
			P2360*	H_17	MZ065249		
	SW Atlantic	Rocha	P2328*	H_2	MZ065216		

	Basin	Collecting site	Sample number	COI haplotype	Accession Number		References
					COI	***Cyt b***	
		(3) N = 17	P2329*	H_2	MZ065217		
			P2330*	H_1	MZ065218		
			P2331*	H_12	MZ065219		
			P2335**	H_2	MZ065220	MZ065203	
			P2336**	H_2	MZ065221	MZ065204	
			P2337**	H_2	MZ065222	MZ065205	
			P2338**	H_13	MZ065223	MZ065206	
			P2339**	H_2	MZ065224	MZ065207	
			P2340**	H_1	MZ065225		
			P2341**	H_2	MZ065212		
			P2342**	H_2	MZ065213		
			P2343**	H_2	MZ065226		
			P2344**	H_2	MZ065227		
			P2354*	H_2	MZ065229		
			P2355*	H_2	MZ065230		
			P2361*	H_2	MZ065228		
	Ocean	Mar Chiquita coastal lagoon (4) N = 11		H_2	EU074543		Mabragaña *et al.* 2011
				H_2	EU074544		
				H_2	EU074545		
				H_2	EU074546		
				H_2	EU074547		
				H_2	EU074548		
				H_2	EU074549		
				H_2	EU074550		
				H_2	FARG596-09[#]		
				H_2	FARG597-09[#]		
				H_2	FARG598-09[#]		
		El Partido de la Costa (5) N = 11		H_2	MK834237		Azpelicueta *et al.* 2019
				H_2	MK834238		
				H_2	MK834239		
				H_2	MK834240		
				H_2	MK834241		
				H_2	MK834242		
				H_2	MK834243		
				H_2	MK834244		
				H_11	MK834247		
				H_10	MK834245		
				H_9	MK834246		
		São Paulo (6) N = 2		H_2	MK834248		
				H_2	MK834249		
P. cromis	Northwestern Atlantic Ocean and Gulf of Mexico	US Atlantic and Gulf of Mexico coasts (7) N = 61		H_3	KP722765	KP722676	Lo *et al*. 2015
				H_3	KX164000		Unpublished
				H_3	EU752164		Yancy *et al.* 2008
				H_3	EU752165		
				H_3	EU752166		
				H_3	EU752167		
				H_3	JQ842654		Weigt *et al.*

Basin	Collecting site	Sample number	COI haplotype	Accession Number		References
				COI	***Cyt b***	
			H_3	JQ842655		2012
			H_3	JQ842656		
			H_3	MH378668		Unpublished
			H_3	MH378669		
			H_3	MH378670		
			H_3	MH379077		Stoeckle *et al*. 2018
			H_3	MT456022		
			H_3	MT456011		
			H_3	MT455865		Unpublished
			H_3	MT455861		
			H_3	MT455667		
			H_3	MW535373		
			H_3	MW535372		
			H_3	MW535374		
			H_3	MW535376		
			H_3	MW535375		
			H_3	MW535371		
			H_3	MW535370		
			H_5	MW535369		
			H_3	MW535368		
			H_3	MW535367		
			H_3	MW535366		
			H_3	MW535365		
			H_3	MW535364		
			H_3	MW535363		
			H_3	MW535362		
			H_3	MW535361		
			H_3	MW535360		
			H_3	MW535359		
			H_6	MW535358		
			H_3	MW535357		
			H_3	MW535356		Unpublished
			H_3	MW535355		
			H_3	MW535354		
			H_3	MW535353		
			H_3	MW535352		
			H_6	MW535351		
			H_3	MW535350		
			H_3	MW535349		
			H_7	MW535348		
			H_3	MW535347		
			H_6	MW535346		
			H_3	MW535345		
			H_3	MW535344		
			H_3	MW535343		
			H_3	MW535342		
			H_3	MW535341		
			H_6	MW535340		
			H_3	MW535339		
			H_8	MW535338		
			H_3	MW535337		
			H_3	MW535336		

Basin	Collecting site	Sample number	COI haplotype	Accession Number		References
				COI	***Cyt b***	
			H_5	MW535335		
			H_4	MT879845	MT879807	Van Nynatten *et al*. 2021

N = indicate the number of individuals for each collection site. * indicate that the sample is deposited in *Sección Genética Evolutiva – Facultad de Ciencias*; ** sample deposited in *Unidad de Gestión Pesquera Atlántica – DINARA.* Numbers with # indicate the sample ID of iBold. The underlined sequences were included in the concatenated phylogenetic dataset.

dataset sequences retrieved from GenBank as follows: 24 sequences of *P. courbina* (11 from the coastal lagoon of Mar Chiquita, 11 from El Partido de La Costa, both from Argentina; and 2 from São Paulo, Brazil); plus 61 sequences of *P. cromis* from the US Atlantic and Gulf of Mexico coasts (Fig. 1 and Table I). The *Cyt b* marker included only two additional available sequences belonging to *P. cromis* from the US Atlantic and Gulf of Mexico coasts (Table I). Moreover, we included sequences of *Pachyurus bonariensisi* (Steindachner 1879), *Pachypops fourcroi* (Lacepède 1802), *Sciaenops ocellatus* (Linnaeus 1766), *Umbrina canariensis* (Valenciennes 1843) and *U. cirrosa* (Linnaeus 1758) as outgroup taxa in the phylogenetic analyses and divergence time estimation. We selected these species due to they are closely related to genus *Pogonias* (Lo *et al*. 2015) and due to their presence in the fossil record, that has been used to calibrate the molecular phylogeny (see below). The sequences were retrieved from GenBank (Table II). We used a combined dataset of 20 sequences to infer phylogenetic relationships, and used the completed dataset of each gene (128 sequences for COI and 20 for *Cyt b*) to estimate the time of divergence (see Table I and II).

Statistical analyses of sequences from COI and Cyt b genes: DNA polymorphism was measured by estimating the number of variable and phylogenetically informative sites, the haplotype diversity (*h*) and nucleotide diversity (π) were assessed using DnaSP v. 6.00 (Rozas *et al*. 2017). For COI dataset, we calculated these measures at three levels: genus (*Pogonias*), species (*P. cromis* and *P. courbina*) and locality samples level (only for *P. courbina*). Due to the lower number of sequences obtained for *Cyt b* gene, we could calculate DNA polymorphism only at locality samples level for *P. courbina*. The genetic differentiation between localities was assessed by calculation the pairwise p-distance for COI and *Cyt b* sequences, implemented in MEGA version X (Kumar *et al*. 2018).

Phylogenetic analyses based on the combined gene dataset: Phylogenetic relationships were performed based on the combined DNA sequence matrix from the COI and *Cyt b* datasets, using three different methodologies. A non-model-based method, Maximum parsimony (MP) was performed in TNT software v 1.5 (Goloboff & Catalano 2016). A strict consensus tree was selected from equally parsimonious topologies. The statistical robustness in the nodes of the resulting trees was determined by 1000 bootstrap pseudoreplicates. Also, two model-based method were assayed: Maximum likelihood (ML) using RAxML 8.2.12 (Stamatakis 2014), implemented in CIPRES (Cyber Infrastructure for Phylogenetic Research) phylogenetic portal (Miller *et al*. 2010), and Bayesian inference (BI), using MrBayes 3.2.7 (Ronquist *et al*. 2012). ModelFinder (Kalyaanamoorthy *et al*. 2017), also implemented in CIPRES, was used to infer the best partition scheme and the nucleotide substitution model that best fit to the dataset used in ML and BI analysis. Partitions and models were defined according to codon position for COI and *Cyt b* datasets. The most suitable nucleotide models were HKY+I (Hasegawa *et al*. 1985) for COI and *Cyt b* at positions 1 and 2, and GTR+G (Tavaré 1986) for COI and *Cyt b* at position 3. The nucleotide substitution model GTR was employed for the analysis because RAxML only provides GTR-related models of rate heterogeneity.

Divergence time estimate for both mitochondrial markers: We conducted multispecies coalescent analysis using the *BEAST option in the computer package BEAST 2 (Bouckaert *et al*. 2014) based on both mitochondrial markers, COI and *Cyt b*, to co-estimate divergence time among *P. cromis* and *P. courbina* and the species-tree. We chose this approximation over the estimate using the concatenated gene-tree due to the *BEAST estimations allow relaxation of the effect of deep coalescences and better accounts for species trees versus gene trees. Selection of calibration points was based on the Sciaenidae phylogenetic tree of Lo

Table II. GenBank accession numbers of sequences belonging to species used as outgroup and their references.

Accession number		Species	Reference
COI	*Cyt b*		
KP722754	KP722665	*Pachyurus bonariensis*	Lo *et al*. 2015
KP722753	KP722664	*Pachypops fourcroi*	
KP722776	KP722687	*Sciaenops ocellatus*	
KP722784		*Umbrina canariensis*	
	EF392638	*Umbrina canariensis*	Unpublished
KC501832		*Umbrina cirrosa*	Keskin & Atar 2013
KP722785	KP722696	*Umbrina cirrosa*	Lo *et al*. 2015

The underlined sequences were included in the concatenated phylogenetic dataset.

et al. (2015). We used the fossil *Umbrina cirrosa* located in the Kienbergnineyard section (14.9-13.7 Mya) to constrain the tMRCA of the clade *Umbrina canariensis* - *U. cirrosa* (Brzobohatý *et al*. 2007), and the oldest fossil of *Pachypops fourcroi* found in the Pebas Formation (23-13.7 Mya) to constrain the tMRCA of the clade *Pachyurus bonariensis* - *Pachypops fourcroi* (Monsch 1998). Then, time of divergence was calculated, under a Yule speciation process, using a strict molecular clock. ModelFinder was also used to infer the best partition scheme and choose the molecular evolution models used in this analysis. For COI dataset, the most suitable nucleotide substitution models were K2P+I (Kimura 1980) at position 1, F81+I (Felsenstein 1981) at position 2 and TIM+G at position 3. The best substitution models for *Cyt b* dataset were K2P+I at positions 1 and 2, and TIM3+G at position 3. We carried out two runs of 400 million generations. Trees and parameters were sampled every 40,000 generations. The results were visualized in the software Tracer v1.7.1 (Rambaut *et al*. 2018) to verify that the stationary has been achieved and that convergence has been reached for all parameters. Both independent runs were combined with LogCombiner after burn-in (10%). Divergence time of each clade was estimated in Mya with a mean and a 95% highest posterior density (HPD). Posterior probabilities and maximum clade credibility trees were calculated using the program TreeAnnotator v2.6.0 from the BEAST package (Bouckaert *et al*. 2014).

Population genetics analyses and historical demography in P. courbina based on COI dataset: In these population and demographic analyses, we only included the COI sequences, because the number of locality samples and the *Cyt b* sequences obtained are considerably lower than for COI dataset.

Haplotype networks were constructed to evaluate the relationships and geographical distribution of haplotypes identified in *Pogonias*, using the Median joining (MJ) algorithm implemented in NETWORK v.5.0.0.0 (Bandelt *et al.* 1999).

Departure from neutrality was examined by means of Tajima's (1989) test, implemented in DnaSP v. 6.00 (Rozas *et al*. 2017) and Fu's (1997) test, implemented in ARLEQUIN v.3.5 (Excoffier & Lischer 2010). Pairwise comparisons of genetic differentiation (F_{ST}) between locations were analyzed employing also the program ARLEQUIN v.3.5. The genetic structure of *P. courbina* samples from the coastal area of Río de la Plata and the SW Atlantic Ocean was investigated using the analysis of molecular variance (AMOVA), which was carried out using ARLEQUIN v.3.5. Different possible groupings of the localities were tested (for more details of the hypotheses tested see Table S1 in the Supplementary material). The significance of variance components was calculated by 1000 pseudoreplicates.

To reconstruct changes in population size through time of species *P. courbina*, extended Bayesian skyline plot analysis was done in BEAST 2 (Bouckaert *et al*. 2014), using an estimated mutation rate for COI of marine fish species (1.2% Myr^{-1}, Henriques *et al*. 2016). Data visualization was implemented in R using ggplot2 library.

Results

Statistical analyses of sequences from COI and Cyt b genes: The present study included 38 new sequences of *P. courbina* corresponding to a fragment of 582 bp for COI and 14 new partial sequences of 799 bp for *Cyt b* (Table I). The total dataset included 128 sequences for COI marker and 20 sequences for *Cyt b* gene. Table III presents estimates of COI polymorphisms per taxa and per sampling geographic localities and estimates of *Cyt b* polymorphisms per sampling geographic localities.

Relatively moderate haplotype diversity and low nucleotide diversity were obtained for the COI marker at genus level (Table III). *Pogonias cromis* presents low values for both diversities; meanwhile *P. courbina* shows relatively moderate haplotype diversity and low nucleotide diversity (Table III). For all Uruguayan localities (Montevideo, Maldonado and Rocha) haplotype diversity is moderate to low, whereas nucleotide diversity is low (Table III). Montevideo presents the highest haplotype diversity for COI marker (0.722). Mar Chiquita coastal lagoon and São Paulo presented null values; meanwhile Partido de la Costa presents moderate haplotype diversity and low nucleotide diversity (Table III). Regarding the estimate of DNA polymorphism in *Cyt b* gene of *P. courbina* (Table III), Uruguayan samples present high to moderate haplotype diversity and a low value for nucleotide diversity.

With respect to pairwise p-distances (Table IV), as it was expected, the higher difference was observed among *P. cromis* and *P. courbina* (ranging 0.0187- 0.0195 for *Cyt b* gene and 0.0107- 0.0115 for COI gene). The differences between southern localities were low (from 0.0000 to 0.0012) for COI, being Partido de la Costa from Argentina and Uruguayan localities that presented the highest values. Similar p-distance values were obtained for *Cyt b* as for COI.

Phylogenetic analyses and divergence time estimate for both mitochondrial markers: Species-tree in Figure 2 shows that the divergence time between *P. cromis* and *P. courbina* occurred in the Pleistocene, approximately 1.1548 Mya (95% HPD = 0.4613–1.8520 Mya).

The MP, ML and BI analyses generated trees with similar topologies (Fig. 3), however, they are different from the topology of the time tree (Fig. 2). Although our combined molecular dataset does not identify the sister group of *Pogonias* with statistical support of MP and BI, all phylogenetic reconstructions support the monophyly of genus *Pogonias* and also supported the monophyly of *P. cromis* and *P. courbina*.

Table III. Estimate of DNA polymorphism in COI and *Cyt b* genes of *Pogonias* species.

	Taxa	Localities	N	S	SP	NH	H	π
COI	*P. cromis*	US Atlantic and Gulf of Mexico coasts	61	3	2	6	0.272 (0.074)	0.0005 (0.0001)
	P. courbina	1. Montevideo	9	4	0	5	0.722 (0.159)	0.0015 (0.0005)
		2. Maldonado	10	2	0	3	0.378 (0.181)	0.0007 (0.0004)
		3. Rocha	17	3	1	4	0.419 (0.141)	0.0008 (0.0003)
		4. Mar Chiquita coastal lagoon	11	0	0	1	0.000 (0.000)	0.0000 (0.0000)
		5. Partido de la Costa	11	3	0	4	0.491 (0.175)	0.0009 (0.0004)
		6. São Paulo	2	0	0	1	0.000 (0.000)	0.0000 (0.0000)
		Total	62	10	3	11	0.401 (0.079)	0.0008 (0.0002)
	Pogonias	Total	123	21	11	17	0.671 (0.028)	0.0058 (0.0001)
Cyt b	*P. courbina*	1. Montevideo	5	1	0	2	0.400 (0.237)	0.0005 (0.0003)
		2. Maldonado	4	5	0	4	1.000 (0.177)	0.0031 (0.0008)
		3. Rocha	5	1	0	2	0.400 (0.237)	0.0005 (0.0003)

N = Number of sequences; S = Number of polymorphic segregating sites; SP = Parsimony informative sites; NH = Number of haplotypes; H = Haplotype diversity (Nei 1987); π = Nucleotide diversity (Nei 1987); Standard deviation in brackets (SD).

Table IV. Pairwise p-distances and standard deviation based on COI (lower left corner) and *Cyt b* sequences (upper right corner) of all locality's samples belonging to *Pogonias courbina* (1-6 localities) and to *Pogonias cromis* (locality 7).

	1	**2**	**3**	**4**	**5**	**6**	**7**
1. Montevideo		0.0017 (0.0008)	0.0004 (0.0004)	---	---	---	0.0187 (0.0060)
2. Maldonado	0.0011 (0.0005)		0.0017 (0.0008)	---	---	---	0.0195 (0.0060)
3. Rocha	0.0012 (0.0004)	0.0008 (0.0002)		---	---	---	0.0187 (0.0060)
4. Mar Chiquita coastal lagoon	0.0008 (0.0004)	0.0003 (0.0002)	0.0004 (0.0002)		---	---	---
5. Partido de la Costa	0.0012 (0.0005)	0.0008 (0.0004)	0.0009 (0.000)	0.0005 (0.0003)		---	---
6. São Paulo	0.0007 (0.0004)	0.0003 (0.0004)	0.0004 (0.0003)	0.0000 (0.0000)	0.0005 (0.0003)		---
7. US Atlantic and Gulf of Mexico coasts	0.0115 (0.0043)	0.0111 (0.0043)	0.0111 (0.0042)	0.0107 (0.0042)	0.0112 (0.0043)	0.0107 (0.0043)	

Standard deviation in brackets (SD).

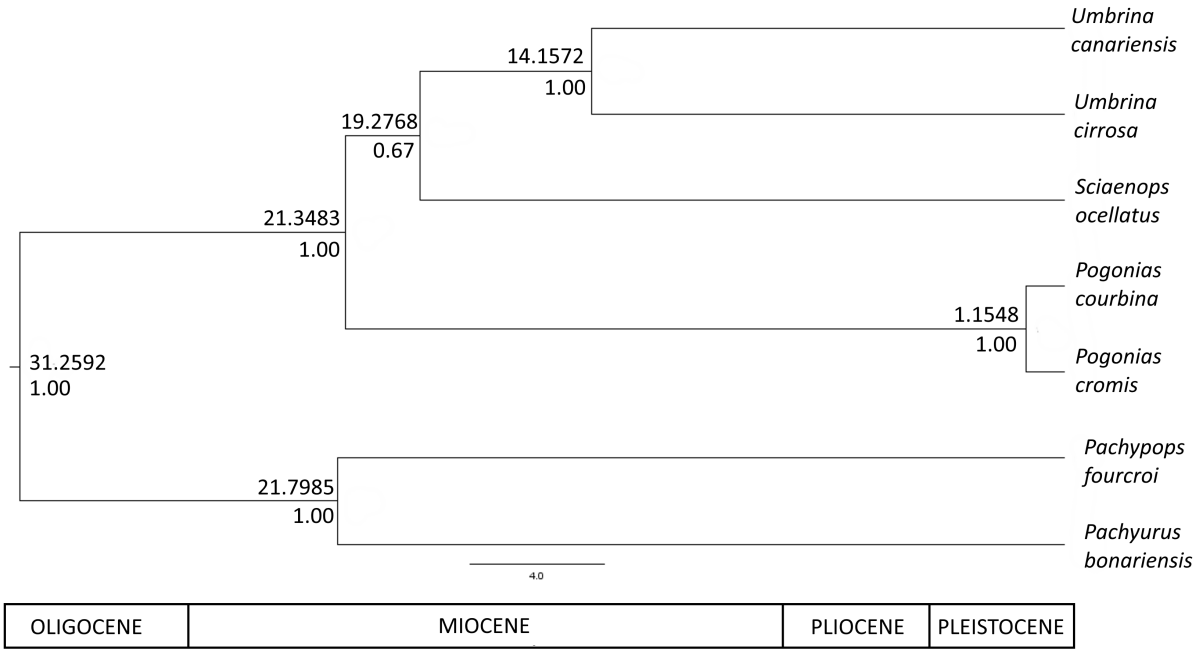

Figure 2. Chronogram obtained under species-tree approach with estimates of divergence time (Million years ago) of genus *Pogonias*. The value on the node represents the estimated mean divergence date inferred using Bayesian coalescent analyses, based on both mitochondrial markers, COI and *Cyt b*, and implemented in BEAST. Bayesian posterior probability of occurrence for clade is given under the branch. The bottom bar summarizes the geologic epochs.

Population genetics analyses and historical demography in P. courbina: The haplotype network based on COI gene is shown in Figure 4. The network exhibited a star-like pattern both for *P. courbina* and for *P. cromis*. The most common haplotype in *P. courbina* is H_2 and is shared between all the southern localities. The remaining haplotypes of *P. courbina* are separated by a single step mutation from H_2. The haplotypes H_10 and H_15 are shared only by samples of two localities, whereas the remaining eight haplotypes were unique per locality. The haplotype H_3 is the most frequent in *P. cromis* and it is separated by six mutation steps from the H_2 of *P. courbina*. The other haplotypes of *P. cromis* (H_4, H_5, H_6 H_7 and H_8) are separated from H_3 by only one mutation step.

Tajima's test was negative and statistically significant (D = -2.18, p < 0.01). Fu's test (Fs) also showed a negative significant value (Fs = -12.28, p = 0.00). Both results suggest a departure from neutrality in *P. courbina.*

Table V shows the pairwise F_{ST} values of COI gene for the seven localities studied with this marker. Low population genetic structure was detected among southern localities. Only Montevideo and Mar Chiquita coastal lagoon present a significant genetic differentiation. As expected, the most divergent values were observed between *P. cromis* and the samples of *P. courbina* (from 0.944 to 0.961). These values are suggesting a very restricted gene flow between these congeneric species.

In the AMOVA, the most variation was detected within populations, and the percentages of variation among groups were low for all hypotheses tested (Table S1 in Supplementary material). Nevertheless, a single hypothesis suggests a weak but significant divergence among the four group tested (9.27% of the total variation among groups), consisting of I- Montevideo; II- Maldonado, Mar Chiquita coastal lagoon; II- Rocha, São Paulo; and IV- Partido de la Costa (hypothesis viii in Table S1). The demographic history of *P. courbina* estimated using the extended Bayesian skyline plot based on COI dataset is presented in Figure 5. The results suggest that the population expanded around 35,000 years before present.

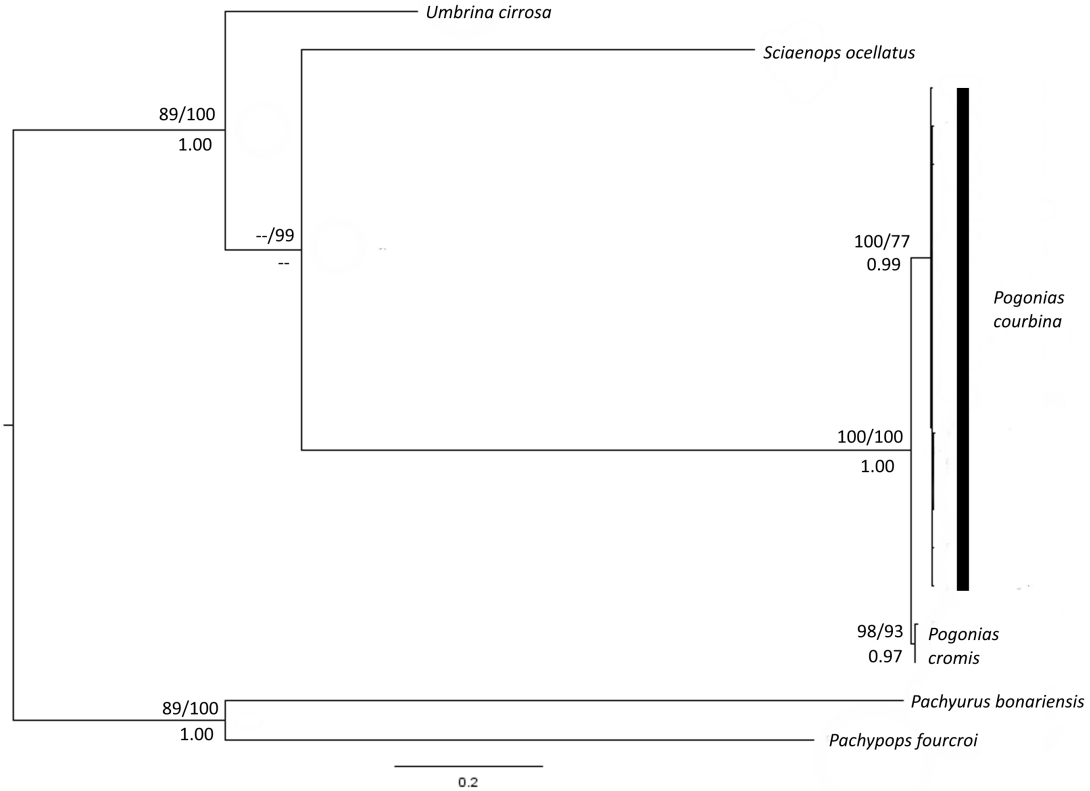

Figure 3. Phylogenetic analyses of the genus *Pogonias* based on the combined dataset. Tree topology obtained using maximum likelihood method based on new 14 sequences of *Pogonias courbina* and two of *Pogonias cromis.* Four sequences corresponding to the outgroup taxa were included. On the right side are indicated the sequences corresponding to each species of *Pogonias* and the outgroup species. Numbers below branches refer to the Bayesian posterior probability of occurrence for clades obtained by MrBayes, while bootstrap support values from Maximum Parsimony/Maximum Likelihood analysis are above branches.

Discussion

The present work contributes to knowledge about the recently redescribed species *P. courbina*, adding more information that can contribute to its conservation in SW Atlantic Ocean coasts. This work is the first to investigate the time of divergence and split of the two species belonging to the genus *Pogonias*, as well as, to employ both sequences of the COI and *Cyt b* genes to investigate the genetic diversity. An examination of population genetic structure of *P. courbina* using COI gene was implemented for evaluating separately several localities from the region. We extended the sampling of *P. courbina* in order to explore in more detail the existence of stocks on the Uruguayan coast.

Phylogenetic analyses and divergence time estimate between Pogonias species based on the mitochondrial COI and Cyt b genes: Phylogenetic analyses based on concatenated data supported the monophyly of the genus *Pogonias*, and the reciprocal monophyly of the two clades: *Pogonias cromis* and *P. courbina*. This is in agreement with the previous findings in Azpelicueta *et al*. (2019) using morphology features and sequences of COI gene. Except for ML, phylogenetic reconstruction implemented did not resolve the sister group of *Pogonias*, due to weak statistical support. In addition, these analyses did not result in the same sister group of the *Pogonias* clade that the chronogram and the phylogenetic position of *Sciaenops ocellatus* did not show a strong statistical support. Further molecular study including nuclear genes would be required to reveal the sister taxa of the *Pogonias* clade.

On the other hand, based on COI and *Cyt b* markers, we found a significant genetic differentiation between *P. courbina* and *P. cromis*. For COI gene, we obtained a genetic distance of approximately 1% between *P. cromis* and *P. courbina*, corroborating the previous results Azpelicueta *et al*. (2019). Genetic distance between *Cyt b* sequences was approximately 2%, which was

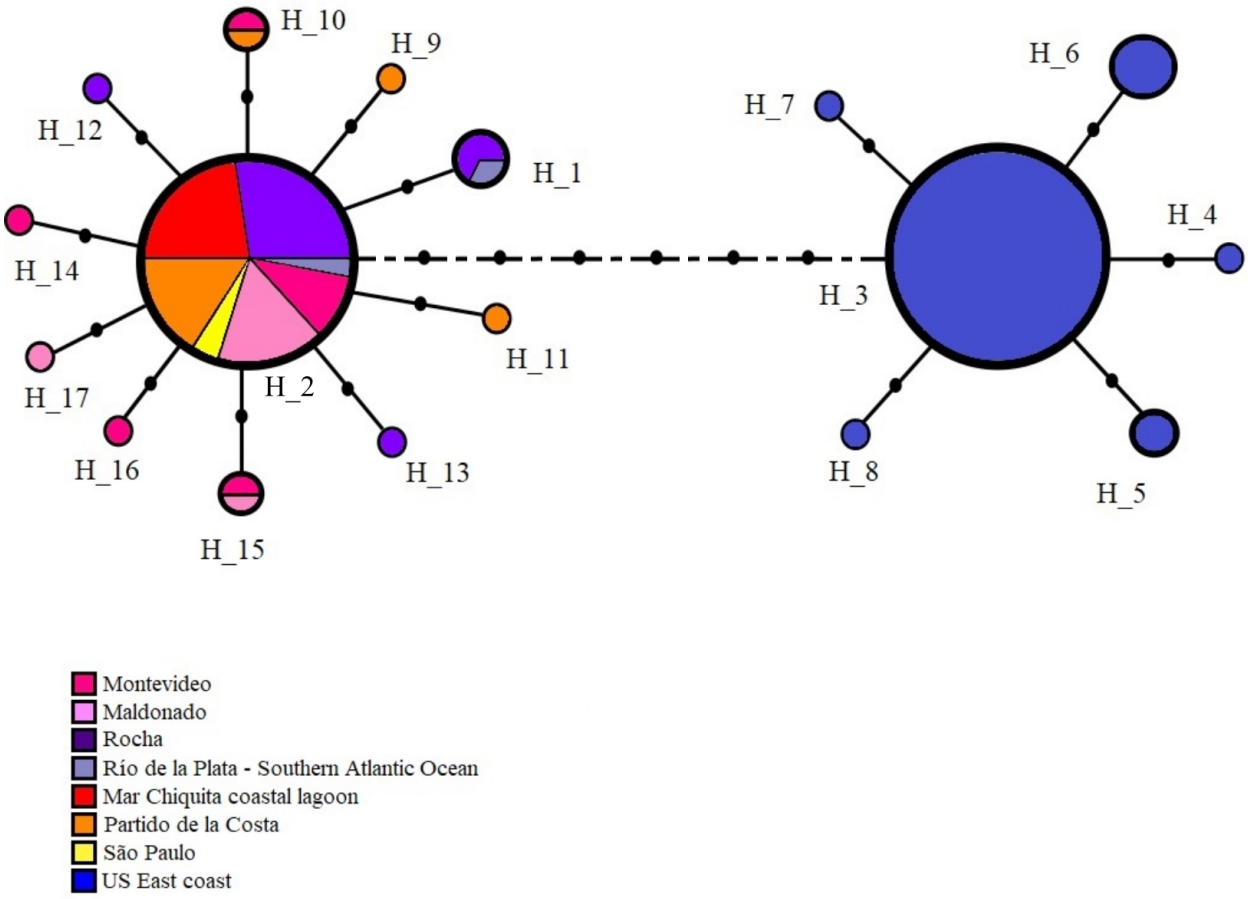

Figure 4. Haplotype network of *Pogonias cromis* and *Pogonias courbina* based on COI gene. Each circle represents a distinct haplotype and circle size is proportional to haplotype frequency. Different colours indicate the sample localities. Dotted line separates the haplotypes of *P. cromis* (right) and *P. courbina* (left). Black circles indicate mutation steps between haplotypes.

Table V. Pairwise F_{ST} values based on COI dataset of *Pogonias courbina* populations per locality (1-6) and *Pogonias cromis* (locality 7).

	1	**2**	**3**	**4**	**5**	**6**	**7**
1. Montevideo	---						
2. Maldonado	-0.032	---					
3. Rocha	0.031	0.014	---				
4. Mar Chiquita coastal lagoon	**0.023**	0.010	-0.001	---			
5. Partido de la Costa	-0.024	-0.001	0.018	0.000	---		
6. São Paulo	-0.321	-0.323	-0.300	0.000	-0.325	---	
7. US Atlantic and Gulf of Mexico coasts	**0.944**	**0.952**	**0.949**	**0.961**	**0.949**	**0.955**	---

F_{ST} significant values are in bold (P = 0.05)

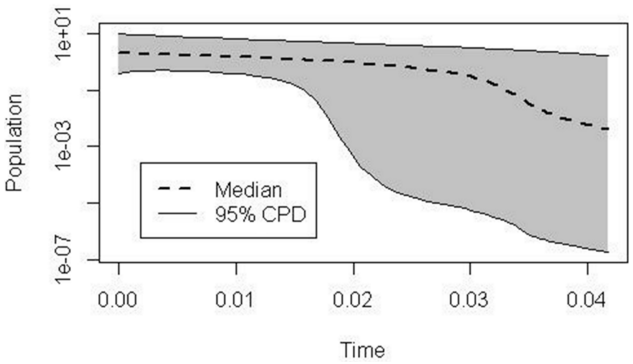

Figure 5. Demographic history of *Pogonias courbina* estimated using the extended Bayesian skyline plot from COI sequences. Times are in million years ago and the y axis to be displayed using a log scale. CPD = Central Posterior Density.

greater than that of COI. Nevertheless, the present genetic distance values are lower than those found between closely related species of sciaenids from the western Atlantic, for instance, species of genus *Macrodon, M. ancylodon* and *M. atricauda* (3.3 - 3.5% for COI and 3.4 - 3.5% for *Cyt b*; Aderne *et al*. 2022) or species of genus *Bairdiella, B. goeldi, B. ronchus* and *B. veraecrucis* (between 1.8 - 3.9% for COI, Marceniuk *et al*. 2019). On the other hand, low interspecies distance using traditional barcoding markers (COI and *Cyt b*) has also been detected in other fish species, as a consequence of recent divergence times or migration (Melo *et al*. 2018, Chagas *et al*. 2020). The fact that we found no evidence of indirect estimates of gene flow between both species of *Pogonias*, it could suggest that the low distances in these markers would be due to a slow evolutionary rate, as has been proposed for two species of stingrays, *Dasyatis brevicaudata* and *D. matsubarai* (Le Port *et al*. 2013). Regarding the divergence time of *P. cromis* and *P. courbina*, it was estimated at 1,154,800 years ago, in the early Pleistocene, similar to the separation of *M. ancylodon* and *M. atricauda* or the species of genus *Bairdiella* mentioned above. The higher genetic distances between these sciaenids could be related to their adaptation to distinct type of environment as they are the different patterns of water temperature between tropical and subtropical clades, differing from the temperate areas where the both species of genus *Pogonias* inhabit. Previous works have pointed out that the water temperature is one of the main factors that enhance the diversification along the evolutionary history of the organisms (Schroth *et al*. 2002, Santos *et al*. 2003). Moreover, metabolic rate decrease with body size and increase with temperature, which might increase mutation rate and induce rapid genetic divergence (April *et al*. 2013). This could be a hypothesis to explain the lower genetic distance between the species of the genus *Pogonias* with respect to the other sciaenids mentioned above, given their larger size and temperate habitats. Testing this hypothesis is a matter of further research.

With respect to the divergence date estimation, it is consistent with divergence time estimated for many other anti-tropical fish taxa (e.g. *Trachurus symmetricus, Sardinops, Scomber japonicus, Scomber australasicus, Sebastomus* and West Pacific *Engraulis*), for which the distribution in the Northern and Southern Hemispheres had been attributed as a result of equatorial crossing during Pleistocene climate changes (Burridge 2002). The mechanism associated with this distribution pattern in the Pleistocene epoch is called glacial dispersal (Ludt 2021). In this epoch occurred repeated glacial cycles, which altered the temperature and sea level (Ludt 2021). The glacial dispersal hypothesis considers that there are organisms which are unable to inhabit tropical water at the present time. However, during glacial maxima, the decrease in sea surface temperature could have allowed the dispersal from one hemisphere to the other of currently antitropical species (Ludt 2021). Ludt & Myers (2021) using ecological niche models could

demonstrate that several anti-tropical species do not have a suitable abiotic habitat in the tropics, however, during the Last Glacial Maximum they found dispersal corridors between hemispheres. For instance, Tea *et al.* (2019) proposed that the species *Microcanthus strigatus* in the Northern Hemisphere has had originated as a result of equatorial crossing during Pleistocene climate changes. This was supported by the reconstructed ancestral ranges of the taxon and divergence-time estimates. Therefore, to determine the probable origin of both species *P. cromis* and *P. courbina* and their trans-equatorial dispersal it would be necessary to reconstruct ancestral ranges and combine ecological, physiological, molecular, and morphological data as proposed by several authors (Ludt *et al*. 2015, Tea *et al.* 2019).

Genetic variation and population genetics analyses in P. courbina: Nucleotide diversity for COI was very low for all samples, ranged from 0.0000 to 0.0015. Except for Montevideo, the results were lower than those found in overfished sciaenids, π ranged from 0.0013 to 0.0043, such as *Nibea albiflora* (Xu *et al*. 2012) and *Miichthys miiuy* (Xu *et al*. 2014). In present study the nucleotide diversity values based on *Cyt b* marker ranged from 0.0005 to 0.0031 and were similar to those found in other economically important marine fish of the SW Atlantic Ocean, between 0.0010 to 0.0050, as *Macrodon atricauda* (Santos *et al*. 2006), *Ocyurus chrysurus* (Da Silva *et al*. 2015) and *Atherinella brasiliensis* (Baggio *et al*. 2017).

The haplotype diversity for COI in present paper (values of H between 0.000 and 0.722) was lower than those obtained for sciaenids mentioned above (ranging from 0.580 to 0.867, Xu *et al*. 2012, Xu *et al*. 2014). The highest haplotype diversity was observed in Montevideo site (H = 0.722), whereas São Paulo and Mar Chiquita coastal lagoon presented the smallest ones (H = 0.000). In São Paulo site, low values of genetic diversity could be because only two sequences were analyzed. However, the values obtained for Mar Chiquita coastal lagoon are particularly worrying, given higher values of genetic diversity for other localities with the same sample number. In addition to extremely low genetic variation found, a recent study suggests that boat noise could affect the vocalization rate of *P. courbina* in Mar Chiquita coastal lagoon, and, thus, affect the reproductive success of this species (Ceraulo *et al.* 2021). Further studies should incorporate highly variable nuclear markers to estimate a critical parameter in conservation as it is the effective population size and determinate if there is loss of genetic diversity in this taxon from this Biosphere reserve.

For *Cyt b* marker, haplotype diversity in the samples analyzed of *P. courbina* varied from 0.400 to 1.000, obtaining a mean of 0.600, that was similar to the value obtained for *O. chrysurus* (H = 0.653, Da Silva *et al*. 2015), but lower than *M. atricauda* (H = 0.831, Santos *et al*. 2006).

Lower nucleotide and haplotype diversities for COI gene can be related to slow evolutionary rate of the *Pogonias* species, as it was discussed above. However, for *Cyt b* gene, we obtained genetic diversity values quite similar to other economically important marine fish of the SW Atlantic Ocean.

On the whole, relatively moderate haplotypic diversity and low nucleotide diversity based on COI gene (H = 0.401, π = 0.001) were detected in *P. courbina*. As expected, our results were lower than those reported by Machado *et al*. (2020) based on the highly variable mtDNA CR in this species (H = 0.959; π = 0.013). However, these assays present a similar pattern of genetic diversity, beyond the difference in the absolute magnitudes. In accordance with Grant & Bowen (1998), this pattern of moderate haplotypic diversity and low nucleotide diversity would indicate, as observed in *P. courbina*, that the population has experienced a rapid expansion after a period of low effective population size. Present results of both neutrality tests showing a significantly negative value also supports this possible demographic scenario in *P. courbina*.

The most frequent COI haplotype had a very extensive distribution, including all the southern localities. Only localities of Uruguay and Partido de la Costa presented unique haplotypes. Remarkably, no haplotypes were shared between *P. cromis* and *P. courbina* reinforcing the separate status of the North and South Atlantic black drum species. The haplotype network presented a star-like topology which, following Slatkin & Hudson (1991), it could be suggesting a past recent population expansion hypothesis after a population reduction in *P. courbina*.

Pairwise F_{ST} values between *P. cromis* and *P. courbina* were the highest, which is in accord with the absence of shared haplotypes and supporting the existence of reproductive isolation. These data are consistent with morphological and genetic differences found by Azpelicueta *et al*. (2019) between both species. Besides, other differences had already previously been found in reproductive behaviour between them (Tellechea *et al*. 2011).

Regarding the demographic history estimate in *P. courbina*, the population expanded around 35,000 years ago during the Pleistocene. This result is similar to that obtained for localities of the Río de la Plata studied by Machado *et al*. (2020), where they found that *P. courbina* in Samborombón Bay and in the Uruguayan coast suffered expansion around 36 and 27 thousand years ago respectively. During this epoch, occurred several climatic and sea level fluctuations that could have enabled the distribution and expansion of marine fishes (Santos *et al*. 2006, Iriarte *et al*. 2011, Rodrigues *et al*. 2014). Several studies have associated such fluctuations along the western Atlantic Ocean with population expansion events on marine fish (e.g., Beheregaray *et al*. 2002, Pereira *et al*. 2009, Da Silva *et al*. 2015, Domingues *et al.* 2018).

At the intraspecies level of *P. courbina*, our AMOVA results based on the COI gene showed a small amount of variation among groups, suggesting a weak population structure of *P. courbina* on the Uruguayan coast and nearby areas. AMOVA only revealed a significant divergence among four groups: I-Montevideo; II- Maldonado, Mar Chiquita coastal lagoon; III- Rocha, São Paulo; and IV- Partido de la Costa. Environmental differences, as salinity, could explain these differences between localities studied. Montevideo corresponds to a central area of Río de la Plata estuary, whereas Maldonado and Mar Chiquita coastal lagoon, given their locations, present more variability as ocean-influenced habitats. On the other hand, Partido de la Costa, Rocha and São Paulo correspond to higher salinity waters from Atlantic Ocean. In fact, Ajemian *et al*. (2018) have shown in *P. cromis* that salinity can strongly influence the movement and distribution of this species. As well as different life history traits exhibited among *P. cromis* populations have suggested an adaptation to the high salinity of the environment as occur in the Upper Laguna Madre, a hypersaline estuary in Texas, USA (Bumguardner *et al*. 1996, Olsen *et al*. 2018). Further studies ought to combine information of life history traits of *P. courbina* in all localities studied which can help to confirm results obtained based on mitochondrial DNA (Williford *et al*. 2021).

On the other hand, the pairwise F_{ST} values obtained were low, suggesting the existence of gene flow between the localities and a genetic homogeneity over the geographic range studied. This result is in agreement with Machado *et al*. (2020) using mtDNA CR, which observed a similar gene pool and high number of migrants between locations studied in the SW Atlantic Ocean in this taxon. These results were consistent with what is expected for a species like *P. courbina* that inhabits estuarine and open marine areas without apparent barriers to migration and with high dispersal capacity (Machado-Schiaffino *et al.* 2009, Baetscher *et al*. 2019).

Nevertheless, our data are preliminary and it is necessary an exhaustive sampling of *P. courbina*, especially along the coast of Brazil and Argentina, in order to include the whole distribution range of the species. Along with this, a larger samples size is also required to reveal if there is population structure in SW Atlantic Ocean. Further approach using multiple nuclear genetic markers could increase the resolving power as suggested by many authors (Labastida-Estrada *et al.* 2019, Li *et al*. 2020, among others).

Conclusions and conservation remarks

Present work reports the time of divergence and split of the two species belonging to the genus *Pogonias* since the Pleistocene, approximately 1,154,800 years ago. The study of two genetic markers encoded by mtDNA revealed a differentiation between northern and southern congeneric species similar to that reported by Azpelicueta *et al*. (2019). In this regard, our study supports the important role of mitochondrial markers to identify fish species. We conclude that *P. courbina* on the Uruguayan coast presents genetic diversity values close to other economically important marine fish of the SW Atlantic Ocean. Taking into account all results obtained (the values of genetic diversities, the significantly negative values of Tajima's test and the haplotype networks characterized by star-like phylogeny), there is strong evidence to suggest a past recent population expansion of *P. courbina* during the Pleistocene in the Río de la Plata. Moreover, our preliminary analysis suggests that there is weak evidence of genetic structure in *P. courbina* from Río de la Plata and the adjacent SW Atlantic Ocean. However, further studies are required to confirm our result. Though the aforementioned demographic scenarios of past recent demographic expansion, some localities have shown low levels of genetic variation, such as Mar Chiquita coastal lagoon, Maldonado, São Paulo. Therefore present work suggests a degree of vulnerability to overexploitation in this endemic SW Atlantic Ocean fish given its longevity and low reproductive potential. Furthermore, based on the situation of southern Brazil where there is a large decline due to many years of intense fishing pressure

(Haimovici *et al*. 2020), conservation measures should be implemented to avoid population decline of *P. courbina* in the Río de la Plata.

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Ethical statement

Fish samples were obtained from commercial fisheries and from the repository of DINARA Fish Collection in La Paloma, Rocha, Uruguay. This investigation did not require approval by an ethical Committee.

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Genetic diversity of the Southern Black Drum *Pogonias courbina* (Teleostei: Sciaenidae) from Río de la Plata and Atlantic Ocean coasts

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Supplementary material

Table S1. Analysis of molecular variance (AMOVA) for *Pogonias courbina* based on COI gene. The tested hypotheses are shown: **i)** localities clustered at four groups: 1- Montevideo, Maldonado, Rocha; 2- Mar Chiquita coastal lagoon; 3 - La Costa Partido and 4 - São Paulo. **ii)** subdivision into three groups: 1- Montevideo, Mar Chiquita coastal lagoon, La Costa Partido; 2- Maldonado, Rocha; 3- São Paulo. **iii)** localities clustered at three groups: 1- Maldonado, Mar Chiquita coastal lagoon, La Costa Partido; 2- Montevideo; 3- Rocha; São Paulo. **iv)** localities grouped in two groups: 1- Montevideo, Mar Chiquita coastal lagoon, La Costa Partido; 2- Maldonado, Rocha, São Paulo. **v)** other three groups were tested: 1-Montevideo, Mar Chiquita coastal lagoon; 2- Maldonado, Rocha, São Paulo; 3- La Costa Partido. **vi)** this hypothesis also clustered the localities at three groups: 1- Montevideo, Maldonado, Rocha, São Paulo; 2- Mar Chiquita coastal lagoon; 3- La Costa Partido. **vii)** the following grouping was also tested: 1- Montevideo, Rocha, São Paulo; 2- Maldonado, Mar Chiquita coastal lagoon; 3- La Costa Partido. **viii)** other four groups were tested: 1- Montevideo; 2- Maldonado, Mar Chiquita coastal lagoon; 3- Rocha, São Paulo; 4- Partido de la Costa. **ix)** Partido de la Costa vs. Montevideo, Maldonado, Mar Chiquita coastal lagoon, Rocha, São Paulo. **x)** 1- Montevideo; 2- Maldonado 3- Mar Chiquita coastal lagoon; 4- Rocha, São Paulo; 5- Partido de la Costa.

Hypothesis	Source of variation	d.f.	Suma of squares	Variance components	Porcentage of variation	Statistics F
i)	Among groups	3	0.345	-0.01450 Va	-6.89	FCT = -0.0689
	Among populations within groups	2	0.608	0.00759 Vb	3.61	FSC = 0.0337
	Within populations	54	11.730	0.21722 Vc	103.29	FST = -0.0329
ii)	Among groups	2	0.270	-0.00563 Va	-2.65	FCT = -0.0265
	Among populations within groups	3	0.683	0.00096 Vb	0.45	FSC = 0.00440

	Within populations	54	11.730	0.217 Vc	102.20	FST = -0.0220
iii)	Among groups	2	0.489	-0.00663 Va	3.07	FCT = 0.0307
	Among populations within groups	3	0.464	-0.00777 Vb	-3.59	FSC = -0.0371
	Within populations	54	11.730	0.217 Vc	100.53	FST = -0.00526
iv)	Among groups	1	0.250	0.0292 Va	1.35	FCT = 0.0135
	Among populations within groups	4	0.704	-0.00458 Vb	-2.12	FSC = -0.0215
	Within populations	54	11.730	0.217 Vc	100.77	FST = -0.00771
v)	Among groups	2	0.432	0.00306 Va	1.42	FCT = 0.0142
	Among populations within groups	3	0.522	-0.00513 Vb	-2.39	FSC = -0.0242
	Within populations	54	11.730	0.217 Vc	100.96	FST = -0.00964
vi)	Among groups	2	0.324	-0.00282 Va	-1.32	FCT = -0.0132
	Among populations within groups	3	0.629	-0.00089 Vb	-0.42	FSC = -0.00411
	Within populations	54	11.730	0.217 Vc	101.74	FST = -0.0174
vii)	Among groups	2	0.408	0.00190 Va	0.88	FCT = 0.00885
	Among populations within groups	3	0.545	0.00425 Vb	-1.98	FSC = -0.0199
	Within populations	54	11.730	0.217 Vc	101.09	FST = -0.0109
viii)	Among groups	3	0.812	-0.02006 Va*	9.27	FCT = 0.0926*
	Among populations within groups	2	0.142	-0.0208 Vb	-9.61	FSC = -0.106
	Within populations	54	11.730	0.217 Vc	100.35	FST = -0.00350
ix)	Among groups	1	0.242	0.00403 Va	1.86	FCT = 0.01856
	Among populations	4	0.712	-0.00427 Vb	-1.97	FSC = -0.02003

	within groups					
	Within populations	54	11.730	0.21722 Vc	100.11	FST = -0.00109
x)	Among groups	4	0.916	0.0487 Va	22.59	FCT = 0.22588
	Among populations within groups	1	0.037	-0.0503 Vb	-23.34	FSC = -0.30144
	Within populations	54	11.730	0.21722 Vc	100.75	FST = -0.00747

* Represents significant values [significance level: α = 0.05; p values calculated from a random permutation test (1000 replicates)]