Marine and Fishery Sciences 38 (3): 405-423 (2025)
https://doi.org/10.47193/mafis.3832025010707

ORIGINAL RESEARCH

Demographic and genetic diversity of the genus *Paralichthys* in the Buenos Aires Coastal Ecosystem of the southwestern Atlantic

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Marine and Fishery Sciences
MAFIS

ABSTRACT. Organisms inhabiting the southwestern Atlantic Ocean shelf have undergone significant population changes as a result of climatic fluctuations during the Pleistocene glaciations. These glaciations led to contractions and expansions in population size. This study investigates three coexisting flatfish species particularly within the Buenos Aires Coastal Ecosystem: *Paralichthys orbignyanus*, *P. patagonicus*, and *P. isosceles*. We analyzed haplotypic and nucleotide diversity, as well as demographic trends, across these species using cytochrome b and mitochondrial control region markers. We hypothesized that habitat loss, sea depth, temperature and salinity variations have differently affected the genetic diversity and demographic histories of these flatfish species. Our results revealed notable differences between species inhabiting shallow and deep waters. Those in shallow and coastal waters, such as *P. orbignyanus* and *P. patagonicus*, generally exhibited lower genetic diversity, signs of population expansion and shorter coalescence (i.e. common ancestor) times. In contrast, species from deeper waters, like *P. isosceles*, displayed higher genetic diversity, population stability, and longer coalescence times. Comparative phylogeographic analyses of these species may offer strong evidence supporting hypotheses about the historical impact of the Last Glacial Maximum on coastal habitats and its effects on the habitat preferences of flatfishes within the Buenos Aires Coastal Ecosystem in the southwestern Atlantic Ocean.

Key words: Flatfishes, Last Glacial Maximum, life history traits, mitochondrial DNA, Phylogeography.

Diversidad demográfica y genética del Género *Paralichthys* en el ecosistema costero de Buenos Aires en el Atlántico Sudoccidental

RESUMEN. Los organismos que habitan la plataforma del Océano Atlántico Sudoccidental han experimentado cambios poblacionales significativos como resultado de las fluctuaciones climáticas durante las glaciaciones del Pleistoceno. Estas glaciaciones provocaron contracciones y expansiones en el tamaño de las poblaciones. Este estudio investiga tres especies de peces planos coexistentes que se distribuyen en el Ecosistema Costero de Buenos Aires: *Paralichthys orbignyanus*, *P. patagonicus* y *P. isosceles*. Analizamos la diversidad haplotípica y nucleotídica y las tendencias demográficas en estas especies utilizando los marcadores de citocromo b y la región control mitocondrial. Planteamos la hipótesis de que la pérdida de hábitat, la profundidad del mar y las variaciones de temperatura y salinidad afectaron de manera diferente la diversidad genética y las historias demográficas de estas especies de peces planos. Los resultados revelaron diferencias significativas entre las especies. Aquellas que habitan en aguas someras y costeras, como *P. orbignyanus* y *P. patagonicus*, suelen presentar una menor diversidad genética, signos de expansión poblacional y tiempos de coalescencia (ancestro

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Received: 6 January 2025
Accepted: 15 May 2025

ISSN 2683-7595 (print)
ISSN 2683-7951 (online)

https://ojs.inidep.edu.ar

Journal of the Instituto Nacional de Investigación y Desarrollo Pesquero (INIDEP)

común) más cortos. En contraste, la especie que habita en aguas más profundas, *P. isosceles*, exhibieron una mayor diversidad genética, estabilidad poblacional y tiempos de coalescencia más largos. Los análisis filogeográficos comparativos de estas especies pueden proporcionar evidencia sólida para respaldar hipótesis sobre el impacto histórico del Último Máximo Glacial en los hábitats costeros y sus efectos en las preferencias de hábitat de los peces planos dentro del Ecosistema Costero de Buenos Aires en el Atlántico Sudoccidental.

Palabras clave: Peces planos, Último Máximo Glacial, rasgos de historia de vida, ADN mitocondrial, Filogeografía.

INTRODUCTION

Marine fish typically exhibit gene flow over considerably greater distances compared to their freshwater counterparts. This phenomenon is primarily attributed to the absence of physical barriers, the influence of ocean currents, high dispersal capabilities, and migratory behaviors (Avise 1992, 2000).

In high-latitude regions, phylogeography is notably shaped by habitat shifts driven by glacial cycles (Bowen et al. 2014). Specifically, in marine settings, benthic organisms inhabiting coastal and rocky shores have experienced recent population bottlenecks due to climate-induced sea-level reductions during the Last Glacial Maximum (LGM), approximately 23,000-25,000 years ago, especially in the southwestern Atlantic Ocean (Rabassa et al. 2005, 2008; González-Wevar et al. 2013; Núñez et al. 2015; Fernández Iriarte et al. 2020; Fainburg et al. 2022).

A thorough understanding of the biology and ecology of organisms under study significantly enhances phylogeographic interpretations (Rabassa et al. 2005). While individual-species analyses cannot alone reveal broad trends, they are essential for establishing the groundwork for comparative phylogeographic studies. Comparative research underscores the influence of Pleistocene sea-level fluctuations in promoting lineage diversification (Barber et al. 2006, 2011; Crandall et al. 2008). When possible, analyzing multiple co-distributed species is advantageous for elucidating biogeographic histories (Carpenter et al. 2011; Toonen et al. 2011). Moreover, temperature is potentially linked to global patterns of intraspecific diversity, particularly within continental shelf regions (0-200 m depth), indicating that genetic analyses can assess demographic dynamics in current populations (Manel et al. 2020).

In the more homogeneous and interconnected marine systems of the southwestern Atlantic Ocean, extensive environmental gradients such as sea surface temperature, ocean currents, salinity, and temperature fronts are prevalent (Acha et al. 2004). These factors are crucial in structuring genetic diversity (Manel et al. 2020) and shaping the marine landscape. For instance, at approximately 38° S latitude, the southwestern Atlantic coast is influenced by the convergence of the warm Brazil Current and the cold Malvinas Current (Piola and Matano 2001; Balech and Ehrlich 2008), which delineate the distribution boundaries of various marine fish and invertebrate species (Cousseau and Perrotta 2013).

During the LGM, climatic changes in the southern hemisphere resulted in lower global temperatures, altered marine current patterns, and the loss of coastal habitats due to a significant sea-level drop of about 120 m. This exposure of the continental shelf in the southwestern Atlantic had profound effects on coastal ecosystems (Ponce et al. 2011; Sérsic et al. 2011; Isla 2012). In this context, climatic shifts have had a substantial impact on coastal habitats, influencing flatfish species that exhibit strong associations with specific environmental conditions, habitat preferences, and life history traits (Vandamme et al. 2020).

The order Pleuronectiforms encompasses significant diversity within the warm temperate southwestern Atlantic province, ranging from 22° S-23° S to 41° S-42° S (Spalding et al. 2007; Walsh et al. 2015). This study focused on the

Buenos Aires Coastal Ecosystem (BACE) (34° S, 41° S-42° S) ecoregion, which includes the continental shelves of Uruguay and Buenos Aires (Milessi 2008; Díaz de Astarloa 2016) (Figure 1).

The genus *Paralichthys* (Paralichthyidae) includes some of the most abundant flounders, such as *P. orbignyanus* (Valenciennes 1839), classified as data deficient by the IUCN (Riestra et al. 2020a); *P. patagonicus* (Jordan 1889), listed as vulnerable (Riestra et al. 2020b); and *P. isosceles* (Jordan 1891), also classified as data deficient (Riestra et al. 2020c). These flatfish species are found together in the BACE, ranging from the Uruguayan coast to San Antonio Oeste Bay in the San Matías Gulf, Argentina (Figure 1). They hold significant commercial value, fetching high market prices and serving as an important resource for both artisanal and demersal fisheries in Argentine and Uruguayan waters (Fabré and Díaz de Astarloa 1996; Díaz de Astarloa and Munroe 1998). Known for their high-quality meat, these flatfishes are often grouped under the name 'fine fish' (Fabré and Díaz de Astarloa 1996, 2001; Díaz de Astarloa and Munroe 1998), yet they are frequently exploited without species identification in fishing records within the BACE (Díaz de Astarloa 2002; Rico 2010; Rico et al. 2011; Fainburg and Fernández Iriarte 2017). Despite being exploited together, these flounders exhibit distinct distribution patterns (Díaz de Astarloa and Fabré 1998) and differ in their tolerance to environmental factors such as temperature, salinity, bathymetry, and substrate type, as well as in traits

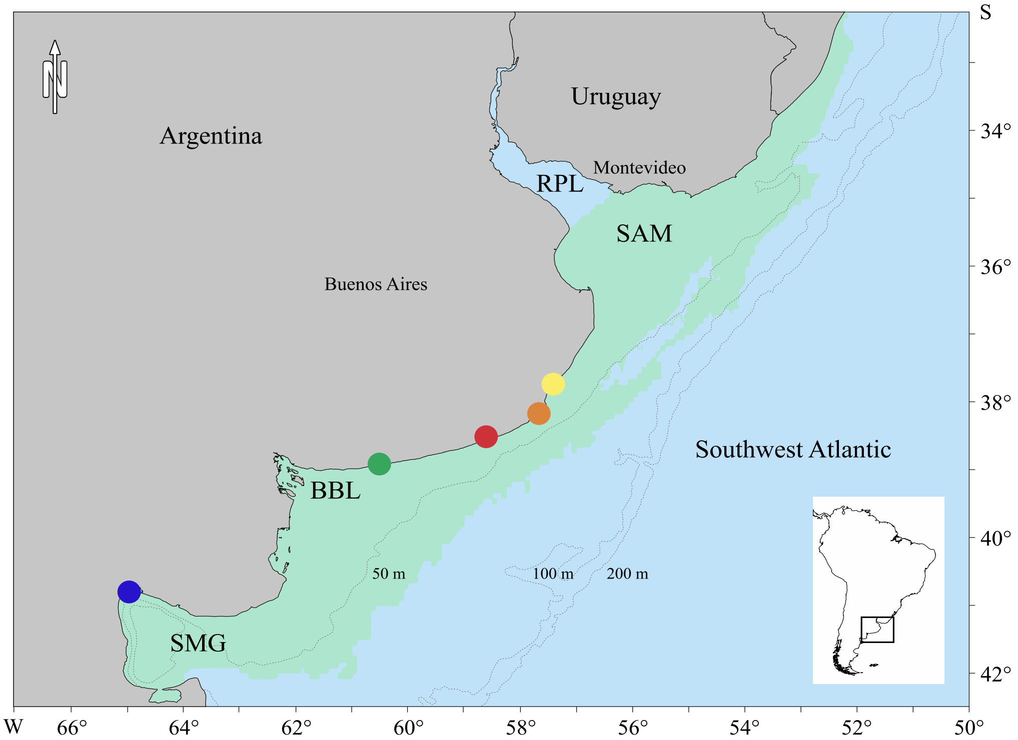

Figure 1. Study area and sampling sites of flatfish species in the Buenos Aires Coastal Ecosystem of the Argentine Biogeographic Province. Lines on the continental shelf indicates 50, 100, and 200 m depths. Mar Chiquita (yellow circle), Mar del Plata (orange), Necochea (red), Marisol (green), San Antonio Oeste (blue). SMG: San Matías Gulf; BBL: Blanca Bay; SAM: Samborombón Bay and RPL (La Plata River). *Paralichthys orbignyanus*, MCH, MAR and SAO sample sites (cytochrome b: N = 60; control region: N = 50). *P. patagonicus*, MDP-NEC sample sites (cytochrome b: N = 15; control region: N = 26). *P. isosceles*, NEC-SAO sample sites (cytochrome b: N = 21; control region: N = 42).

like body size (Díaz de Astarloa and Munroe 1998; Díaz de Astarloa 2002; Díaz de Astarloa and Fabre 2003; Munroe 2015; Riestra et al. 2020a, 2020b, 2020c).

Paralichthys orbignyanus is the most frequently fished species on muddy bottoms in coastal banks targeted by trawling fleets (Rico 2010). This shallow-water flatfish ranges from Rio de Janeiro, Brazil (23° S), to the San Matías Gulf, Argentina (41° S). Within this range, it inhabits estuarine areas, typically found in warm, low-salinity waters from the shoreline to about 20-30 m in deep (Appendix, Figure A1). Its greatest abundance occurs around 20 m depth in temperate and euryhaline coastal estuarine areas, with individuals reaching sizes of 60-110 cm (male and female, respectively) (Díaz de Astarloa and Munroe 1998). Additionally, this species is found in parts of continental shelves where mud and clay sediments are prevalent, but is scarce where the substrate is sandy or gravelly (Díaz de Astarloa and Munroe 1998) (Figure 1). *Paralichthys patagonicus* has a wide distribution from Rio de Janeiro to at least as far south as northern Patagonia (43° S), typically inhabiting sandy bottoms in coastal marine waters at depths of 40-70 m (Díaz de Astarloa and Munroe 1998; Fabré and Díaz de Astarloa 2001; Díaz de Astarloa 2002) (Appendix, Figure A2). It has a medium body size of 48-67 cm (male and female, respectively), preferring marine sandy bottoms and avoiding muddy substrates (Díaz de Astarloa and Munroe 1998). While its distribution range partially overlaps with that of *P. orbignyanus*, the presence of estuaries and low-salinity rivers with muddy substrates seems to limit the occurrence of *P. patagonicus*. In addition, these two flatfish species are considered phylogenetically sister species (Mabragaña et al. 2011) and are chromosomally very similar (Azevedo et al. 2007). *Paralichthys isosceles* is distributed from Paraná, Brazil (26° 30′ S), to San Jorge Gulf, Argentina (46° S), typically found on sandy or rocky bottoms, and rarely on muddy substrates, at depths of 70-100 m (Walsh et al. 2005) (Appendix, Figure A3). It has a smaller body size, measuring 32-38 cm for males and females, respectively (Díaz de Astarloa and Munroe 1998).

The main hypothesis tested is that the three marine-coastal flatfish species may have been differently affected by Pleistocene climatic changes, which could have caused habitat loss, historical population bottlenecks, and a reduction in genetic diversity. The objective of comparative phylogeographic analyses using cytochrome b and the mitochondrial DNA control region in these flatfish species was to gain insight into the historical effects of the LGM on coastal habitats in the BACE within the southwestern Atlantic Ocean.

MATERIALS AND METHODS

Sampling sites

Samples of *P. orbignyanus* were collected from the Mar Chiquita coastal lagoon (MCH) (37° 44′ S), Marisol (MAR) (38° 55′ S) at the Quequén Salado River's mouth, and the San Antonio Oeste bay (SAO) (40° 48′ S) in the San Matías Gulf. Samples of *P. patagonicus* were obtained from the coastal trawl fleet in the Argentine Sea, with landings in Mar del Plata (MDP) (36° 11′ S) and Necochea ports (NEC) (38° 58′ S). Samples of *P. isosceles* were collected from landings of the coastal fleet in NEC and SAO (Figure 1). No approvals were required for tissue samples obtained from commercial catches, nor were permissions necessary for those collected from artisanal fisheries. Samples were identified using taxonomic keys (Rico and Lagos 2009; Díaz de Astarloa 2016) and stored in absolute alcohol. Specimens were deposited in the fish collection of the Facultad de Ciencias Exactas y Naturales at the Universidad Nacional de Mar del Plata. Partial fragments of cytochrome b were amplified using primers Glu-L-CP (5' TGACTTGAAGAACCACCGTTG 3') and CB2-H (5' CCCTCAGAATGATATTTGTCCTCA 3') (Aboim et al. 2005). The mitochondrial control region in *P. orbignyanus* was amplified using the primer Porc1-F (5’-GTTAGAGCGCCAGTCTTG-TA-3’) (Fainburg et al. 2022) and the universal primer E (5’-CCTGAAGTAGGAACCAGATG-3’) (Lee et al. 1995). Universal primers A (5’ TTC-CACCTCTAACTCCCAAAGCTAG 3’) and E (5’ CCTGAAGTAGGAACCAGATG 3’) were used for the control region in other flatfish species (Lee et al. 1995). The amplification of cytochrome b and the control region in all flatfish species was carried out following previously used protocols (Fainburg et al. 2022). The amplification process was conducted in a final volume of 25 µl, containing 1X TAQ polymerase buffer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.3 µM of each primer, 1 U Taq DNA polymerase (Inbio Highway), 7.5 µl of DNA (20-30 ng), and double-distilled water to make up the volume. The polymerase chain reactions (PCR) were carried out in a 2,700 Thermal Cycler (Applied Biosystems). The cycling parameters consisted of an initial denaturation at 94 °C for 5 min, followed by 5 cycles of 94 °C for 60 s, annealing at 47 °C for cytochrome b and 45 °C for the control region for 60 s, and extension at 72 °C for 60 s. This was followed by 34 cycles at 94 °C for 60 s, annealing at 55-58 °C for 60 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 5 min. Amplifications were visualized on 1.5% agarose gels stained with Red Gel (Biotium) and purified using the AccuPrep PCR purification kit (Bioneer) before being sequenced on an ABI 3500 automatic sequencer at the INTA Genomics Service (Castelar, Argentina).

Sequences were manually edited using Proseq v.2.91 (Filatov 2002) and aligned with ClustalX2 (Larkin et al. 2007). The concatenation of the cytochrome b and control region datasets resulted in a reduction in both the number of individuals and sampling locations for the three flatfish species, leading to inconclusive data. Consequently, results of subsequent analyses are not presented. The cytochrome b and control region sequences of *P. orbignyanus* were deposited in GenBank (Access No: MW504172-MW50418), and those of the other flounder species will be deposited (Access No: PP506021-PP506082).

Statistical analyses

Haplotype diversity (Hd) and nucleotide diversity (π) for both markers were estimated using DNAsp (Ramos Onsins and Rozas 2002). The genealogical relationships between sequences were inferred by haplotype median-joining network constructed with Network 4.6 (Bandelt et al. 1999). Genetic divergence between population pairs (F_{ST}) was estimated with 10,000 permutations in ARLEQUIN (Excoffier and Lischer 2010). Neutrality test deviations were assessed with Tajima’s D (Tajima 1983) and Fu’s F (Fu 1997) using DNAsp. Mismatch distribution was estimated and compared with the expected distribution under the sudden population expansion model using 10000 simulations in DNAsp (Librado and Rozas 2009). Tajima’s D and Fu’s Fs tests were performed to discriminate mutation/drift equilibrium and to evaluate the hypothesis of population expansion through the significant excess of low-frequency haplotypes. For neutral markers, significant negative values of D and F can be expected in cases of population expansion. Moreover, the demographic history of the control region was investigated using mismatch distributions, which are the distribution of pairwise differences among haplotypes (Rogers and Harpending 1992). This method can discriminate whether a collecting site and species have undergone a sudden population expansion or has remained stable over time. Expansion time and demographic changes in effective number weighted by generation time (Ne t) were evaluated using the Bayesian Skyline Plot model in BEAST 1.7 (Drummond and Rambaut 2007; Drummond et al. 2012). The nucleotide substitution model was determined under the Akaike information criterion in jModelTest 0.1.1 (Posada 2008). A Bayesian Skyline Plot analysis was then performed for the control region using a relaxed molecular clock with an intra-specific substitution rate of 6%/Ma, as estimated in marine fish (Bowen et al. 2006; Ruzzante et al. 2008; Fainburg et al. 2022). To account for uncertainty in this estimate, results were presented assuming within-lineage per-site mutation rates of 4%/Ma and 8%/Ma. Three independent runs of 10 million steps were conducted using Markov Chain Monte Carlo (MCMC), with trees and parameters sampled 1,000 times. The runs were visualized in Tracer, ensuring convergence to the same MCMC distribution when the Effective Sample Size (ESS) exceeded acceptable values (> 200). Only one of the three runs was plotted, with the 95% confidence interval estimated as well (Drummond and Rambaut 2007; Drummond et al. 2012). The comparison of Bayesian skyline plots was focused on the control region marker since all flatfish species could be compared. Conversely, no significant convergence was reached in cytochrome b runs.

RESULTS

Mitochondrial DNA sequencing

A partial fragment of cytochrome b was obtained, measuring 351 bp in *P. orbignyanus* (N = 60), 404 bp in *P. patagonicus* (N = 15), and 471 bp in *P. isosceles* (N = 21) (Table 1). A partial fragment of the control region was obtained, measuring 552 bp in *P. orbignyanus* (N = 50), 411 bp in *P. patagonicus* (N = 26), and 408 bp in *P. isosceles* (N = 42) (Table 2).

Genetic diversity

In cytochrome b, *P. orbignyanus* and *P. patagonicus* exhibited low haplotype and nucleotide diversity, whereas *P. isosceles*, which inhabits deeper marine coastal regions, displayed moderate-to-high haplotype diversity and nucleotide diversity that was higher than that of the other species (Table 1). In the control region, all flatfish species demonstrated very high haplotype diversity, with *P. isosceles* exhibiting nucleotide diversity an order of magnitude greater than that of the other species inhabiting shallower coastal regions (Table 2)

Population structure

Pairwise genetic differences in cytochrome b among collecting sites were not significant for all flounders: *P. orbignyanus* between MCH-SAO ($F_{ST} = 0.001$, $P = 0.607$) and MAR-SAO ($F_{ST} = 0.008$, $P = 0.438$); *P. patagonicus* between MDP-NEC ($F_{ST} = 0.020$ $P = 0.256$); and *P. isosceles* between NEC-SAO ($F_{ST} = 0.001$, $P = 0.99$). In the control region, pairwise genetic differences among sample sites of *P. orbignyanus* were not significant between MAR and MCH ($F_{ST} = 0.003$, $p > 0.05$), but were significant between MCH-SAO ($F_{ST} = 0.287$, $p < 0.05$) and MAR-SAO ($F_{ST} = 0.349$, $p < 0.05$) (Fainburg et al. 2022). As far as *P. patagonicus* is concerned, the comparison between MDP-NEC ($F_{ST} = 0.013$, $p > 0.05$) and for *P. isosceles* between NEC-SAO ($F_{ST} = 0.001$, $p > 0.05$) indicated no significant genetic structure in either species.

Demographic analysis

Neutrality tests, Tajima's D and Fu's F, conducted on cytochrome b, were negative and significant only in *P. orbignyanus* (Table 1). The cytochrome b pairwise differences among all DNA sequences resulted in a unimodal distribution for all flounders (data not shown). Regarding the control region, Fu's F neutrality tests yielded negative and significant values for *P. orbignyanus* and *P. patagonicus* (Table 2). In the control region, pairwise differences among all DNA sequences showed a unimodal distribution for *P. orbignyanus* and *P. patagonicus*, while *P. isosceles* displayed multimodal distributions (Figure 2 A-C).

Haplotype network

Paralichthys orbignyanus showed a star-like

Table 1. Genetic variability and demographic parameters index of the cytochrome b in *Paralichthys orbignyanus* (PO), *P. patagonicus* (PP) and *P. isosceles* (PI). N = number of individuals; h = number of haplotypes; Hd = haplotypic diversity; π = nucleotidic diversity; D = Tajima's D; F = Fu's F. * P < 0.05; **P < 0.01. MCH: Mar Chiquita, MDP: Mar del Plata, NEC: Necochea, MAR: Marisol, SAO: San Antonio Oeste.

PO	N	h	Hd	π	D	F
MCH	22	2	0.173 ± 0.101	0.0005 ± 0.0008	-0.641	-0.176
MAR	16	2	0.125 ± 0.106	0.0004 ± 0.0006	-1.162	-0.700
SAO	22	4	0.333 ± 0.124	0.0010 ± 0.0011	-1.471	-2.262**
Total	60	5	0.219 ± 0.060	0.0006 ± 0.0008	-1.601	-4.193*
PP						
MDP	8	2	0.250 ± -0.180	0.0006 ± 0.0009	-1.055	-0.182
NEC	7	1	0.000 ± 0.000	0.0000 ± 0.0000	N/A	N/A
Total	15	2	0.133 ± 0.112	0.0003 ± 0.0006	-1.160	-0.649
PI						
NEC	8	3	0.464 ± 0.200	0.0014 ± 0.0014	-0.448	-0.478
SAO	13	5	0.692 ± 0.119	0.0020 ± 0.0016	-0.964	-1.963
Total	21	5	0.595 ± 0.108	0.0017 ± 0.0014	-0.799	-1.631

network in cytochrome b with five haplotypes, one central haplotype shared by 88% of the samples (53/60) across all sites (PO, Figure 3 A). *Paralichthys patagonicus* presented two cytochrome b haplotypes, with a central one (14/15 of the sample size, 93%) shared by both sites (MDP and NEC) (PP, Figure 3 A). Finally, *Paralichthys isosceles* exhibited five cytochrome b haplotypes, with one central haplotype (7/21; 33% of the sample size) shared by both sites (NEC-SAO) (PI, Figure 3 A).

The control region haplotype network for *P. orbignyanus* revealed 22 haplotypes, with two frequent haplotypes, one shared across all sites and another shared between MCH and MAR (17/50; 34% of the sample size) (PO, Figure 2 B). The control region haplotype network for *P. patagonicus* showed 15 haplotypes in a star-like pattern, with a central haplotype shared by MDP and NEC (6/26; 23% of the sample size), separated from several unique haplotypes (PP, Figure 2 B). The control region haplotype network for *P. isosceles* revealed 17 haplotypes, including two frequent ones found in NEC and SAO (16/21; 76% of the sample size), which are not clearly connected in the network. Additionally, several unique haplotypes from both sites were observed (PI, Figure 2 B). Notably, PI exhibited a significantly longer line length among haplotypes, proportional to the number of mutations separating them.

Bayesian Skyline Plot

The substitution model used for the control region sequences was Hasegawa, Kishino, and Yano with invariant sites and a gamma distribution (HKY + I + G). The Bayesian Skyline Plot (Figure 4)

Table 2. Genetic variability and demographic parameters of the control region in *Paralichthys orbignyanus* (PO), *P. patagonicus* (PP), and *P. isosceles* (PI). N = number of individuals; h = number of haplotypes; Hd = haplotypic diversity; π = nucleotidic diversity; D = Tajima's D; F = Fu's F. * P < 0.05; **P < 0.01. MCH: Mar Chiquita, MDP: Mar del Plata, NEC: Necochea, MAR: Marisol, SAO: San Antonio Oeste.

PO	N	h	Hd	π	D	F
MCH	20	13	0.953 ± 0.028	0.0062 ± 0.0037	-0.906	-5.921**
MAR	14	7	0.758 ± 0.116	0.0031 ± 0.0022	-1.168	-2.523*
SAO	16	7	0.817 ± 0.073	0.0038 ± 0.0025	-0.515	-1.615
Total	50	22	0.923 ± 0.019	0.0056 ± 0.0032	-1.466	-12.645**
PP						
MDP	15	9	0.914 ± 0.052	0.0079 ± 0.0048	-0.900	-2.516
NEC	11	8	0.927 ± 0.067	0.0053 ± 0.0036	-1.493	-4.137**
Total	26	15	0.920 ± 0.041	0.0069 ± 0.0042	-1.728*	-8.266**
PI						
NEC	23	11	0.905 ± 0.035	0.0109 ± 0.0062	0.027	-1.588
SAO	19	11	0.889 ± 0.058	0.0124 ± 0.0070	-0.576	-1.905
Total	42	16	0.891 ± 0.028	0.0115 ± 0.0064	-0.703	-2.919

revealed recent demographic changes for *P. orbignyanus* and *P. patagonicus*: about 35,000 years ago (ranging from 23,000 to 51,000 years) for *P. orbignyanus* (Fainburg et al. 2022) and 25,000 years ago (ranging from 17,000 to 38,000 years) for *P. patagonicus*. During these periods, *P. orbignyanus* saw a significant increase in effective population size per generation (Ne t), while *P. patagonicus* experienced a smaller increase. Genetic structure analysis showed that only *P. orbignyanus* exhibited differentiation between the SAO site in the San Matías Gulf and MCH and MAR sites, with coalescence occurring approximately 10,000 years ago (ranging from 7,000 to 15,000 years ago) (data not shown). Analyses including or excluding SAO samples revealed a consistent trend for *P. orbignyanus*, with coalescence around 35,000 years ago. Conversely, *P. isosceles* showed no recent demographic changes, maintaining a stable trend from about 98,000 years ago (ranging from 65,000 to 147,000 years ago) (Figure 3).

DISCUSSION

Climatic changes during the LGM in the southern hemisphere led to a significant drop in global temperatures, alterations in marine current patterns, and the eradication of coastal habitats, exposing the continental shelf of the southwestern Atlantic (Rabassa et al. 2005; Ponce et al. 2011). Following the end of the last Pleistocene glaciation, sea levels began to rise from their lowest point (-120 m), marking the onset of the last major marine transgression. During the Pleistocene's regressive pe-

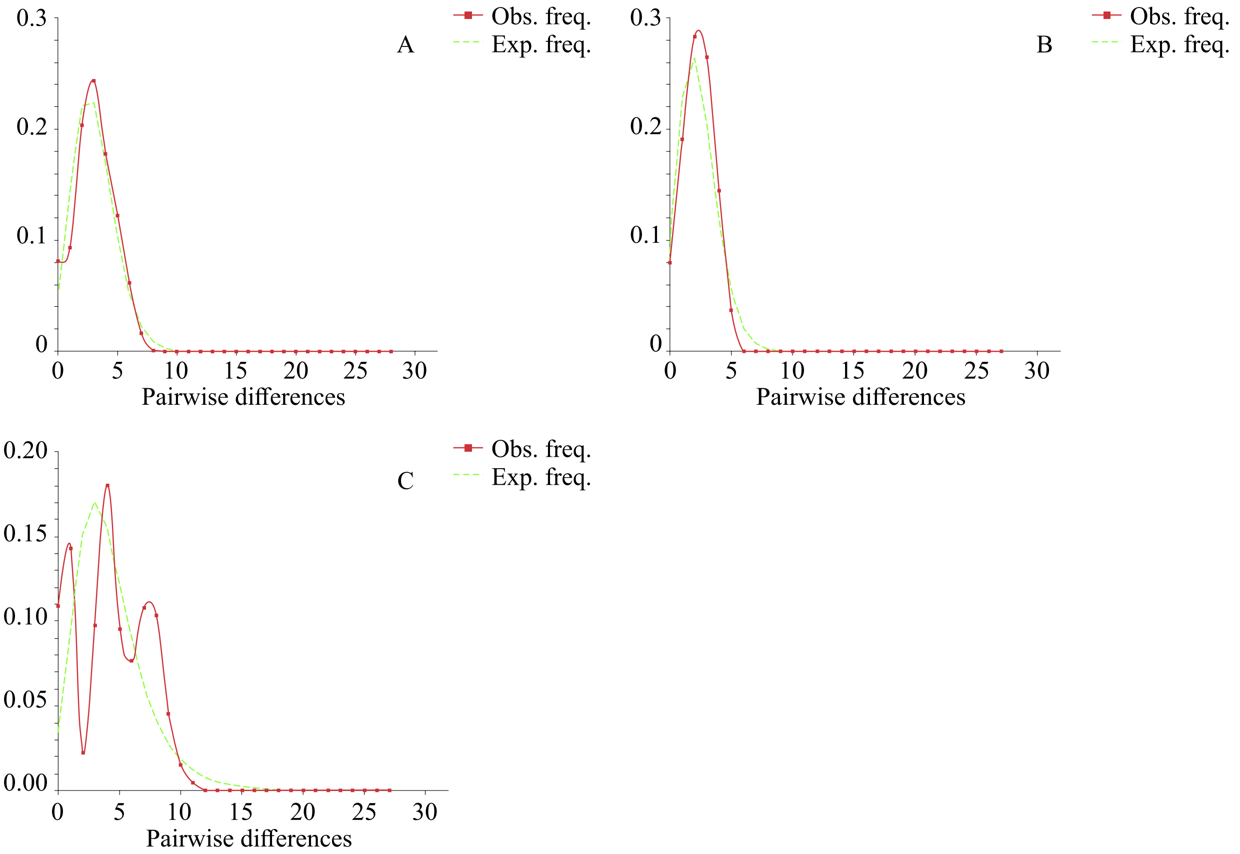

Figure 2. Mismatch distributions of the control region marker. A) *Paralichthys orbignyanus*. B) *P. patagonicus*. C) *P. isosceles*. Obs. freq.: observed frequency; exp. freq: expected frequency.

riod, the continental shelf transformed into a vast coastal plain featuring numerous river systems that extended to the shelf's edge, forming a series of complex deltas (Isla and Madirolas 2009). As sea levels subsequently rose, there was significant flooding of coastal plains, deltas, estuaries, and other transitional environments. In contrast, sea-level surges during the Holocene had more abrupt effects. When the sea surpassed a critical threshold, it rapidly inundated the depression of the San Matías Gulf, approximately 10,000 to 12,000 years ago (Ponce et al. 2011; Isla 2012). The fluctuation in sea levels throughout the Holocene (10,000 years to the present-post-LGM) led to the formation of new geographical areas within the BACE, altering factors such as salinity regimes, habitats, and substrates. The post-LGM sea level rise provided ample time for the development of new coastal territories, affecting the distribution, isolation, and speciation of various coastal organisms (Beheregaray and Sunnucks 2001; Ponce et al. 2011; Isla 2012). The LGM is recognized as a critical event that influenced several marine fish species (Bowen et al. 2016), with benthic and rocky coastal species exhibiting a stronger association with recent bottlenecks than pelagic species (Janko et al. 2007).

Benthic coastal and rocky shore organisms in marine environments show evidence of recent bottlenecks due to population size expansions and contractions (Rabassa et al. 2005; González-Wevar 2013; Núñez et al. 2015; Fernández Iriarte et al. 2020; Fainburg et al. 2022). During the LGM,

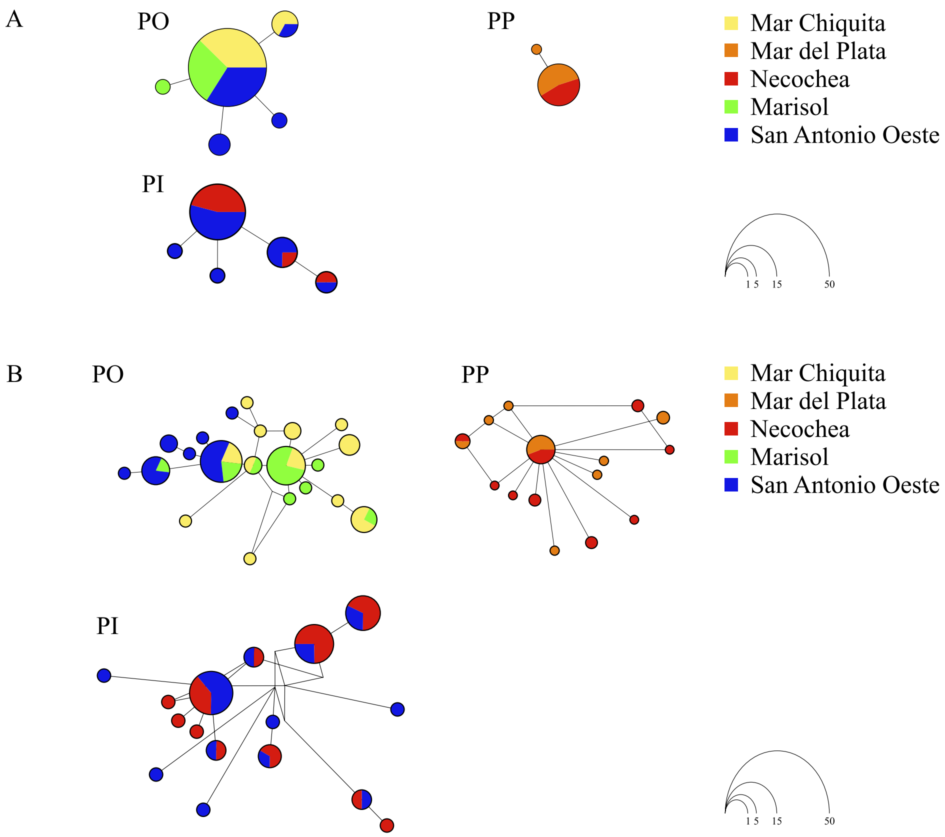

Figure 3. Mitochondrial DNA networks of cytochrome b (A), and control región (B) haplotypes for each flounder species: *Paralichthys orbignyanus* (PO), *P. patagonicus* (PP), and *P. isosceles* (PI) collected in the Buenos Aires Coastal Ecosystem. Circle size is proportional to haplotype frequency and line length is proportional to the number of mutations separating haplotypes.

refuges for flounder *P. orbignyanus* and crabs *Neohelice granulata* would have been more abundant in the BACE region between 34° S-37° S (Ituarte et al. 2012; Fainburg et al. 2022). These species could have recolonized this area following deglaciation and subsequent sea level rise. The distribution of benthic flatfish species on the continental shelf of the BACE was likely notably influenced by the LGM, affecting genetic diversity, haplotype networks, and demographic processes, including recent bottlenecks and population expansions. *Paralichthys orbignyanus* and *P. patagonicus*, residing in warm coastal areas, exhibit lower haplotypic diversity in the cytochrome b marker and higher diversity in the control region, but both species show lower nucleotide diversity across these markers. In contrast, *P. isosceles*, found in deeper marine coastal regions, displays higher haplotypic and nucleotide diversity in both markers compared to the other species. Genetic diversity patterns in flounders are significantly affected by demographic history and body size. Coastal and larger flatfish species, notably impacted by the last Pleistocene glaciation, exhibit lower genetic diversity, likely due to habitat loss on the marine shelf of the BACE, particularly affecting *P. orbignyanus* and, to a lesser extent, *P. patagonicus*. Conversely, *P. isosceles*, which inhabits deeper environments with rocky bottoms, high salinity, and cold temperatures, may experience more stable conditions (Fabré and Díaz de Astarloa 2001).

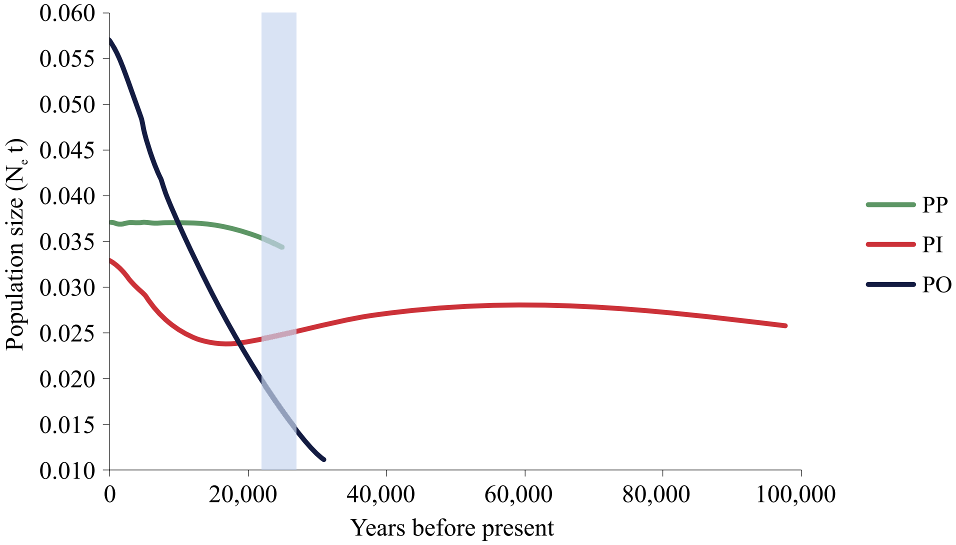

Figure 4. Bayesian Skyline Plot based on mitochondrial control region marker. Demographic reconstruction of flounders: *Paralichthys orbignyanus* (PO), *P. patagonicus* (PP) and *P. isosceles* (PI). The y axis represents the product of the effective population size (N_e) and generation length (t) in a log scale.

Mismatch analysis of pairwise sequence differences for *P. orbignyanus* and *P. patagonicus* revealed unimodal distributions, possibly indicating recent demographic or range expansion events (Rogers and Harpending 1992). Fu's F test supports this, showing evidence of population expansion through an excess of rare variants in *P. orbignyanus* (across both markers) and *P. patagonicus* (in the control region). This suggests a historical bottleneck followed by mutation accumulation during population expansion (Grant and Bowen 1998). Mismatch analysis also showed a unimodal distribution for the cytochrome b sequence and a multimodal distribution for the control region in *P. isosceles*, indicating a bimodal demographic pattern and a long period of stability. This pattern could potentially reflect secondary contact between lineages or stable populations with a long evolutionary history (Grant and Bowen 1998).

The Bayesian Skyline Plot analysis indicated that *P. orbignyanus* experienced a shorter bottleneck around 35,000 years before present, while *P. patagonicus* faced a similar event approximately 25,000 years ago, with both species expanding following approximately the Pleistocene LGM. In contrast, *P. isosceles* displayed a longer coalescence period of stability, occurring around 98,000 years before present (see results and Figure 3). However, it is important to note that these estimates are approximations, as mutation rates are not constant over time, even within a single species (Ho et al. 2005; Ruzzante et al. 2008). Nevertheless, coalescent times align reasonably well with those calculated using a 5-6% substitution rate in fishes (Bowen et al. 2006; Ruzzante et al. 2008; Fainburg et al. 2022). This outcome is consistent with the habitat loss on the continental shelf, along with temperature and salinity changes that occurred during and after the LGM in the BACE. Overall, coalescent estimates for marine organisms should be considered as approximations of the environmental changes that occurred during recent climatic shifts, particularly those related to Pleistocene glaciations (Ruzzante et al. 2008). However, caution is necessary when interpreting these results. While they align with the proposed hypotheses, several limitations must be considered, including the relatively small sample sizes, the limited number of sampling sites, and the mutation rate used. Given these factors, it is advisable to interpret the findings with care, especially since the confidence intervals for the Bayesian Skyline Plot results are broad (Appendix, Figure A4).

Flatfish distribution is influenced by various factors, including past demographic history, geographical coastal changes, and environmental variables like depth, bottom type preferences, temperature, and salinity all of which play crucial roles (Díaz de Astarloa and Munroe 1998; Díaz de Astarloa and Fabré 2003). The Pleistocene demographic pattern of population expansion and contemporary environmental factors are associated with coastal features such as lagoons and gulfs, contributing to the moderate historical and slight contemporary population structure observed in flatfishes. Significant genetic differences in *P. orbignyanus* within the control region between population sites (MDP-MCH versus SAO in the San Matías Gulf) can be attributed to historical and ecological factors, such as temperature and salinity changes, or behavioral aspects like philopatry and larval retention, shaping genetic population structure (Fainburg et al. 2022). *Paralichthys orbignyanus* likely settled in the San Matías Gulf approximately 10,000-12,000 years ago, post-LGM, due to the formation of this littoral habitat (Isla 2012; Ponce et al. 2012). Similar genetic structures linked to specific gulfs have been observed in *P. lethostigma* (Anderson and Karel 2012; Anderson et al. 2012; Wang et al. 2015) and *P. olivaceus* (Shigenobu et al. 2013; Sun et al. 2022). Flatfish employ a periodic spawning strategy, finely tuned to exploit significant variations in environmental quality across both temporal and spatial scales (Winemiller et al. 1992). This strategy includes the ‘lottery effect’ of diffusion spawning, a bet-hedging tactic (Hedgecock and Pudovkin 2011). In this framework, larger flatfish species such as *P. orbignyanus* and *P. patagonicus*, which primarily inhabit shallow waters, may utilize this bet-hedging strategy, potentially leading to reduced fecundity. Additionally, if the age at maturity decreases from *P. orbignyanus* to *P. patagonicus* to *P. isosceles* (Figueiredo and Menezes 2000; López Cazorla 2005), and if these traits correlate negatively with genetic variation (Martínez et al. 2018), this may contribute to decreased genetic diversity as observed in *P. orbignyanus* and *P. patagonicus* in this study.

Contemporary gradients such as sea surface temperature, ocean currents, salinity, and temperature fronts are prevalent in the southwestern Atlantic Ocean (Acha et al. 2004). These gradients suggest a wide range of individual outcomes, including the possibility of synchronizing reproductive activities with favorable oceanographic conditions (Acha et al. 2004). Such synchronization can enhance gamete maturation, fertilization, larval development, settlement, and recruitment into the adult spawning population. Consequently, these factors play a crucial role in shaping the marine biodiversity of flatfishes. Genetically discrete populations or species may exhibit overlapping distributions at various life stages within a single management zone. In such cases, populations with lower productivity are at higher risk of local overexploitation (Reiss et al. 2009; Roy et al. 2012). Our findings highlight the complex interplay between historical events, habitat preferences, and life history traits that contribute to the observed patterns of genetic diversity in these flounder species. Understanding these features is essential for scientists to better comprehend marine organisms’ lives and their roles in marine ecosystems, which is critical for the conservation and sustainable management of marine resources.

ACKNOWLEDGEMENTS

The authors express their gratitude to the artisanal and sport fishermen and researchers who assisted in sampling flounders: Ceferino Traverso, Julia Alarcos, and Juan Timi. The authors explicitly wish to thank the reviewers and editor for their insightful comments and suggestions, which substantially enhanced the quality of this article. We are also deeply appreciative of M.P. Oteiza for her English revision of the manuscript. This is INIDEP contribution no 2433.

Funding

This research was supported by the Universidad Nacional de Mar del Plata under grant EXA1076/22, and by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) under grant PIP 798 awarded to Pedro Fernández Iriarte. Leandro Fainburg received a PhD scholarship from CONICET.

Data availability

DNA sequences generated in this study were submitted to GenBank. The cytochrome b and control region sequences of *P. orbignyanus* were deposited in GenBank (Access No: MW504172-MW50418), and those of the other flounder species will be deposited (Access No: PP506021-PP506082).

Conflict of interest

The authors declare no competing interests.

Ethical approval

The authors declare that no ethical approval was required for the writing of this article.

Author contributions

Leandro A. Fainburg: conceived and designed the experiment; generated data; conducted the primary data analysis; wrote the initial draft of the manuscript. David E. Sabadin: helped in data collection and wrote the initial draft of the manuscript. Juan M. Díaz de Astarloa: contributed to data interpretation; revised the manuscript critically for important intellectual content. Pedro Fernández Iriarte: agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved; drafted the final version of the manuscript. All authors reviewed and approved the manuscript.

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APPENDIX

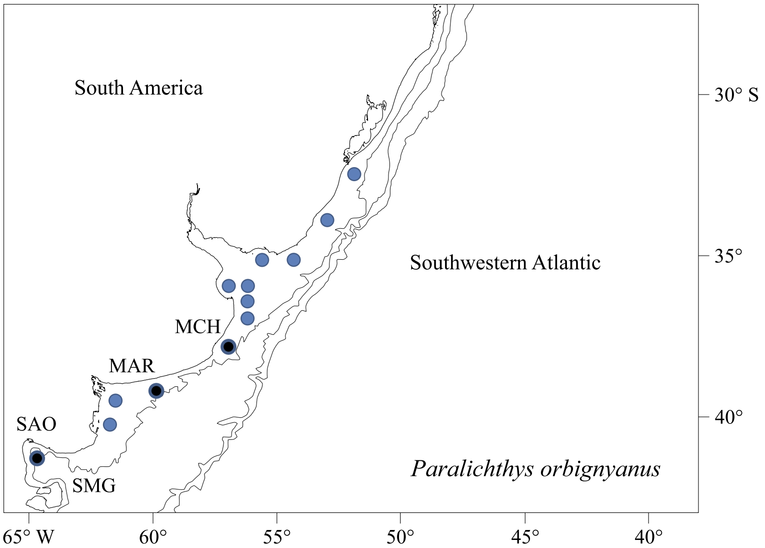

Figure A1. Principal concentrations of *Paralichthys orbignyanus* in the Buenos Aires Coastal Ecosystem (BACE) (modified from Fabré and Díaz de Astarloa 1996; Díaz de Astarloa and Fabré 2003). MCH: Mar Chiquita, MAR: Marisol, SAO: San Antonio Oeste, SMG: San Matías Gulf.

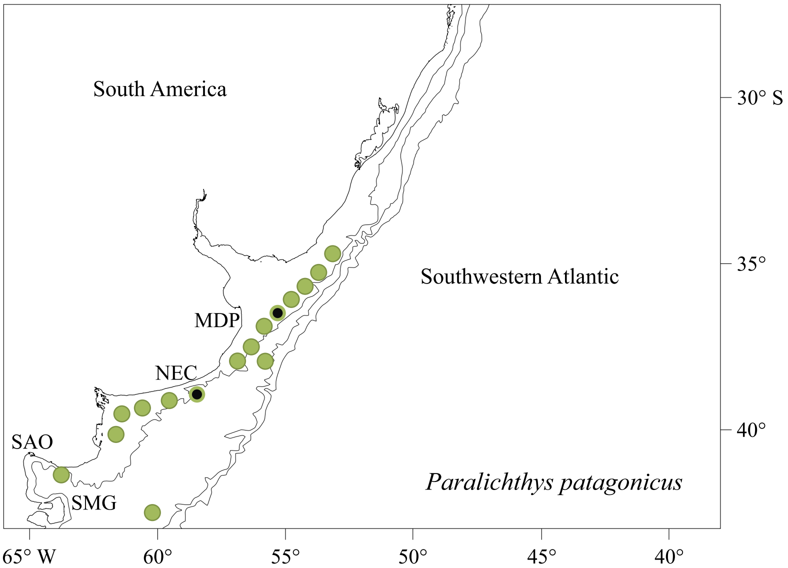

Figure A2. Principal concentrations of *Paralichthys patagonicus* in the Buenos Aires Coastal Ecosystem (modified from Fabré and Díaz de Astarloa 1996; Díaz de Astarloa and Fabré 2003). MDP: Mar del Plata, NEC: Necochea, SAO: San Antonio Oeste, SMG: San Matías Gulf.

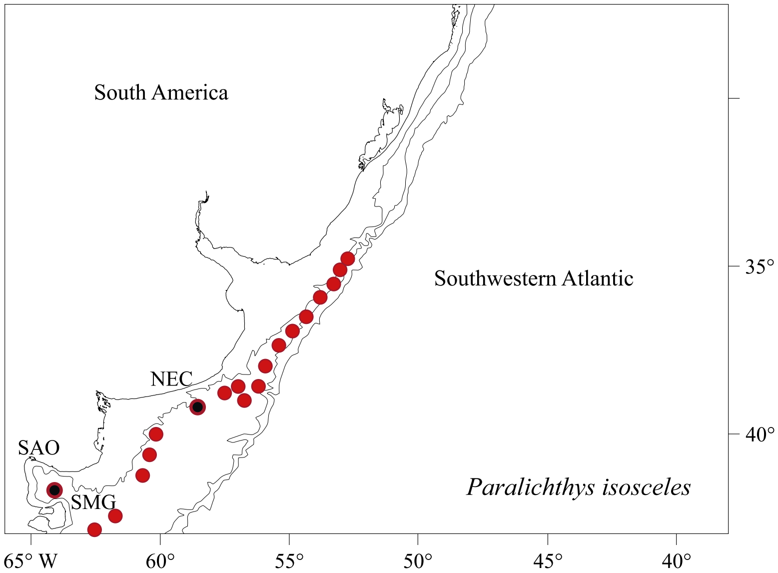

Figure A3. Principal concentrations of *Paralichthys isosceles* in the Buenos Aires Coastal Ecosystem (modified from Fabré and Díaz de Astarloa 1996; Díaz de Astarloa and Fabré 2003). NEC: Necochea, SAO: San Antonio Oeste, SMG: San Matías Gulf.

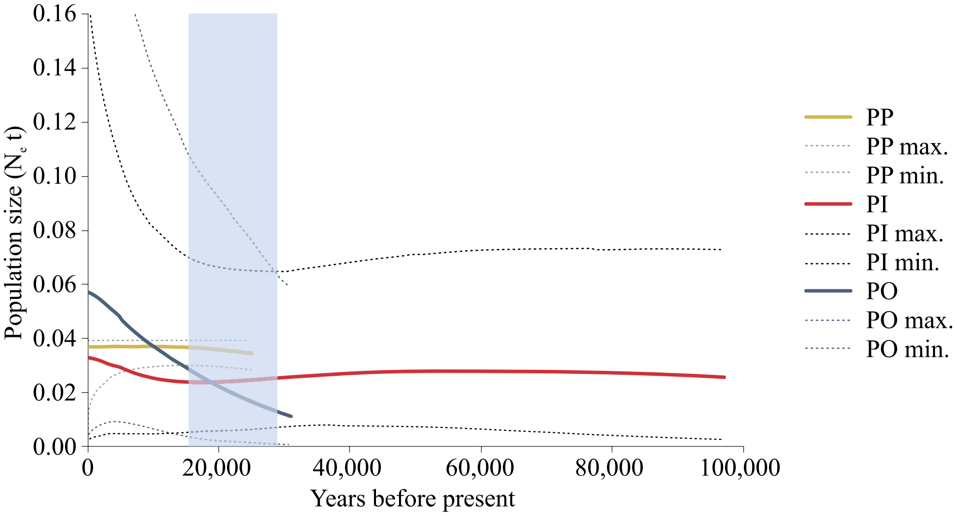

Figure A4. Bayesian Skyline Plot based on mitochondrial control region marker. Demographic reconstruction of flounders: *Paralichthys orbignyanus* (PO, blue line and dashed line in blue), *P. patagonicus* (PP, yellow line, dashed in grey) and *P. isosceles* (PI: red line, dashed line black). The y axis represents the product of the effective population size (N_e) and generation length (t) in a log scale. The maximum and minimum confidence intervals (95%) are included.