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Genetic and morphometric analyzes of *Paralichthys* species confirm the presence of *P. brasiliensis* in the Uruguayan waters

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ABSTRACT

Paralichthys species have high commercial value and constitute a resource for demersal fisheries in the Southwestern Atlantic Ocean. Although six species have been described based on morphological characters for the Uruguayan and Argentinean waters, only the presence of three species is accepted, being their distribution still uncertain. Genetic tools are useful for species identification as a complement of morphological characters. This paper determines the genetic and morphometric variation between three species of Paralichthys that inhabiting Uruguayan waters. The interorbital width (IN) and the length from the mouth to the dorsal fin base (SD) of *P. patagonicus* differed significantly from the other two species (*P. orbignyanus* and *P. brasiliensis*). From a morphological perspective, individuals identified as *P. orbignyanus* formed two different groups. Clade Po_A included those belonging to oceanic coastal waters of Uruguay and deeper waters of the Rio de la Plata together with individuals of *P. brasiliensis* from ItajaÃ, Santa Catarina (Brazil). Individuals collected in the Rio de la Plata coastal and beach zone formed the clade Po_B group. Genetic analysis confirmed for the first time the presence of *P. brasiliensis* in Uruguayan and Rio de la Plata shelf waters and extends its known distribution area further south. Discriminant scores for *P. orbygnianus* separate individuals in two groups corresponding to the presence of two species. Molecular and morphological analysis showed individuals in the clade Po_A includes those collected in Uruguayan waters together with those collected in northern Brazilian waters belonging to the species *P. brasiliensis*.

Along the Southwestern Atlantic Ocean, the genus *Paralichthys* is distributed between 34° and 47° S [1]. Although six species have been described based on morphological characters for Uruguayan and Argentinean waters [2], only the presence of three species is accepted (*P. isosceles, P. patagonicus, P. orbignyanus*) [3,4], however their distribution is still uncertain [1]. According to Figueiredo and Menezes [5], and Diaz de Astarloa and Munroe [1], *P. brasiliensis* does not occur in Argentinean or in Uruguayan waters. For Nion et al. [6], its presence in Uruguayan waters is considered debatable but more studies are needed for its confirmation. *Paralichthys* species have high commercial value and constitute a much-appreciated resource for the artisanal and industrial demersal fisheries in the Southwestern Atlantic Ocean [7–12]. Additionally *Paralichthys orbignyanus* has been the subject of numerous studies related to the development of aquaculture [13–19]. Accurate identification and the knowledge of differences between species is relevant from the biological point of view, particularly in aquaculture and breeding programs, because invasion risks of hybrids into natural environments [20–22]. Also, misclassifications generate errors in defining stocks and lead to biased fishing quotas which can have repercussions in the conservation and management of the flatfish resources [23]. Identification of evolutionary significant units has been traditionally based on morphological characters [24]. However, there are limitations to relying primarily on morphological characters when individuals of a given species vary hugely in their morphology. In this sense, the use of molecular markers has become a useful tool for species identification, independent of morphological characters [25]. Mitochondrial DNA has been widely used as a tool in evolutionary and population studies in many eukaryotes [26], given its maternal line. The aims of this work were to determine the genetic and morphometric variation between three species of *Paralichthys* (*P. orbygnianus, P. patagonicus,* and *P. isosceles*) that inhabit Uruguayan waters, and find the most suitable morphological characters that can be used for a corrected identification.

A total of 33 individuals from flatfishes of *P. orbygnianus* (*n* = 19), *P. patagonicus* (*n* = 5), and *P. isosceles* (*n* = 9) were collected from Uruguayan waters for genetic and morphometric analysis and five individuals of *P. brasiliensis* from Itajaí, Santa Catarina (Brazil) for genetic analysis. Samples of muscles of the flatfish *Oncopterus darwinii* (*n* = 2) from the Uruguayan coast were collected to be used as outgroup in genetic analysis (Figure 1). All species were identified according to Diaz de Astarloa [3] and Figueiredo and Menezes [5]. All muscle samples were preserved in 96° ethanol for subsequent molecular analyzes. DNA extraction was performed using the phenol-chloroform method according to Maniatis et al. [27]. A fragment of the cytochrome b gene of approximately 400 pb was amplified using primers L14735 and H15149AD. PCR reaction was performed as follows: for 50 ml of the PCR reaction were used 50 ng of DNA, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM MgCl$_2$, 2.5 units of Taq DNA polymerase, 200 mM each dNTP, and 0.5 mM of each primer. Amplification was performed under the following conditions: 1 cycle at 94°C for 5 min, 35 cycles at 94°C for 45 s, 45 s at 52°C and 45 s at 72°C and a final extension at 72°C for 7 min. PCR products were sequenced in an automated sequencer at Macrogen Inc. (Korea). Sequences were aligned with Clustal W (using default parameters) [28] implemented in MEGA7 [29]. A neighbor joining tree of genetic distances was constructed to evaluate de phylogenetic relationships of sequences in the software MEGA7 [29]. The best evolution model of data was estimated using the Modeltest 3.7 program [30] and the support of clades was evaluated with a bootstrap of 1000 pseudoreplicates. Morphometric variables (mm), total length (TL), standard length (SL), interorbital width (IN), pectoral fin length (PL), length from the mouth to the base of the anal fin (MA), length from snout to dorsal fin base (SD), and head length (HL), were obtained for all individuals according the criteria proposed by Diaz de Astarloa [3]. To infer how morphometric variables would discriminate the different species, we conducted a canonical discriminant analysis (CDA) [31,32]. These analyzes assess the separation between species through discriminant and canonical axes, evaluating the groups presence into a multivariate space by maximizing the variation between them [32]. Before performing multivariate analysis, the size and allometry effect on the variables analyzed were removed according to Lleonart et al. [33], and all six variables were standardized regarding the total length (TL). We used standard regression model $Y = aX^b$ adjusted by logarithmic transformation of the variables X and Y to homogenize the residuals. Each measure Y_{ij}, where i and j is the individual variable was transformed into Z_{ij} according to $Z_{ij} = Y_{ij} (X_0 /X_j) b_i$ where X_j is the total length of individual j, X_0 is the total length of reference (we used the average total length) and b_i is the allometric parameter relating the dependent variable Y_i with the independent
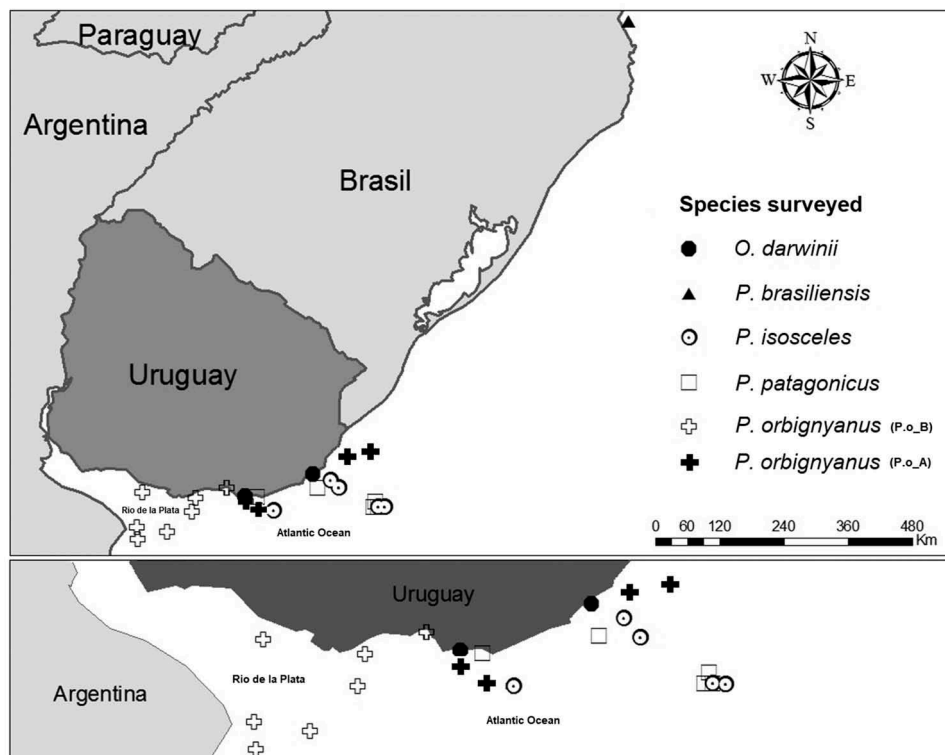

Figure 1. Sampling sites where the individuals of different species were collected (white crosses: *P. orbignyanus* Río de la Plata estuary subgroup (P.o_B); black crosses: *P. orbignyanus* Atlantic Ocean subgroup P.o_A).

variable X. Z_{ij} is the value that would measure Y_{ij} if the fish length were X_0, considering its allometric growth and residual [33]. The CDA were performed using Past program (Version 3.19; March 2018) [34].

The best substitution model for all the species studied was HKY+G with α = 0, 26. All individuals morphologically identified as *P. patagonicus* and *P. isosceles* group together using neighbor joining as grouping algorithm (Figure 2). Nevertheless, individuals morphologically identified as *P. orbignyanus* formed two different groups with a 99% bootstrap support at each node. Individuals in the clade Po_A include those collected in oceanic coastal waters of Uruguay and the deeper waters of the Rio de la Plata together with individuals of *P. brasiliensis* from Itajaí, Santa Catarina (Brazil). Individuals collected in the Rio

de la Plata coastal and beach zone formed the clade Po_B group (Figure 2). The CDA of morphometric variables showed significant differences (Wilks λ = 0, 1391, F $_{18,72}$ = 3,514, p = 0, 000112 < 0.05; Pillai = 1,349; F $_{18,72}$ = 3,269, p = 0, 0001819 < 0.05) between the groups *P. isosceles, P. patagonicus* and the group determined as *P. orbygnianus* composed for two subgroups named Po_A (n = 11) and Po_B (n = 8) (Figure 3). The first canonical factor explained 52.09% and the second 41.33% of the total discrimination, respectively. The morphometric variables that contributed to discriminate groups were: PL with positive sign and SL and HL with negative sign to the canonical axis 1 and, IN and MA with positive signs and SD with negative sign to the canonical axis 2 (Table 1). *P. patagonicus* would have lower values of SL and HL
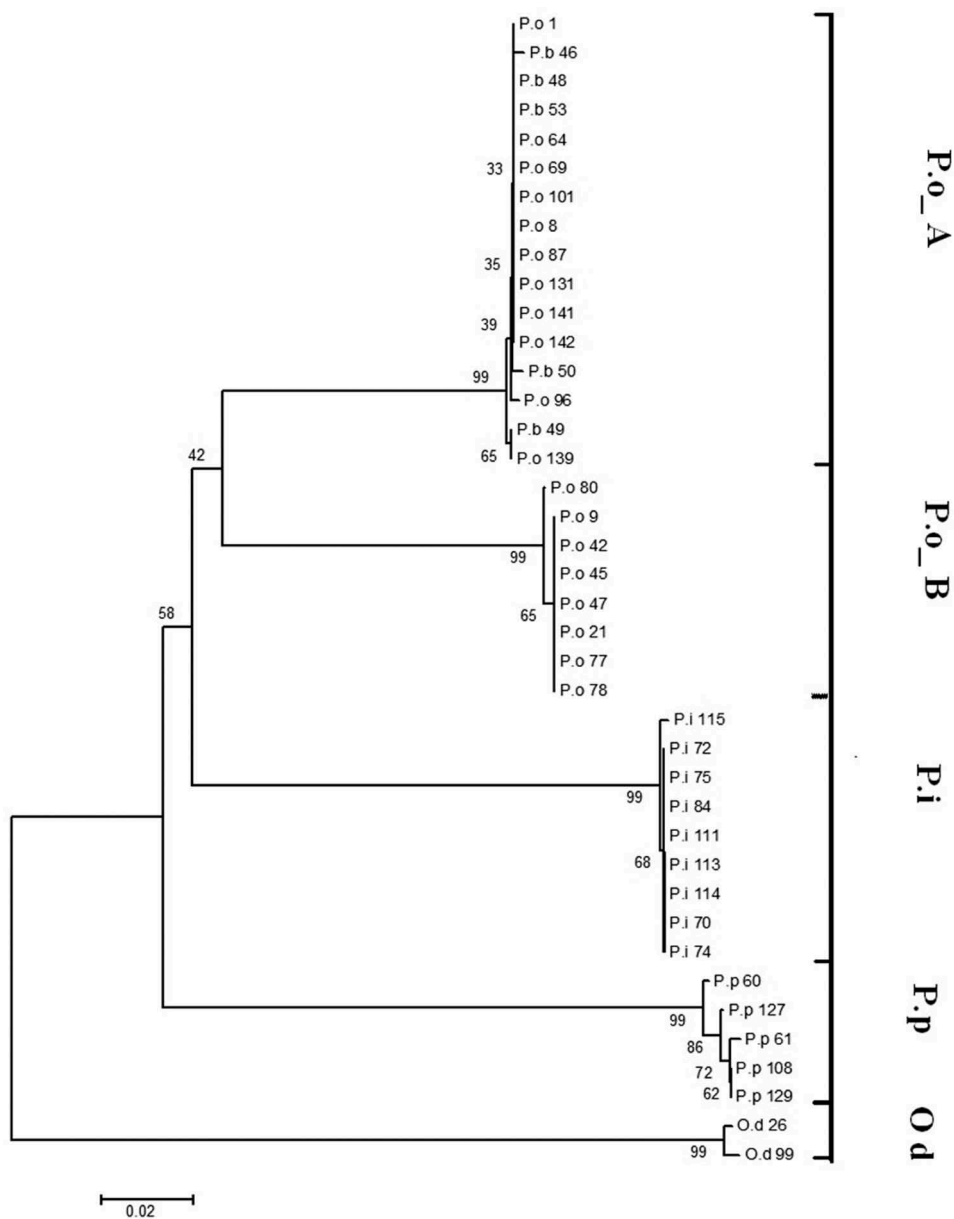

Figure 2. Phylogenetic relationship of individuals analyzed using Neighbor Joining as clustering algorithm (P.o_A: *Paralichthys orbignyanus* A, P.o_B: *Paralichthys orbignyanus* B, P.b: *Paralichthys brasiliensis*, P.i: *Paralichthys isosceles*, P.p: *Paralichthys patagonicus*, O.d: *Oncopterus darwinii*). The numbers corresponds to different individuals. On each node the percentage bootstrap support it is shown.
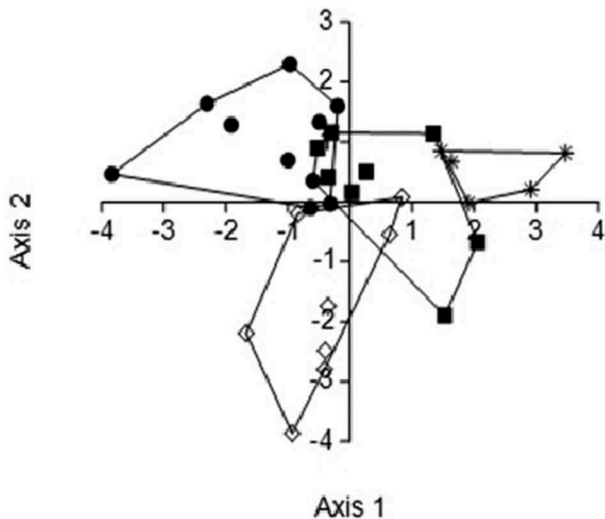
Figure 3. Plot of axis 1 and 2 results of canonical discriminate analysis (CDA) showing the groups of individuals by species (star *: *P. patagonicus*; fill square ■: *P. isosceles*; dot ●: *P. orbignyanus* (named Po_A) and diamond ◊: *P. orbignyanus* (named Po_B). (According to genetic results Po_A correspond to *P. brasiliensis* and Po_B correspond to *P. orbygnianus).*

Table 1. Results of Canonical discriminant analysis (% = percentage of canonical discrimination) and contributions of variables analyzed to factor 1 and 2 (standard length (SL), head length (HL), pectoral fin length (PL), interorbital width (IN), length from the mouth to the base of the anal fin (MA), and length from snout to dorsal fin base (SD).

	Factor 1	Factor 2
Eigenvalues	1,616	1,282
Percentage	52,09	41,33
Variables		
SL	−3,038	2,241
HL	−2,567	−1,376
PL	1,799	−0,385
IN	0,392	0,612
MA	1,231	3,085
SD	−2,539	−5,528

but higher values of IN and PL than Po_A and Po_B. On the other hand Po_A would have lower values of HL and SD than Po_B.

Genetic analysis confirms for the first time the presence of *P. brasiliensis* in Uruguayan and Rio de la Plata shelf waters and extends its known distribution area further south. Discriminant scores for *P. orbygnianus* separate individuals in two groups, which according to the results of the genetic analyzes with a good bootstrap support, corresponded to the presence of two species: *P. brasiliensis* (named Po_A) and *P. orbygnianus* (named Po_B). In the sense, the genetic molecular analysis shows individuals in the clade Po_A includes those collected in Uruguayan waters together with those collected in northern Brazilian waters belonging to the species *P. brasiliensis*. Individuals collected in coastal and beach zone of the Rio de la Plata and in the mouth of Solis Grande Creek named Po_B group, belong to the species *P. orbignyanus*. As several studies in the family Pleuronectidae have shown, the use of morphological

and molecular tools is the most accurate approach to identify and/or delimit species within the family [21,23,25,35–38]. Menni et al [2], mentioned that *P. brasiliensis* inhabits Uruguayan and probably Argentinean waters, particularly the oceanic environment. Nevertheless Diaz de Astarloa and Munroe [1], defined as valid species for Uruguay and Argentina continental shelf only *P. orbignyanus, P. patagonicus* and *P. isosceles* stating that *P. brasiliensis* does not occur in Argentinean-Uruguayan waters. According to Figueiredo and Menezes [5] *P. orbignyanus* differs from *P. brasiliensis* only by the number of gill rankers and crosswise scales on the straight part of lateral line rows. These authors also suggest that *P. brasiliensis* is not present in Uruguayan waters and that the distribution range limits from San Luis de Maranhao (Maranhao) to Ilha Grande Bay (Rio de Janeiro), Brazil. However *P. brasiliensis* has been cited for the State of San Pablo waters [39] and also confirmed its presence for Paranaguá and Parana coastal waters of Brazil [40,41], respectively. In practice, *P. orbignyanus* and *P. patagonicus* were the species most difficult to differentiate morphologically onboard, although the most noticeable features to differentiate these species are their scales (cycloid in *P. orbignyanus* and ctenoid in *P. patagonicus*) and the beginning of the anal fin [42] as well as other morphological characteristics [4]. However the interorbital width (IN) and the length from the mouth to the dorsal fin base (SD), contributed significantly to separate *P. patagonicus* from other species (*P. orbignyanus* and *P. brasiliensis)*. Thus these variables should be considered when getting morphometric information to help facilitate the separation between species. Our work shows evidence for the presence of *P. brasiliensis* in Uruguayan waters and contributes to the knowledge of its current geographical distribution.

Several fish species have been registered southernmost from their previously location reported . These events have been associated with the warm circulation from Brazil Current regarding a putative factor to explain the occurrence of different species [43, 44, 45, 46, and 47]. This could be the case of *P. brasiliensis*, but other studies would be necessary to confirm this hypothesis.

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