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RESEARCH ARTICLE

The genus *Basilichthys* (Teleostei: Atherinopsidae) revisited along its Chilean distribution range (21° to 40° S) using variation in morphology and mtDNA

El género *Basilichthys* (Teleostei: Atherinopsidae) analizado a lo largo de su distribución en Chile (21° a 40° S), utilizando rasgos morfológicos y variabilidad del ADN mitocondrial

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ABSTRACT

There is still doubt as to the number of species of the freshwater Chilean ichthyofauna, 64 % of which have conservation problems. One of the groups is that of the silversides of the genus *Basilichthys*. Three morphological species of this genus have been described in Chile with disjoint distributions: *Basilichthys semotilus*, *B. microlepidotus* and *B. australis*; the latter two overlap in distribution only in the Aconcagua River and are not easily distinguishable by morphological and meristic characters. In order to evaluate the efficacy of identification of these species by molecular techniques, we analyzed the sequence of 9 % of the mitochondrial DNA (Control Region and COI) of individuals from the Loa River (21°41' S) to the Valdivia River (39°50' S), adding meristic features for *B. microlepidotus* and *B. australis* in order to study population variation to clarify the taxonomy of the native species of the genus. The phylogenetic analysis showed that the individuals of *Basilichthys semotilus* form an haplogroup separated from the other species of the genus; however, *B. australis* and *B. microlepidotus* form a monophyletic group that shares the most common haplotypes. An analysis of meristic information showed no statistically significant differences in the number of lateral line scales or number of rays in the fins between *B. microlepidotus* and *B. australis*. These results do not support the current classification for the latter two species; there appears to be one group in the extreme north of the country (*Basilichthys semotilus*) and a second group in central Chile which should be called *B. microlepidotus*. This information will be useful to review the conservation status of the Chilean fauna.

Key words: COI, Control Region, drainages, mtDNA, Silverside fish.

RESUMEN

Si bien aún existen dudas sobre el número de especies descritas en el país, se reconoce que el 64 % de la ictiofauna dulceacuícola chilena se encuentra en alguna categoría de peligro de conservación. Uno de los grupos categorizados como vulnerable y en peligro de extinción es el de los pejerreyes del género *Basilichthys*. A lo largo Chile, este género posee tres especies morfológicas con distribución disjunta: *Basilichthys semotilus*, *B. microlepidotus* y *B. australis*. Las dos últimas sobreponen su distribución en el río Aconcagua y no son fácilmente diferenciables morfológicamente. Para evaluar la eficacia en la identificación de estas especies al utilizar marcadores moleculares, se analizó el 9 % del ADN mitocondrial (Región Control y COI) de organismos obtenidos desde el río Loa (21°41' S) al río Valdivia (39°50' S) y adicionando un análisis merístico en organismos pertenecientes a las especies *B. microlepidotus* y *B. australis*. El análisis filogenético muestra que los individuos de *B. semotilus* forman un haplogrupo separado de las otras especies del género, sin embargo, *B. australis* y *B. microlepidotus* serían parte de un mismo grupo monofilético. Un segundo análisis, el cual incluye información merística, no muestra diferencias estadísticas significativas en la cantidad de escamas de la línea lateral, y número de rayos en las aletas entre *B. microlepidotus* y *B. australis*. Estos resultados no sustentan la clasificación actual, separando claramente un grupo presente en el extremo norte del país (*B. semotilus*) y un segundo grupo en Chile central el cual debería ser llamado *B. microlepidotus*. Esta información será importante para revisar el estado de conservación de la ictiofauna chilena.

Palabras clave: COI, cuencas hidrográficas, mtDNA, pejerreyes, Región Control.

INTRODUCTION

The freshwater fauna of Chile, because of its geographical isolation, is rather different and has a low diversity compared to the other American regions. In order to formulate effective conservation plans for this fauna, it is necessary to determine the real diversity of species present in specific places and their geographic distributions. In Chile, this has begun first with foreign expeditions and more recently in conservation programs by making lists of species with conservation problems (Eigenmann 1927, Ministerio del Medio Ambiente 2011). However, there are still limitations imposed by species identification, both from the presence of as yet undescribed species and in the assignment of species level to the geographically isolated populations.

Even though 64 % of the species of Chilean freshwater fishes have been reported to have conservation problems (Habit et al. 2006, Vila et al. 2006), the low number of morphological differences in characters among species has caused difficulties with this assessment (Campos 1982, Gajardo 1985, Arratia 1990, Dyer 2000b). The study of fishes by Eigenmann (1927) contributed notably to their classification and a reduction in the number of described species; however, debates about the validity of some of them have long existed and still exist (Dyer 2000a).

In terms of abundance and distribution, one of the most representative Chilean freshwater fish genera is *Basilichthys* (Girard, 1855). This genus belongs to the South American subfamily Atherinopsidae, characterized by having a non-protractile mouth with the skin interrupted over the middle of the snout (Eigenmann 1927). Three allopatric species of this genus have been described: *Basilichthys semotilus* (Cope, 1874) in the Loa River (21°41' S), *B. microlepidotus* (Jenyns, 1841), found from the Huasco River (28°30' S) to the Aconcagua River (32°20' S) (Dyer 2000a) and *B. australis* (Eigenmann, 1928) from the Aconcagua River to Chiloé Island (42°18' S) (Campos et al. 1984).

In a complete phylogenetic revision of the Atherinopsinae, Dyer (1997) remarked that the species composition of *Basilichthys* has been problematic since its description. Dyer (1997) recognized two species groups: *Semotilus*, which includes the species from southern Perú and northern Chile *B. semotilus*, *B. beardsleei* (Abbott, 1899) and *B. archaeus* (Cope, 1878) and the *Microlepidotus* group, with two species: *B. microlepidotus* and *B. australis*. Within the *Microlepidotus* group, the species do not have clear diagnostic morphological characters that allow their identification. Eigenmann (1927) found that these species are scarcely distinguishable, differing only in the scale numbers of the lateral line. Later, Gajardo (1985), by using large sample sizes, showed that the number of scales overlaps; no other clear and significant morphological or meristic differences have been found between the species. Furthermore, molecular analysis did not find differences in allele composition for 37 allozymic loci (Gajardo 1988). At present the only evidence that supports these species is a difference in the modal number of chromosomes (2n = 46 for *B. microlepidotus* and 2n = 48 for *B. australis*) (Gajardo 1992). Furthermore, both species have been indicated to coexist in the Aconcagua basin (Gajardo 1985).

In recent years new molecular techniques have been developed that are useful to complement the morphological determination of species and/or the presence of isolated populations of the same species. These kinds of techniques have proved successful in different groups of organisms (i.e. Hebert et al. 2003a, 2003b, Ward 2009), providing precision and repeatability (Hebert & Gregory 2005). In the case of freshwater fishes, molecular markers have proved very useful because they tend to show clear phylogenetic patterns and their relation to the history of each watershed (Bernatchez & Wilson 1998, Bermingham & Martin 1998, Avise 2000). In recent years, sequencing of the cytochrome c oxidase I gene (COI) has been used in the taxonomy of freshwater fish; this gene has proved to be useful in identifying species in accordance with morphology (e.g., Ward et al. 2005, Hubert et al. 2008, Lara et al. 2010).

The objective of this study is to evaluate the taxonomy of the *Basilichthys* species in Chile. In order to determine the species diversity of this group, samples from ten geographically isolated rivers from 27° to 40° S were analyzed using two fragments of mitochondrial DNA (COI and Control Region), and the meristic counts used currently in *Basilichthys* taxonomy. With this information the taxonomic status of these

species will be updated, providing additional information for better conservation plans.

METHODS

Sampling

Specimens of *Basilichthys* were caught by netting, angling and electrofishing from different rivers ranging from the Loa River (21°41' S; 69°35' W) to the Valdivia River (39°50' S; 72°44' W). Geographical locations and putative species names used in this study are listed in Table 1 and shown in Fig. 1. Voucher specimens were deposited in the Museo Nacional de Historia Natural (MNHN, Chile; Table 1). Humane standards were followed in the handling of the fish; all fish were euthanized using 100 mg L^{-1} tricaine methanosulfonate.

DNA extraction, amplification and mtDNA sequencing

Total genomic DNA was extracted from ethanol-preserved fin clips using the salt-extraction method (Aljanabi & Martínez 1997). Pure DNA was stored at -20 °C in 50 μL of water until analysis. Using the mitochondrial sequence of *Hypoatherina tsurugae* (GenBank AP004420; Miya et al. 2003), silverside-specific primers for the control region were designed as follows: forward (5'-CCT AAC TCC CAA AGC TAG GAT-3') and reverse (5'-TGC GGT ACT TGC ATG TGT AA-3'). Amplification from the template DNA used the following conditions: 1x buffer, 3.2 nM $MgCl_2$, 0.2 U μL^{-1} dNTP (Invitrogen), 5 pmol forward and reverse primers, and 0.1 U μL^{-1} Taq DNA polymerase (Invitrogen). The PCR reaction (in 25 μL final volume) included a denaturing step of 94 °C for 3 min followed by 30 cycles of 94 °C for 30 sec, 60 °C for 90 sec, and 72 °C for 90 sec with a final elongation step of 10 min at 72 °C. The primers and PCR conditions for the COI amplification followed Folmer et al. (1994). PCR products of both the COI and the Control Region were cleaned using QIAQuick columns (QIAGen, Mississauga, Ontario, Canada) and sequencing in both directions was performed in Macrogen Inc. (http://www.macrogen.com). Sequences were aligned using ProSeq software (Filatov 2002) and checked using Multalign online software (Corpet 1988).

Analysis

In order to determine genetic relationships among specimens sampled from the 10 different rivers we performed two analyses: (a) a neighbor joining-based relationship (NJ) performed in Mega 4.0 (Tamura et al. 2007) for haplotypes of both COI and the Control Region separately. (b) Using the same sequences, a Maximum Parsimony (MP) analysis run using PAUP

TABLE 1

Geographic locations, putative species and sample size for genetic analyses.

Sitios de estudio, nombre putativo de las especies y número de muestras para el análisis genético.

Putative species	Site	Geographic coordinates	Sample size	Voucher number
Basilichthys semotilus	Loa River	21°41' S; 69°35' W	5	MNHN CP7391
Basilichthys microlepidotus	Huasco River	28°29' S; 71°07' W	3	MNHN CP7377, CP7375, CP7376
Basilichthys microlepidotus	Limarí River	31°37' S; 71°24' W	3	MNHN CP7378, CP7379, CP7380
Basilichthys microlepidotus	Combarbalá River	31°09' S; 70°59' W	2	MNHN CP7386, CP7387
Basilichthys microlepidotus	Choapa River	31°49' S; 71°00' W	3	MNHN CP7381, CP7382, CP7383
Basilichthys microlepidotus	Aconcagua River	32°44 S; 70°44' W	3	MNHN CP7388, CP7389, CP7390
Basilichthys australis	Maipo River	33°47' S; 70°43' W	3	MNHN CP7373, CP7372, CP7374
Basilichthys australis	Mataquito River	34°59' S; 71°47' W	1	
Basilichthys australis	Maule River	35°23' S; 71°36' W	3	MNHN CP7384, CP7385
Basilichthys australis	Valdivia River	39°50'S; 72°44' W	5	MNHN CP7392
Odonthestes regia	Iquique	20°42' S; 70°11' W	1	

4.0b10 software (Swofford 2002). For both NJ and MP, the consistency of branches was tested using a bootstrap re-sampling with 1000 replicates. All sequences used in this analysis were published in Genbank with the following accession numbers: Control Region: FJ380091 to FJ380105 and COI: FJ380197 to FJ380116. Sequences of *Odontesthes regia* (Humboldt 1809) sampled from Iquique (20°42' S; 70°11' W) were used as outgroup for these analyses (Genbank Accession Number: COI: FJ380117, Control Region: FJ380106).

Meristic counts analysis

To evaluate possible meristic differences among populations of *B. microlepidotus* and *B. australis*, samples from nine rivers (Fig. 1 and Table 2) were analyzed. For each specimen we counted the number of lateral line scales and rays of the fins. An ANCOVA analysis was performed for each measured character by using the GLM procedure of SAS (SAS Institute 1998) with the standard length as a covariate in all analysis. Normality and homocedasticity was tested by using the Shapiro and Bartlett tests, respectively. When the assumptions were violated, the Box Cox analysis was used to find the best transformation to the data. Finally, when the ANCOVA was significant the LSMeans pairwise analysis was performed as the a posteriori test. The $\alpha = 0.01$ was used to control a possible type I error.

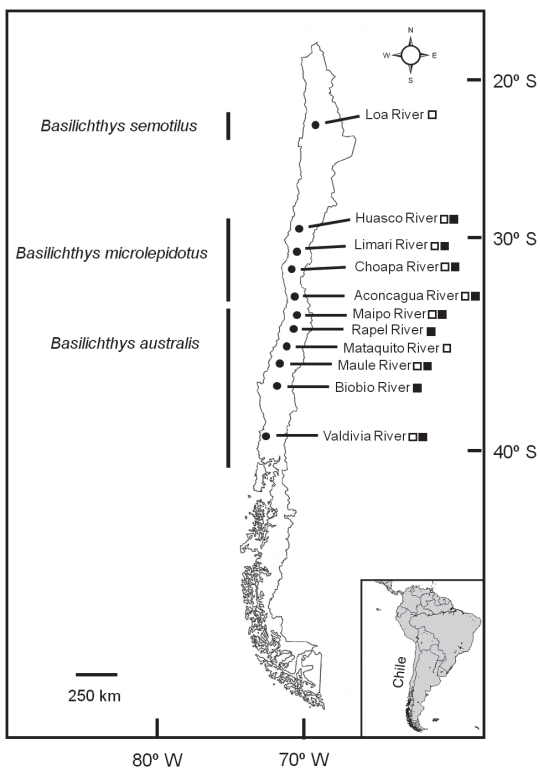

Fig. 1: Sample sites of *Basilichthys semotilus*, *B. microlepidotus* and *B. australis*. □ = samples for genetic analysis ■ = samples for meristic analyses.

Sitios de muestreo de *Basilichthys semotilus*, *B. microlepidotus* y *B. australis*. □ = muestras para análisis genéticos ■ = muestras para análisis merísticos.

RESULTS

For the COI gene, 687 bp were sequenced, obtaining 10 haplotypes in the 31 individuals analyzed. No insertions or deletions were detected, so alignment was straightforward. There were 97 polymorphic sites of which 19 were parsimony informative characters. The MP COI analysis retained one most parsimonious tree with the following parameters: length = 20, consistency index (CI) = 1.00 and retention index (RI) = 1.00.

The NJ and MP analyses gave similar results, separating the haplogroup of *B. semotilus* from the other species of the genus. There was a mean difference of 19.5 bp (SD = 1.26) between the haplotypes of *B. semotilus* and the group with *B. microlepidotus* and *B. australis*. These analyses showed that *B. australis* and *B. microlepidotus* belong to a single haplogroup with bootstrap support > 99 %. Also, the latter two species shared haplotype 3 (H3), which was found in most of the studied watersheds (Fig. 2A and 2B, Table 3).

For the 731 bp of the control region sequenced we found 15 haplotypes in the 31 organisms analyzed. This sector also did not present alignment problems; only one insertion was present, in the individuals from the Valdivia River. 145 sites were polymorphic and 138 were parsimony informative characters. The MP COI analysis retained 17 trees and the most parsimonious tree presented the following parameters: length = 164, consistency index (CI) = 0.91 and retention index (RI) = 0.93.

Both the NJ and MP analyses produced results similar to those found for the COI region. The analyses showed one haplogroup composed of *B. semotilus,* clearly separated from a second haplogroup composed of *B. australis* and *B. microlepidotus* by an average of 29.4 bp differences (SD = 2.23) (2.5 %). This region of mitochondrial DNA showed high statistical support (bootstrap support 100 %) for one haplogroup containing all the sequences of *B. australis* and *B. microlepidotus*. These two species shared 4 of the 13 haplotypes found in the haplogroup (haplotypes 3, 5, 6 and 7; Fig. 2C and 2D, Table 3).

The meristic analyses showed that the inclusion of the standard length in all ANCOVA analyses was statistically significant ($P < 0.001$). The number of scales ranged from 50 (Choapa

TABLE 2

Fish length, number of scales of the lateral line and fin rays by river for *Basilichthys* group microlepidotus. Table includes average, standard deviation and minimum-maximum range for the character obtained for each site. Means sharing the same letter are not significantly different (P < 0.01). N = number of fish analyzed.

Tamaño de los peces, número de escamas de la línea lateral y rayos de las aletas por río para *Basilichthys* del grupo microlepidotus. La Tabla incluye promedio, desviación estándar y rango máximo y mínimo para el carácter para cada sitio. Los promedios que comparten la misma letra no son significativamente diferentes (P < 0.01). N = número de peces analizados.

		Fish length		Scales	Fin rays				
River	N	Standard	Total	number	Pectoral	Ventral	Anal	Dorsal	Caudal
Huasco	16	61.34 ± 20.15 (36.1-100.8)	74.19 ± 23.72 (43.2-118.3)	76.75 ± 11.31 (54-90) abc	13.13 ± 1.75 (11-16) ab	5.20 ± 0.41 (5-6) a	13.63 ± 0.92 (13-15) a	10.88 ± 0.96 (10-13) abc	17.81 ± 0.98 (16-20) a
Limarí	27	73.46 ± 9.71 (54.6-95.6)	88.35 ± 12.05 (68.1-117.5)	80.63 ± 5.33 (70-91) b	13.74 ± 1.29 (12-15) ab	5.04 ± 0.19 (5-6) a	13.74 ± 1.56 (11-17) a	11.07 ± 0.55 (9-12) abc	19.74 ± 1.70 (18-23) b
Choapa	149	47.23 ± 8.88 (23.3-71.3)	56.38 ± 11.09 (28.2-85.3)	69.40 ± 8.25 (50-92) ab	13.34 ± 1.39 (8-16) a	5.12 ± 0.33 (5-6) a	13.59 ± 1.26 (7-17) a	10.93 ± 0.50 (8-12) c	18.21 ± 1.80 (15-23) ab
Aconcagua	30	77.78 ± 11.34 (51.9-99.9)	93.49 ± 12.85 (64.5-116.0)	84.47 ± 8.94 (72-95) ab	13.36 ± 1.52 (10-16) ab	5.47 ± 0.51 (5-6) b	13.47 ± 1.55 (11-16) a	10.80 ± 1.19 (8-12) b	19.30 ± 1.60 (17-23) ab
Maipo	31	52.24 ± 17.77 (26.12-94.4)	62.55 ± 21.30 (32.3-113.8)	75.90 ± 11.41 (56-103) cde	12.65 ± 1.08 (11-15) b	5.13 ± 0.34 (5-6) a	13.90 ± 1.33 (12-17) a	10.65 ± 0.66 (9-12) ab	18.52 ± 1.39 (17-22) ab
Rapel	21	50.66 ± 16.23 (36.8-100.6)	59.65 ± 19.01 (43.2-118.1)	73.95 ± 8.99 (61-93) a	12.76 ± 0.70 (11-14) ab	5.19 ± 0.40 (5-6) a	14.00 ± 0.95 (13-15) a	11.00 ± 0.00 (11-11) ac	17.57 ± 1.57 (16-22) a
Maule	28	63.89 ± 10.74 (46.7-84.3)	74-21 ± 12.99 (53.3-99.1)	87.18 ± 8.54 (74-103) e	12.46 ± 1.29 (10-15) b	5.11 ± 0.31 (5-6) a	13.78 ± 1.12 (11-16) a	11.21 ± 0.91 (9-12) ac	18.46 ± 1.40 (17-23) ab
Biobío	13	65.01 ± 8.40 (48.3-73.9)	75.35 ± 10.01 (55.8-87.3)	82.0 ± 11.95 (61-104) ad	13.31 ± 0.95 (12-15) ab	5.31 ± 0.48 (5-6) a	14.46 ± 1.51 (13-17) a	10.92 ± 0.29 (10-11) abc	17.77 ± 1.09 (16-19) a
Valdivia	12	40.56 ± 11.98 (27.3-49.1)	43.44 ± 6.00 (32.1-51.3)	78.58 ± 10.01 (54-89) c	12.92 ± 1.08 (12-15) ab	5.08 ± 0.29 (5-6) a	13.92 ± 1.24 (13-16) a	10.92 ± 0.51 (11-12) ac	19.00 ± 1.21 (17-21) b

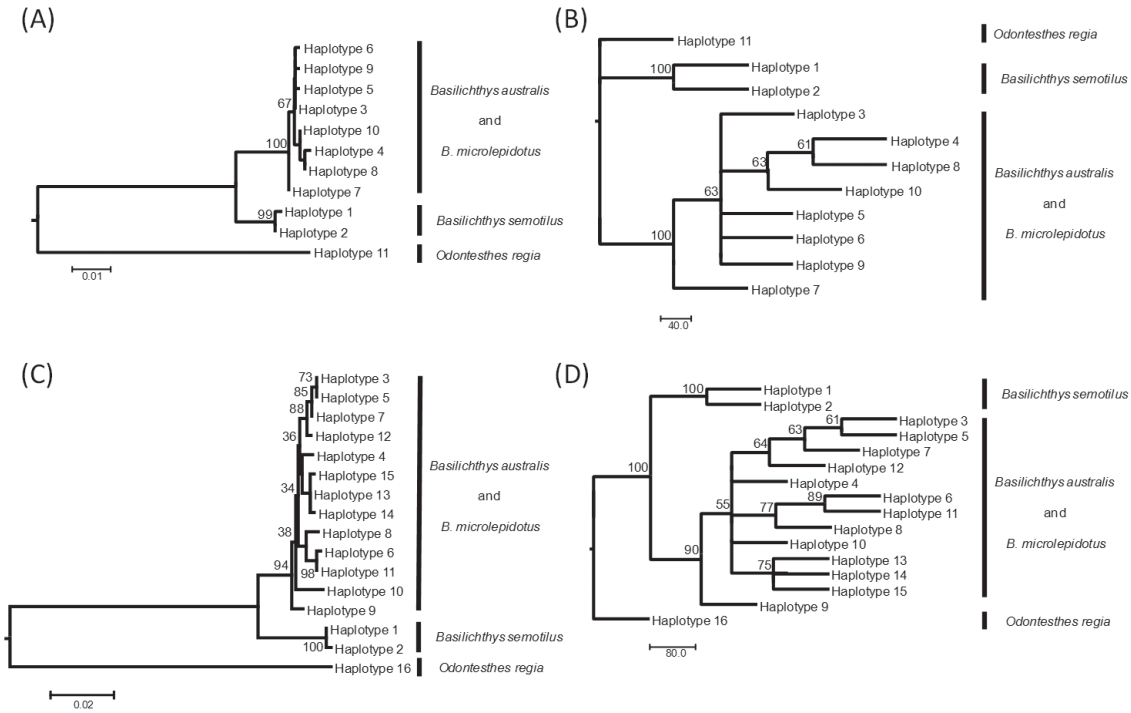

Fig. 2: Trees constructed with COI and Control Region sequences for species of the genus *Basilichthys*. (A) Neighbor joining analysis and (B) Maximum Parsimony for COI sequences. (C) Neighbor joining analysis and (D) Maximum Parsimony for Control Region sequences. In all cases, numbers above branches are bootstrap values (only values > 50 % are shown). See Table 2 for location of each haplotype.

Árbol realizado con secuencias del gen COI y Región Control para las especies del género *Basilichthys*. (A) análisis Neighbor joining y (B) Maximum Parsimony realizado con secuencias del gen COI. (C) análisis Neighbor joining y (D) Maximum Parsimony realizado con secuencias de la Región Control. En todos los casos, el número sobre los brazos representa el valor del boostrap (se muestran solo valores sobre 50 %). Ver Tabla 2 para la ubicación geográfica de cada haplotipo.

TABLE 3

Name of each haplotype found in each River.

Denominación de cada haplotipo encontrado en cada río

	Haplotype	
River	COI	Control Region
Loa	H1, H2	H1, H2
Huasco	H3	H3
Limarí	H3	H3, H4, H5
Combarbalá	H3	H4
Choapa	H4, H5	H6, H7
Aconcagua	H3, H6, H7	H5, H7, H8
Maipo	H3, H8, H9	H3, H6, H7
Mataquito	H3	H12
Maule	H3, H8, H10	H9, H10, H11
Valdivia	H3	H13, H14, H15

River) to 104 (Biobío River), including most of this range in all rivers (Fig. 3). The ANCOVA analysis showed significant differences among sites ($F_{1,8}$ = 11.92, P < 0.001). The pairwise analysis reveals that specimens from the Limarí River had a greater mean number of scales than the Maipo, Maule, Rapel and Valdivia rivers (P < 0.01); Maule River had specimens with a larger number of scales than specimens from Huasco (P < 0.001), Limarí (P < 0.001), Choapa (P < 0.001), Aconcagua and Maipo (P = 0.003). From the same analysis, Maipo specimens had a lower mean number of scales than Limarí (P < 0.01), Choapa (P = 0.003), Aconcagua (P = 0.006), Maule (P = 0.003), Rapel (P = 0.001) and Valdivia (P = 0.001).

The pectoral fin rays ranged from 8 to 16, showing statistical differences among sites ($F_{8,317}$ = 3.81; P =0.0003). Pairwise analyses revealed that the Choapa River had silversides with a mean ray number larger than that observed in the samples from Maipo and Maule (P < 0.001). All other paired comparisons did not show statistical differences. The ventral fin rays ranged from five to six for all sites studied. For this character, the ANCOVA analysis found differences among sites ($F_{8,317}$ = 3.95; P = 0.0002); specimens from the Aconcagua River were larger than those of all other sites (P < 0.01). The rays of the anal fin ranged from 11 to 17 and the means were not different among sites ($F_{8,295}$ = 1.96; P = 0.051). The dorsal fin showed between 8 and 12 rays and the ANCOVA analysis detected statistical differences among sites ($F_{8,316}$ = 3.29; P = 0.001). The pairwise analyses showed that samples from Maipo River had a lower mean number of rays than samples from Choapa (P = 0.004) and that the Aconcagua River sample had fewer rays than the Choapa River (P < 0.001), Rapel River (P = 0.001), Maule River (P < 0.001) and Valdivia River (P = 0.003). Rays in the caudal fin ranged from 15 to 23. In the ANCOVA analysis this character showed differences in means among sites ($F_{8,317}$ = 3.29; P = 0.001). Pairwise analyses showed that Huasco and Biobío had fewer caudal fin rays than Limarí (P = 0.003 and P = 0.001, respectively) and Valdivia (P = 0.003 and P = 0.001, respectively). Also, this analysis detected that fish from Limarí more rays than the sample from the Rapel River (P = 0.006). Overall, all meristical analysis performed for specimens obtained

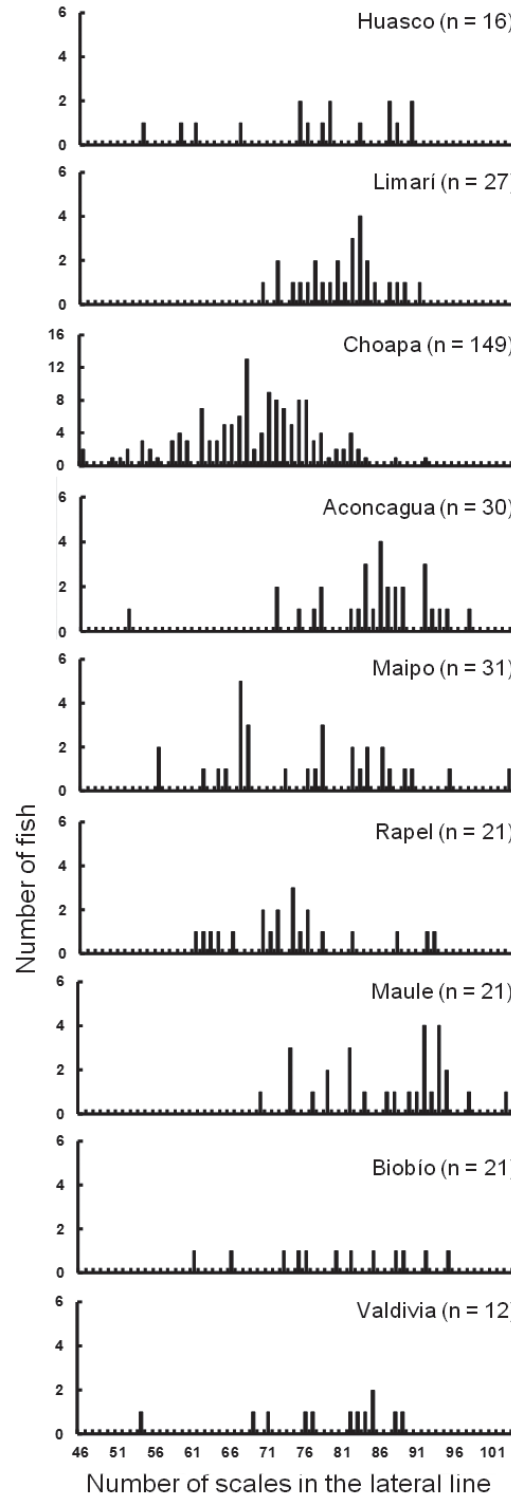

Fig. 3: Distribution of scale number by specimen in the lateral line. Each graph represents a sampled River.

Distribución del número de escamas de la línea lateral por individuo analizado. Cada gráfico representa un río analizado.

from different drainage basins showed no statistical differences in their means between *B. micrilepidotus* and *B. australis*, as most fish usually have a wide range in their meristics characters (e.g., Parenti 1984).

DISCUSSION

Historically the recognition and determination of freshwater fish species has been especially difficult; few morphological characters have been used to discriminate between species. This has been recognized as a problem in the Trichomycteridae (e.g., Arratia 1990), Cheirodontidae (e.g., Campos 1982) and Atherinidae (e.g., Dyer 1997, 2000a). Our results in the genus *Basilichthys* indicate clear genetic differences between the *semotilus* and *microlepidotus* groups proposed by Dyer (1997). Specifically, within the *microlepidotus* group the meristic and genetic information indicated the presence of a monophyletic group without apparent morphological differences.

Since the work of Gajardo (1985, 1988, 1992) and recent discussion by Dyer (1997), classical morphological description has been controversial for *B. microlepidotus* and *B. australis*. Although organisms from the extremes of their geographic distribution show a few morphological differences, individuals from the central zone of the country have very similar characteristics where they also have been described living in sympatry (Gajardo 1985, Dyer 2000b, 2006). A recent analysis indicated possible migration after the formation of four watersheds (Limarí, Choapa, Aconcagua and Maipo) in central Chile (Quezada-Romegialli et al. 2010), which also suggests that the two species previously described in this area may really be only one.

One of the most relevant characters described is the number of lateral line scales, whose range is from 74 to 96 (mean = 85.70) in *B. microlepidotus* and from 86 to 115 (mean = 99.46) in *B. australis* (Gajardo 1985). This author pointed out that most of the variance was within localities and he did not find significant differences between the species. By using samples from nine rivers, the present study found similar results to those obtained for two rivers by Gajardo (1985), indicating that the differentiation of *B. microlepidotus* and *B. australis* does not have meristical support.

Further analysis using the gene products of 37 allozyme loci did not show differences between the individuals of *B. microlepidotus* and *B. australis* (Gajardo 1988). Probably the only supporting evidence that both are valid species is a karyological analysis performed by Gajardo (1992). In this analysis, he found that *B. microlepidotus* from the Petorca River (32°20' S) had a modal number of 2n = 46 (NF = 62), while *B. australis* from the Angostura River (33°06' S) had a modal number of 2n = 48 (NF = 57) (Gajardo 1992). Although for some authors a difference in chromosome number is a good character to distinguish species, it may also be indicative only of chromosomal races. There are examples of chromosome races in various groups of Chilean organisms such as the catfish *Trichomycterus areolatus* (Valenciennes, 1840) which has 2n = 54, 55 and 56 in different rivers in the south of the country (Colihueque et al. 2006). These variations have also been reported in mice (e.g., Nachman & Searle 1995) and reptiles (e.g., Lamborot et al. 2003, Olmo 2005). In these terms, chromosome differences may not imply reproductive isolation in some vertebrata taxa.

All of the previously compiled information with respect to recognizing both species has not been conclusive. For this reason, the present study complements the meristic information usually utilized in the group with mtDNA sequences to present more evidence to help elucidate their taxonomy.

Mitochondrial DNA studies, especially those of the COI region, have proved to be a standardized method that also helps in species identification in cases where taxonomic diversity is undetected (Hebert et al. 2004, Pegg et al. 2006). mtDNA analysis in fish biodiversity has been increasing in the last years (Ward et al. 2005, Hubert et al. 2008, Lara et al. 2010), showing that this technique concurs with previous morphological analyses. Ward & Holmes (2007) showed in a study performed in 338 fish species that this region gave conclusive results except for one pair of species that may hybridize (Ward & Holmes 2007). Ward (2009) demonstrated, using sequences of COI of 1088 fish species, that the probability that two identical sequence samples belong to the same species is 98-99 %. Indeed, in this case the presence of a haplotype (H3) in most of the

studied rivers is evidence that these two species must be only one specific unit.

The variation of mitochondrial DNA in our analysis does not support the separation of *B. microlepidotus* and *B. australis* as different species. The statistical support in the tree analyses and the presence of shared haplotypes for both the Control Region and COI indicate that there is one species with a wide distribution. Based on this evidence and according to the zoological nomenclature code, the name *B. microlepidotus* (Jenyns, 1841) has priority and should be used in the future.

On the other hand, it is interesting that *B. semotilus*, with northern distribution, is genetically different from the organisms found in the central area of Chile, with an average difference of 19.5 base pair differences in the COI region and 29.4 bp differences in the Control Region compared to *B. microlepidotus*. *B. semotilus* is found in Peru from the Reque River, Lamberque (7° S) to the Sama River, Tacna (18° S), and in Chile it has been found in Codpa (19° S) and Loa River (22° S). Dyer's study (2000a) indicated that the large distance between the Sama River, Perú and the Loa River, Chile (about 1000 km) might indicate that the Chilean individuals belong to a different species and this is presently under study (Dyer 2009, personal communication).

The ichthyic fauna of Chile has a low richness (N = 44 species) compared to other South American regions (Arratia 1997), the principal characteristic being its high endemism (Arratia et al. 1981, Ruiz & Berra, 1994, Vila et al. 1999, Habit et al. 2006). Because of this high endemism, better knowledge of the real number of species, their geographic distributions and population sizes are presently necessary in order to develop effective conservation plans and a new conservation classification status. In the case of the silverside *B. microlepidotus*, individuals may currently be moving between watersheds using marine roots (Quezada-Romegialli et al. 2010), thus this species may be able to re-colonize watersheds naturally after ecological disasters. However, this process of natural re-colonization may be reduced by barriers to the fish dispersal. Due to the presence of dams which limit the natural requirements of reproduction, feeding and migration of fluvial species, restoration plans for populations will have to include planned and directed translocation of individuals. Other aspects such as habitat deterioration and the introduction of invasive species such as salmonids in the country's freshwater systems have also had an important negative impact on native fishes (Habit et al. 2005, Pardo et al. 2009). Overall, our research shows that conservation plans must be performed case by case. While *B. microlepidotus* could be translocated from different central Chilean drainages to allow the survival of the species under a perturbation event, *B. semotilus* has low alternatives of translocation and therefore requires a specific plan of protection.

The new molecular tools, together with morphological characters, will help to bring up to date taxonomic information of species to unify criteria between the investigators who work in conservation. These methods that are not invasive (if samples are obtained from fin pieces), complemented with morphological characters will be the key to identify adults, larval stages and eggs, as well as forensic work looking for habitat and its fauna conservation.

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