



## Phylogeography of the ancient catfish family Diplomystidae: Biogeographic, systematic, and conservation implications



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### ABSTRACT

The catfish family Diplomystidae is one of the earliest branching lineages within the diverse order Siluriformes and shows a deep phylogenetic split from all other extant and extinct major catfish groups. Despite its relevance in the evolution of siluriforms, phylogenetic relationships within the Diplomystidae are poorly understood, and prior to this study, no phylogenetic hypotheses using molecular data had been published. By conducting a phylogeographic study across the entire distribution of the family, that encompasses river systems from Central-South Chile and Argentina, we provide the first molecular phylogenetic hypothesis among all known species of Diplomystidae, and in addition, investigate how their evolutionary history relates to major historical events that took place in southern South America. Our phylogenetic analyses show four main lineages and nine sub-lineages strongly structured geographically. All Pacific basin populations, with one exception (those found in the Baker basin) clustered within three of the four main lineages (clades I–III), while all populations from Atlantic basins and those from the Baker basin clustered in a single main clade (clade IV). There was a tendency for genetic diversity to decrease from north to south for Pacific basins consistent with an increasing north-south ice coverage during the last glacial maximum. However, we did not find a statistically significant correlation between genetic diversity and latitude. Analysis of molecular variance (AMOVA) showed that river basins and the barrier created by the Andes Mountains explained a high percentage of the genetic variation. Interestingly, most of the genetic variation among drainages was explained among Pacific basins. Molecular phylogenetic analyses agree only partially with current systematics. The geographical distribution of main lineages did not match species distribution and suggests a new taxonomic hypothesis with support for four species of *Diplomystes*, three species distributed allopatrically from the Rapel to the Valdivia basin, and only one species distributed in Baker and Atlantic basins. High genetic differentiation among river basins suggests that conservation efforts should focus on protecting populations in each basin in order to preserve the genetic diversity of one of the oldest groups of catfishes on the earth today.

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### 1. Introduction

Recent phylogeographic studies in temperate zones of South America have highlighted the important role of two major historical events: the Andean Mountains uplift, separating biotas in two areas (west and east), and the Pleistocene glacial cycles, impacting mostly the southernmost portion of South America during the last 2.5 million years (e.g., Morando et al., 2007; Victoriano et al., 2008;

Ruzzante et al., 2006, 2008; Xu et al., 2009; Sérsic et al., 2011; Unmack et al., 2009, 2012; Vera-Escalona et al., 2012). During the last glacial maximum (LGM) (~18,000–23,000 years ago; Clapperton, 1993; Hulton et al., 2002), an extensive ice sheet covered areas from southern Chile and Argentina from  $-38^{\circ}\text{S}$  to  $-56^{\circ}\text{S}$  (Fig. 1). Ice free areas, hypothesized as refugia, were located in coastal zones in western Chile as well as in the eastern flank of the Andes and in the Argentinean Patagonian steppe (Muellner et al., 2005). Recent phylogeographic studies have shown patterns of postglacial dispersal in agreement with refugial hypothesis for plants (Allnutt et al., 1999; Premoli et al., 2000; Pastorino and Galló, 2002; Muellner et al., 2005; Nuñez-Avila and Armesto, 2006), lizards (Morando

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**Fig. 1.** Map showing the sampling locations for *Diplomystes*. The light grey area corresponds to the extension of continental shelf exposed during low sea levels. The dark grey area is the North Ice Field, a remnant of the last glacial sheet. The dotted line corresponds to the political boundary between Chile and Argentina, which goes along the highest peaks of the Andes. The segmented line shows the extension of the ice sheet at the LGM (Clapperton, 1993). Areas of endemism are based on Dyer (2000).

et al., 2007; Victoriano et al., 2008), amphibians (Nuñez et al., 2011), mammals (Smith et al., 2001; Palma et al., 2005; Lessa et al., 2010; Himes et al., 2008), and as reviewed by Sérsic et al. (2011). Alternatively, other studies have suggested cryptic refugia within the ice sheet (Ashworth et al., 1991; Xu et al., 2009; Unmack et al., 2012; Vera-Escalona et al., 2012) challenging the traditional view of a massive and continuous ice sheet.

Both Andean orogenesis and glacial cycles have strongly influenced the hydrological landscape of southern South America. The major impact of the Andes uplift was to split hydrological drainages into Pacific and Atlantic basins, acting as a potential vicariant event for many aquatic species. Accordingly, phylogeographical patterns of freshwater fishes have shown significant levels of genetic structure between both sides of the Andes (Ruzzante et al., 2006, 2008; Zemlak et al., 2008, 2010; Unmack et al., 2009).

However, these studies have also suggested a more complex history in Patagonia, with drainage rearrangements and temporary connections between Atlantic basins and between Atlantic and Pacific basins. Genetic patterns in two fish species, the catfish *Trichomycterus areolatus* (Unmack et al., 2009, 2012) and the perch *Percichthys trucha* (Ruzzante et al., 2006), showed high levels of phylogeographic structure between Pacific drainages, but lower phylogeographic structure and divergence among populations from Atlantic drainages. The same was observed for *Galaxias platei* (Zemlak et al., 2008) and *G. maculatus* (Zemlak et al., 2010), but in addition, higher levels of genetic flow between southernmost Atlantic and Pacific drainages suggested dispersal across the southern Andes. These studies have postulated that interconnections between basins could have been possible during the Pleistocene due to temporary formation of extensive paleolakes in the eastern flank

of the Andes and drainage reversal between Atlantic and Pacific basins due to melting of large ice blocks that acted as barriers between them (connections between Atlantic and Pacific basins).

The catfish family Diplomystidae (Teleostei, Siluriformes), distributed in several Pacific and Atlantic basins from southern South America, offers a good opportunity to test these phylogeographic hypotheses. Diplomystidae is considered by most ichthyologists to be one of the first lineages to diverge from the ancestor of all living Siluriformes (Lundberg and Baskin, 1969; Lundberg and Case, 1970; Arratia, 1987; Grande, 1987; Fink and Fink, 1996; de Pinna, 1998; Sullivan et al., 2006). The family is endemic to southern South America and is distributed on both sides of the Andes in rivers and lakes in Chile and Argentina between 34°S and 47°S (Fig. 1). Six species are currently recognized (Arratia, 1987; Azpelicueta, 1994). Whether one (*Diplomystes*) or two genera (*Olivaichthys*) are recognized has been controversial as researchers have differing opinions (Eschmeyer and Fong, 2013), here we place all species in *Diplomystes*. Three of the six recognized species are endemic to Chile (Pacific drainages): *D. chilensis*, *D. nahuelbutaensis*, and *D. camposensis*, while the other three (which are placed in the genus *Olivaichthys* by some authors) are endemic to Argentina (Atlantic drainages): *D. viedmensis*, *D. cuyanus*, and *D. mesembrinus* (Table 1; Fig. 1). Morphologically, diplomystids show little obvious external differences among species, and the most important characters for separating the species are osteological (Arratia, 1987; Azpelicueta, 1994). For this reason, researchers typically use geographic criteria to identify species. Diplomystids are carnivorous, feeding mainly on small crustacean decapods and adults and larvae of aquatic insects (Arratia, 1983; Beltrán-Concha et al., 2012). They use habitats with fast flowing, well-oxygenated water. Adults occupy deeper midstream zones, while juveniles prefer stream margins with shallower depth and slow flowing water (Arratia, 1983). They are rarely collected in small rivers, being more commonly found in

higher order rivers. Reproductively, diplomystids have low fecundity, which along with high habitat specificity contributes to their naturally small-sized populations (Arratia, 1983; Vila et al., 1996; Habit, 2005; Habit et al., 2009). This is accentuated in populations from Pacific basins, because these basins are noticeably smaller than Atlantic basins. Small population sizes make diplomystids especially vulnerable in terms of conservation. Species distributed in Chile are considered endangered (Campos et al., 1998), while species from Argentina are considered rare (Bello and Ubeda, 1998).

Diplomystids from Pacific drainages occur across three biogeographic sub-provinces (or areas of endemism following Dyer (2000)) for fishes within the Chilean Province: *Diplomystes chilensis* in the Central Area, *D. nahuelbutaensis* in the South-Central Area and *D. camposensis* in the Southern Area (Fig. 1). Boundaries among these areas have not been defined precisely because of the lack of more comprehensive fish surveys in rivers in between these areas (Dyer, 2000; Habit et al., 2006). Recently, new populations of unidentified diplomystids have been recorded in some Pacific basins, including populations from basins that are between the areas of endemism (Mataquito and Toltén basins; Dyer, 2000), as well as populations from the Baker basin in the Chilean Patagonia (Muñoz-Ramírez et al., 2010) and the Manso basin (Puelo Lake, Argentina; Baigún and Ferriz, 2003).

Using DNA sequences, we reconstructed the phylogeny for all known diplomystid species and performed several analyses to investigate their phylogenetic relationships and how their evolutionary history relates to major historical events that took place in southern South America. First, we tested for the impact of glaciations on the genetic diversity of populations in glaciated and unglaciated basins. Due to the increase in glacial coverage from north to south in Pacific drainages, which would have reduced habitat availability and then population sizes (Fig. 1), we predict a

**Table 1**  
Sampling locations and individual codes for *Diplomystes* analyzed in this study.

Site no.	Locality	Basin	Species	Latitude	Longitude	N° of sequences	Repository	Vouchers
1	Las Cabras	Rapel	<i>D. cf. chilensis</i>	−34.2759	−71.3633	1	1	DTi93
2	Puente Tinguiririca	Rapel	<i>D. cf. chilensis</i>	−34.6158	−70.9870	6	1	DTi86, 88–92
3	Puente Negro	Rapel	<i>D. cf. chilensis</i>	−34.6774	−70.8776	6	1	DTi129–134
4	Los Queñes	Mataquito	Unknown	−34.9943	−70.8538	12	1	DTe177, 184–194
5	Puente Yacal abajo	Mataquito	Unknown	−35.1722	−71.1377	11	1	DLon166–176
6	Pte Claro	Maule	<i>D. nahuelbutaensis</i>	−35.7062	−71.0779	8	1	DMau144–151
7	El Pejerrey	Maule	<i>D. nahuelbutaensis</i>	−36.0654	−71.3986	11	1	DAch217, 218, 230–238
8	Vado Azul	Maule	<i>D. nahuelbutaensis</i>	−36.1644	−71.3378	2	1	DAch219–220
9	Estación 4	Itata	<i>D. nahuelbutaensis</i>	−36.6422	−72.4478	5	1	DIt120–123, 249
10	IT-1	Itata	<i>D. nahuelbutaensis</i>	−36.6685	−72.4411	3	1	DIt124–126
11	Huepil	Itata	<i>D. nahuelbutaensis</i>	−37.2339	−71.9211	1	1	DHue195
12	Puente Llacolen	Biobío	<i>D. nahuelbutaensis</i>	−36.8295	−73.0701	5	1	DBII113–117
13	El Pangal	Biobío	<i>D. nahuelbutaensis</i>	−37.1895	−72.1576	10	1	DLa198–206; DTu05
14	Puente Laja	Biobío	<i>D. nahuelbutaensis</i>	−37.2676	−72.7154	4	1	DTu01–04
15	Rucue	Biobío	<i>D. nahuelbutaensis</i>	−37.3451	−71.8296	9	1	DTu06–08, 20, 22, 24–27
16	Negrete	Biobío	<i>D. nahuelbutaensis</i>	−37.5778	−72.5445	11	1	DNe101, 102, 104–107; DBb94–97, 99
17	Puente Villacura	Biobío	<i>D. nahuelbutaensis</i>	−37.5851	−72.1490	5	1	DDu110, 112, 119; DBio127, 128
18	Puente Villa Cautín	Imperial	<i>D. nahuelbutaensis</i>	−38.4164	−72.0698	3	1	DCau152–154
19	Puente Cautín	Imperial	<i>D. nahuelbutaensis</i>	−38.4667	−71.9337	11	1	DCau155–165
20	Puente Pitrunco N°2	Toltén	Unknown	−39.0495	−72.0906	9	1	DCol221–229
21	Balsa Coipue	Toltén	Unknown	−39.0839	−72.4437	9	1	DTol01–06, 08; CoiDip01; DcT04
22	Puente Copihuelpe	Valdivia	<i>D. camposensis</i>	−39.3938	−72.3635	9	1	DCru240–248
23	Calle Calle 2	Valdivia	<i>D. camposensis</i>	−39.7878	−72.7111	8	1	DCZ416–419, 421–424
24	Camping	Valdivia	<i>D. camposensis</i>	−39.8532	−72.7524	5	1	DMa43, 54, 60, 82; DCZ415
25	Balsa Enco	Valdivia	<i>D. camposensis</i>	−39.9120	−72.1603	8	1	DEn209–216
26	Balsa Colonia	Baker	Unknown	−47.2033	−72.6321	1	1	DBk143
27	Balsa Baker	Baker	Unknown	−47.3168	−72.8630	7	1	DBk136–142
28	Reserva Ullum	Colorado	<i>D. cuyanus</i>	−31.5068	−68.8395	10	2	MLP9849–57 (DUllm01–10)
29	Bocatoma	Negro	<i>D. viedmensis</i>	−39.8953	−65.0475	1	3	DipBoca.1
30	Los Altares	Chubut	<i>D. mesembrinus</i>	−43.8349	−67.7709	10	2	MLP9840–47 (DAIt01–10)

Repository legend: 1. Laboratory of Ecology and Conservation of Fish, EULA Center, Universidad de Concepcion. 2. Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata. 3. J.B. Johnson laboratory fish collection, Brigham Young University, Provo, UT 84602, USA.

**Table 2**Results of the population expansion test Fu's  $F_s$  (Fu, 1997) for each of the nine clades in the mitochondrial Bayesian tree.

Fu's $F_s$ test	I.a	I.b	I.c	II	III.a	III.b	III.c	IV.a	IV.b
No. of individuals	36	24	7	43	37	14	14	10	19
Theta_pi	2.71	1.93	13.62	2.07	1.47	2.73	2.15	2.67	4.35
Exp. no. of alleles	7.70	5.51	5.82	6.90	5.34	5.39	4.81	4.58	7.73
$F_s$	-26.48	-27.15	-1.04	-27.04	-27.80	-15.41	-17.51	-8.93	-19.39
$F_s$ p-value	0.00	0.00	0.17	0.00	0.00	0.00	0.00	0.00	0.00

negative correlation between latitude and genetic diversity, along with signals of recent population expansion at higher latitudes. Second, we tested the hypotheses of more recent connectivity between Atlantic drainages and between Atlantic and southernmost Pacific drainages. If interconnections have occurred recently (and recurrently) between Atlantic basins during Pleistocene glacial cycles, our prediction is that species from those drainages should show shallower relationships than species from Pacific drainages, which remained isolated longer. Likewise, if the Baker basin, that currently drains into the Pacific Ocean, has been historically connected to the Atlantic basins through recurrent drainage reversals, we predict that populations from the Baker basin should be more closely related to Atlantic populations than to Pacific populations. Finally, we also test how well areas of endemism in Pacific drainages match the phylogenetic relationships within *Diplomystes*.

## 2. Material and methods

### 2.1. Sampling

For this study we used a total of 201 individuals collected between 1998 and 2009, covering the known geographic range of the group. This included 30 localities from 12 river basins, nine from Chile (Pacific basins) and three from Argentina (Atlantic basins) (Fig. 1; Table 1). We found no individuals from either the Aconcagua or the Maipo basins, which is consistent with their hypothesized extinction in these areas (Arratia, 1987; Muñoz-Ramírez et al., 2010). In addition, no samples were obtained from Puelo Lake (Manso basin) as they are only very rarely captured there (V. Cussac pers. obs.). Specimens were fixed and preserved in 95% ethanol and deposited in the collection of institutions as indicated in Table 1.

When possible, specimens were previously assigned to a species name based on their geographic distribution and external diagnostic characters described in Arratia (1987), such as the pre-orbital length and the pre-pelvic length both relative to the standard length. Individuals from the Mataquito, Tolten, and Baker basins were left without assignment because these populations are relatively new findings with geographic distributions that fall outside the limits of other known species distributions (Arratia, 1987; Muñoz-Ramírez et al., 2010) (Fig. 1 and Table 1). Thus, the relationship of these populations to the known species and their identity can be hypothesized and discussed at the light of our results.

### 2.2. Laboratory protocols

We extracted genomic DNA from muscle tissue from each specimen using the DNeasy Tissue Kit (QIAGEN Inc., Chatsworth CA). All individuals were amplified for the mitochondrial cytochrome *b* (*cytb*) gene and the control region (CR). A subset of fifteen individuals representing both the geographic and genetic diversity (based on *cytb*/CR results), were sequenced for additional mitochondrial and nuclear genes in order to perform additional phylogenetic analyses, including a species tree coalescent analysis (Heled and Drummond, 2010). This subset included a single

individual from each river system sampled, except Itata and Tolten basins, for which three and two individuals were included, respectively, in order to capture broader mitochondrial genetic diversity we found there. The additional mitochondrial genes included were NAD4L, ND4, ND5, and ND6. The amplified nuclear genes consisted of S7 (intron one) and GH (growth hormone, exons three through five and introns three and four). For *cytb* we additionally used two internal primers in combination with the external primers in the few cases where the flanking primers failed to amplify. Details of all primers and polymerase chain reaction (PCR) combinations are in Supplementary Figure S1 and Supplementary Table S1. *Cytb* and CR were amplified with final concentrations for PCR components per 25  $\mu$ L reaction as follows: 25 ng template DNA, 0.25  $\mu$ M of each primer, 0.625 units of GoTaq DNA polymerase (Promega, Madison, WI, USA), 0.1 mM of each dNTP, 2.5  $\mu$ L of 10 $\times$  reaction buffer and 2.5 mM MgCl<sub>2</sub>. Amplification parameters were as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 90 s, and 72 °C for 7 min. Nuclear genes were obtained by nested pcr using primers flanking the region of interest (shown in bold in Supplementary Table S1), followed by internal primers. These were conducted as 10  $\mu$ L reactions with 25 ng template DNA, and two fifths of the quantities of the other ingredients, but using Ex taq (Takara, Madison, WI, USA). Products from the first PCR reaction were then diluted to 1:49, and 1  $\mu$ L of this product was added to the second 25  $\mu$ L reaction. We examined PCR products on a 1% agarose gel using SYBR safe DNA gel stain (Invitrogen, Eugene, OR, USA). We purified PCR products using a Montage PCR 96 plate (Millipore, Billerica, MA, USA). Sequences were also obtained via cycle sequencing with Big Dye 3.0 dye terminator ready reaction kits using 1/16th reaction size (Applied Biosystems, Foster City, CA). Sequencing reactions were run with an annealing temperature of 52 °C following the ABI manufacturer's protocol. We purified sequenced products using sephadex columns. Sequences were obtained using an Applied Biosystems 3730 XL automated sequencer at the Brigham Young University DNA Sequencing Center. All sequences obtained in this study were deposited in GenBank, Accession numbers (JX648663–JX649064; KJ174950–KJ175007).

### 2.3. Analysis of DNA sequences

Chromatograms were edited in CodonCode Aligner 3.0.3 (Dedham, MA, USA). Sequences were imported to BioEdit 7.0.5.2 (Hall, 1999) and aligned by eye. *Cytb* sequences were checked via amino acid coding in MEGA5 (Tamura et al., 2011) to test for unexpected frame shift errors or stop codons. We performed the Xia test (Xia et al., 2003) to evaluate the degree of sequence saturation. Additionally, we conducted the McDonald & Kreitman test of neutrality (McDonald and Kreitman, 1991) in DnaSP 5.1 (Librado and Rozas, 2009) for protein coding sequences to evaluate possible deviations from neutrality that may bias our analyses. To characterize genetic divergence, average between-species and between-populations genetic distances were calculated based on the proportion of shared differences (*p*-distance) using MEGA5.



## 2.4. Phylogenetic analysis

We conducted separate phylogenetic analyses for the mitochondrial data set and the nuclear data set to check whether they produced similar phylogenetic topologies. Additionally, we conducted a species tree analysis using an extended mitochondrial data set (ND4L, ND4, ND5, ND6, and *Cytb* genes) plus fragments of two nuclear markers (GH and S7 regions) for a subset of 15 individuals selected to represent all main lineages found in the mitochondrial analyses and all hydrological basins. The species tree coalescent analysis was preferred over concatenation given that this analysis accounts for differences between gene histories due to coalescent stochasticity and it does not require outgroups for rooting the tree. The last property is particularly useful in our case since Diplomystidae presents deep phylogenetic relationships with any other group of catfishes, making phylogenetic reconstruction more challenging due to the large differences in branch lengths of any potential outgroups relative to the ingroup (Felsenstein, 1978; Philippe and Forterre, 1999; Graham et al., 2002).

Phylogenetic relationships of haplotypes from the combined *cytb* and CR sequences were reconstructed by Bayesian inference using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). We included each mitochondrial region (*cytb* and CR) as separate partitions in the Bayesian analysis. We identified the best-fitting model of molecular evolution using jModeltest 0.1.1 (Posada, 2008). Under the Bayesian Information criterion the model for *cytb* and CR was HKY + I, and GTR + I + G respectively. We conducted two independent runs to check for convergence in the estimated parameters. Four chains were used for phylogeny estimation, starting with a random tree and running for 5,000,000 generations, sampling every 1000 trees. The initial 10% of the resulting trees was discarded as burn-in using the 'sumt' command. Once convergence of the two independent runs was confirmed, parameter estimates from both runs were combined to obtain a total of 9002 trees. A 50% majority-rule consensus tree with branch lengths was reported. In addition, we also constructed a haplotype network for the mitochondrial sequence data using the median joining algorithm implemented in Network 4.610 (Bandelt et al., 1999). A haplotype network is useful for showing the mutation process in shallow phylogenetic relationships within closely related taxa.

Phylogenetic reconstruction of the nuclear genes was performed similarly to the complete mitochondrial data set, with both genes combined, but separated into two partitions in order to allow an appropriate substitution model for each gene. We ran a Bayesian analysis with the models F81 + I for the GH gene and F81 + G for the S7 gene. Due to the difficulty in obtaining both nuclear markers from the same single individual from the Baker basin, we combined sequences from two different individuals that shared the same mitochondrial haplotype (DBk142 [GH] and DBk141 [S7]).

To estimate the root of Diplomystidae, we conducted a separate phylogenetic analysis based on *cytb* only, including the same subset of diplomystids considered previously for the nuclear gene tree and the species tree, and using several outgroups obtained from GeneBank that included six catfish species (accession numbers: NC\_008280, NC\_008232, NC\_004698, NC\_004697, NC\_006381, AP012026) and two non-catfish species (accession numbers: NC\_015840 and NC\_015754). We performed a Bayesian analysis with the same settings described previously for the combined mitochondrial dataset, using the corresponding model for *cytb* (see above).

The species tree analysis was conducted in BEAST (Heled and Drummond, 2010) using the three independent loci (four mitochondrial genes combined and the two nuclear loci GH and S7). Individual assignments into species were done by following our re-

sults from the mitochondrial phylogenetic analysis. The analysis was run specifying the HKY + G model of sequence evolution with four categories. A constant population size coalescent prior on gene trees, and a Yule prior on species trees were used. MCMC chains were run for 50 million generations, sampling every 5000 to produce 10,000 sampling values. Parameter estimates were checked for convergence in Tracer v1.4 (Rambaut and Drummond, 2007), discarding the first 10% of the samplings as burn-in. The species tree was finally obtained by summarizing the sampled trees in the TreeAnnotator application, distributed with the BEAST package.

## 2.5. Demography, genetic diversity and genetic structure

In order to test for population expansion, we calculated Fu's  $F_s$  test of neutrality (Fu, 1997) in Arlequin 3.1 (Excoffier et al., 2005). This test detects excesses of low frequency haplotypes that may be due to past bottlenecks and population size change or selection. Fu (1997) found that  $F_s$  value is sensitive to demographic expansion, which usually leads to large significant negative values under a neutral assumption. To test the hypothesis of a glacial impact on *Diplomystes* from Pacific drainages, we conducted correlation analyses between latitude and genetic diversity, calculated for both populations and basins. Due to an increase in glacial coverage on basins from north to south, a negative and significant correlation is expected under the hypothesis of glacial impact. DnaSP was used to calculate, haplotype richness (S), haplotype diversity (H), and nucleotide diversity ( $\pi$ ). To test for correlations between genetic diversity and latitude we used Spearman's rank correlation tests for H and  $\pi$ . For correlations at the population level, we only included those populations with five or more individuals to avoid possible biases. For correlations at the basin level, we used all individuals from each basin and the latitude at the basin mouth as the latitude for each basin.

To calculate genetic structure between basins,  $F_{st}$  values were calculated in Arlequin (Negro basin, with only one individual, was excluded from this analysis). Analyses of molecular variance (AMOVA; Excoffier et al., 2005) were also conducted in Arlequin to test for historical patterns of isolation. Predictions of historical isolation among basins or group of basins should result in high levels of genetic differentiation among these groups. We used this analysis to examine levels of genetic structure between lineages separated by the Andean Range and among drainages. According to the hypothesis of a strong role of the Andes as a geographical barrier, high levels of genetic differentiation are expected between Pacific and Atlantic basins. In addition, we also test for the hypothesis of areas of endemism in the Chilean Province (Dyer, 2000). To do this, we compared genetic variation explained among areas of endemism vs. genetic variation explained among drainages. Assuming that areas of endemism are the result of major historical events that separated geographic regions over long time periods, we expect high levels of genetic differentiation to be explained by those areas. AMOVA groups were constructed as follow (numbers in parenthesis indicate populations grouped): comparison 1, (west of Andes vs. east of Andes), (1–27)(28–30); comparison 2, Chile vs. Argentina + Baker, (1–25)(26–30); comparison 3, each individual river basin, (1–3)(4–5)(6–8)(9–11)(12–17)(18–19)(20–21)(22–25)(26–27)(28)(29)(30); comparison 4, areas of endemism, (1–3)(6–19)(22–25); comparison 5, Pacific drainages (excluding Baker basin), (1–3)(4–5)(6–8)(9–11)(12–17)(18–19)(20–21)(22–25).

## 3. Results

A total of 87 haplotypes from 201 individuals were obtained from the combined mitochondrial data set (2073 bp; *cytb* and CR,

1138 bp and 935 bp, respectively). Contrary to our expectations, divergence among species was low. The largest average number of pairwise differences between any pair of populations was 58.2 nucleotide differences ( $p$ -distance = 0.028) and it was between the localities of Puente Negro (Rapel basin, west of the Andes) and the localities of either San Juan (Colorado basin) or Bocatoma (Negro basin) both east of the Andes. Divergence among Pacific lineages vs. among Atlantic lineages was markedly different, being more than four times higher among Pacific ones. While average number of pairwise differences among Pacific drainages was 32 ( $p$ -distance = 0.015), it was only 7.2 ( $p$ -distance = 0.0035) among Atlantic drainages.

### 3.1. Phylogenetic analysis

Phylogenetic relationships among the 87 mitochondrial DNA haplotypes show four well supported major clades with strong geographic structuring (Fig. 2A; clades I–IV). Clades I–III group all haplotypes distributed in Pacific drainages, except those from the Baker basin which clustered in clade IV, associated entirely with populations from Atlantic drainages (populations 26–30; Figs. 1 and 3). Clade I was associated with the northern distribution of Chilean *Diplomystes*, from the Rapel basin to the Itata basin. This clade splits into three sub-clades well supported (I.a–c). Sub-clade I.a was associated with Rapel and Mataquito basins, the two northernmost river systems in the distribution of diplomystids from the west of the Andes. Sub-clade I.b was mainly associated with Maule basin, although haplotypes 1 and 36 were also present in the next basin to the south, Itata. Clade I.c was mainly associated with the Itata basin, with only one haplotype (35) shared with the Biobío basin.

Clade II was strongly associated with the Biobío basin with only one haplotype (33) from a single individual coming from Itata basin. Clade III was associated entirely with southern Pacific drainages distributed south of Biobío, from the Imperial to Valdivia basins, and divided into three well supported sub-clades (III.a–c). Sub-clade III.a was mainly associated with Imperial basin with one haplotype (18) shared with the Toltén basin. Sub-clade III.b was mainly associated with the Toltén basin with only one haplotype (22), coming from the Imperial basin. Sub-clade III.c, the sister clade of clades III.a and III.b, was associated to the Valdivia basin with one haplotype (71) found only in the Toltén basin. Additionally, the two most frequent haplotypes (29 and 32) in the Valdivia basin were also found in the Toltén.

Clade IV, with two sub-clades, was geographically the most widely distributed. A well supported sub-clade IV.a groups all haplotypes from Colorado basin, while a less supported sub-clade IV.b contained all haplotypes from the Negro, Chubut and Baker basins.

Phylogenetic analyses for the nuclear genes were broadly congruent with mtDNA results, especially at deeper nodes (Fig. 2B). The nuclear gene tree showed three major clades with clade (I + II) representing clades I and II from the mitochondrial gene tree, while clades III and IV were concordant with the mitochondrial gene tree. Species tree coalescent analysis was consistent with the general topology of the mitochondrial gene tree and the tree with the two nuclear genes concatenated (Fig. 2C). This reconstruction showed *D. viedmensis* as the sister species of all three Chilean species. Among the three Chilean species, *D. camposensis* was placed as the sister species of both *D. chilensis* and *D. nahuelbutaensis*. This analysis was also consistent with the separate analysis conducted to investigate the root of Diplomystidae (Supplementary Fig. S2).

The haplotype network revealed the same four major haplogroups that were recovered from the Bayesian phylogenetic reconstruction (Fig. 3). Similarly, each haplogroup consists of one to three sub-haplogroups strongly structured geographically. West

of the Andes, there was mix of different patterns in haplogroup shapes and relative haplotype frequency. Haplogroups from Biobío and Maule basins showed a clear star-like topology, each with one high-frequency haplotype in a central position, and several low frequency haplotypes derived from it. Haplogroup from Valdivia and Toltén basin also resembled a star-like topology, but the one from Valdivia showed two (instead of one) high-frequency haplotypes, whereas the one from Toltén showed one high-frequency haplotype, linked to some more distant singleton haplotypes. Haplogroups from Rapel, Mataquito, Itata, and Imperial basins showed a less obvious star-like topology, with a tendency for more uniform haplotype frequencies. East of the Andes, on the other hand, haplotypes clustered in a single haplogroup with uniform haplotype frequencies, which also included all the haplotypes from the Baker basin.

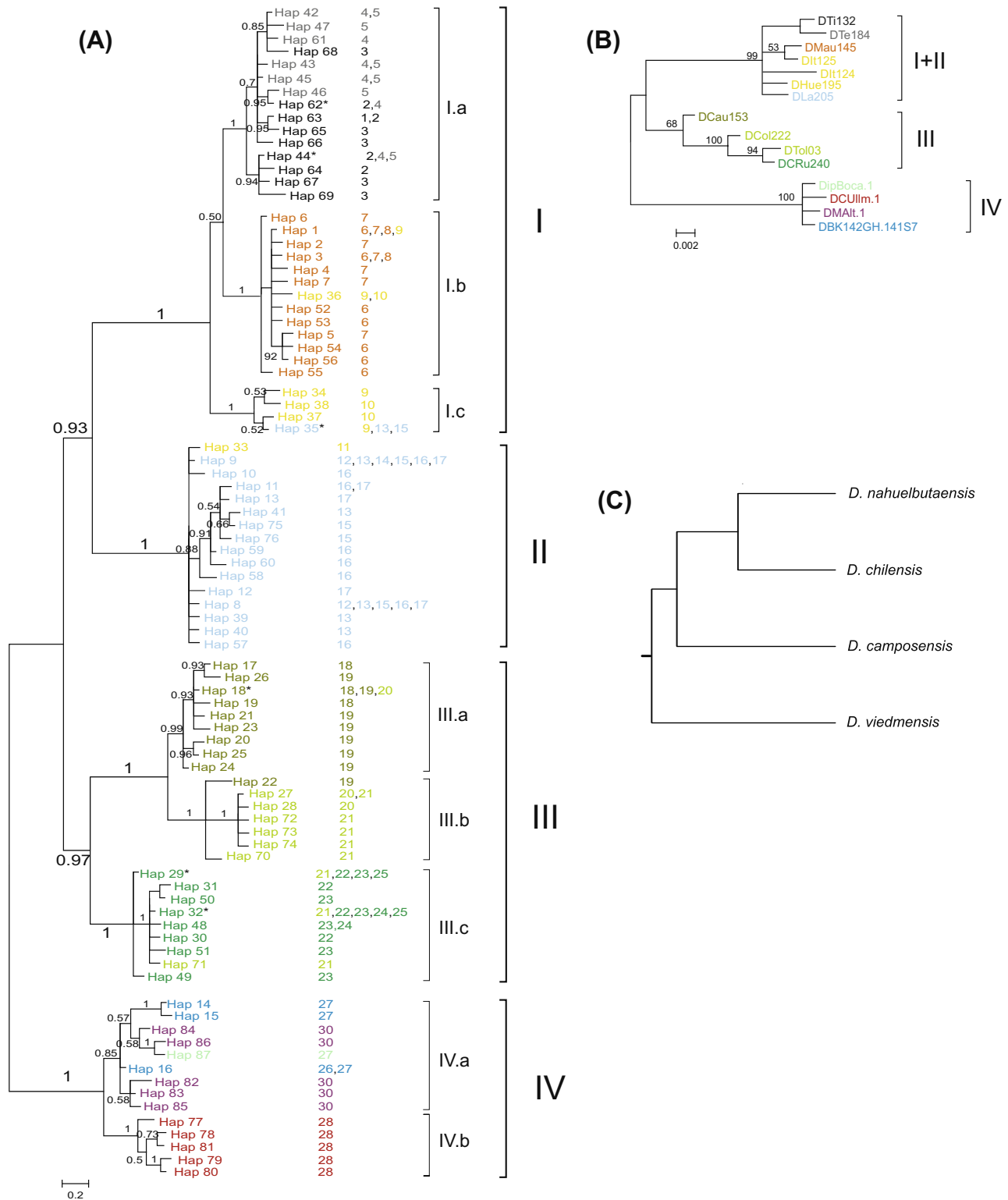
### 3.2. Demography, genetic diversity and genetic structure

To test for population expansion we calculated Fu's  $F_s$  statistic for each of the nine sub-clades/lineages in the mitochondrial gene tree (clades I.a–c, II, III.a–c and IV.a–b), which were concordant with haplogroups in the haplotype network and strongly associated with drainages.  $F_s$  values were negative and significant in all but one lineage (sub-clade I.c) (Table 2). Sub-clade I.c, represented by the lowest sample size (seven individuals), was the only lineage with no significant  $F_s$  value ( $F_s = -1.04$ ,  $p = 0.17$ ). Results of this test were consistent with the star-like shape observed in the haplotype network, which is often indicative of population expansion.

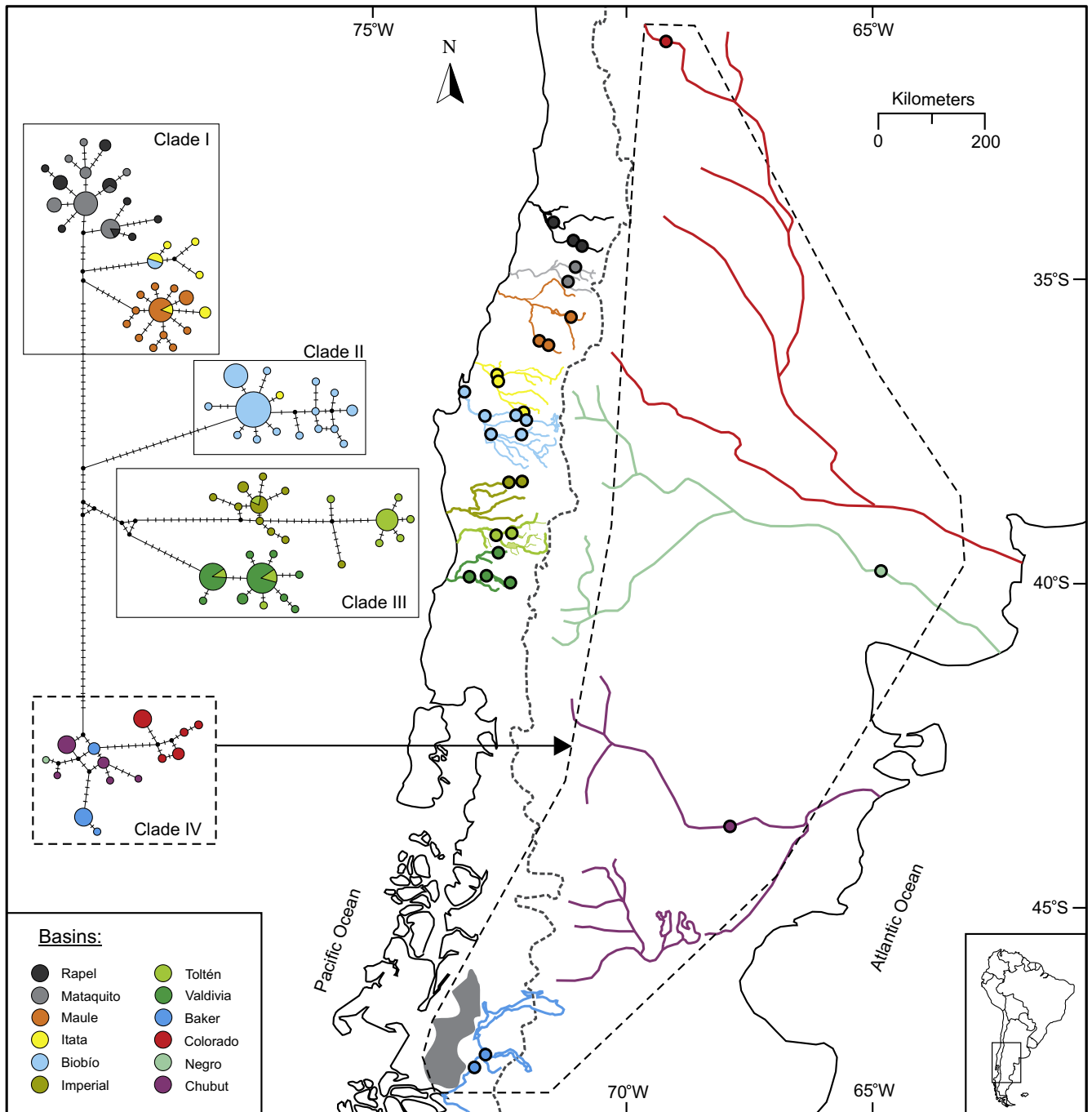
Genetic diversity, measured as haplotype diversity ( $H$ ), was relatively high in northern populations with a tendency to decrease towards southern populations (Tables 3 and 4). The highest haplotype diversity were found in the northern populations Puente Negro ( $H = 0.93$ ), Puente Claro ( $H = 0.96$ ), Balsa Coipue ( $H = 0.97$ ) and Puente Cautín ( $H = 0.93$ ), while the lowest were found at the southern populations of Puente Pitrunco ( $H = 0.42$ ), Balsa Enco ( $H = 0.54$ ) and Balsa Baker ( $H = 0.52$ ) (Fig. 1). However, correlations between haplotype diversity and latitude were only marginally insignificant both for the population and basin level analysis (basin level:  $R = -0.63$ ,  $p = 0.067$ ; basin level:  $R = -0.43$ ,  $p = 0.059$ ; Fig. 4). Nucleotide diversity was generally low in almost all populations often being below 0.003 (Table 3). Exceptions of higher values of nucleotide diversity were found in some populations from the Itata (populations 9 and 10), Laja (population 13) and Toltén basins (population 21), which contain haplotypes from different mitochondrial lineages (Figs. 2A and 3), and whose values ranged between 0.005 and 0.01.

In terms of genetic structure, we found strong genetic differentiation among populations across the entire geographical range ( $F_{st} = 0.851$ ,  $p = 0.00$ ). The highest variation was among populations (85.1%), while only a small portion was observed within populations (14.9%). Between basins, population structure was generally high in all comparisons (Table 5).  $F_{st}$  values ranged from 0.40 to 0.97 except between the Rapel and Mataquito basins where it was quite low, but still significant ( $F_{st} = 0.06$ ,  $p = 0.04$ ). Genetic differentiation between west and east of the Andes was high, and it explained approximately 78% of the total molecular variance. When populations from the Baker basin were incorporated to the group "east of the Andes" according to an eastern origin for Baker basin populations (drainage reversal hypothesis), the explained variance increased to 82.3%. The comparison among all basins and the comparison among just the basins from west of the Andes both explained 96.6% of variation, indicating that most variation was due to differentiation among drainages from the west of the Andes.

Relative to the hypothesis of areas of endemism in the Chilean Province (Dyer, 2000), AMOVA results show that areas of ende-



**Fig. 2.** (A) Bayesian phylogenetic tree based on the combined mitochondrial genes cytochrome *b* and control region. (B) Bayesian phylogenetic reconstruction based on the combined nuclear genes *S7* and growth hormone. (C) Species tree analysis based on the multispecies coalescent model using the three independent DNA regions (mitochondrial DNA combined, and the nuclear genes *GH*, and *S7*). Numbers on nodes in A and B are Bayesian posterior probabilities, and numbers in front of terminal branches are sampling locations. Colors in labels from A and B represent basin of origin and match those in Fig. 3. Asterisks next to terminal labels in A indicate those haplotypes that are present in two different basins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** Median joining network of the 201 individuals of *Diplomystes* based on the combined mitochondrial DNA genes *cytb* and *CR*. Haplotype sizes are proportional to their frequencies. Colors match with the basin where the haplotype is present. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mism explained 45.5%, less than half the genetic variation explained among basins within those areas (93.0%; Table 6). Therefore, even though variation explained by areas of endemism was high, it can be explained by variation among basins between these areas of endemism.

## 4. Discussion

### 4.1. Genetic diversity and glaciations

During the LGM, an extensive glacial sheet covered at least some proportion of the headwaters of the drainages west of the

Andes, from the Imperial basin (38°S) south to the Maullin basin, with drainages south of that completely covered (Clapperton, 1993). Therefore, availability of aquatic habitat in the southern drainages is likely to have been reduced due to glaciation. Aquatic populations likely underwent strong reductions due to habitat loss, surviving in refugia at the west portion of drainages. Our results do not show a clear pattern of increased signal of population expansion and genetic reduction from north to south, except for haplotype diversity that shows a trend for southern populations to be less diverse than northern ones, although marginally not significant. More strikingly, nucleotide diversity tended to be generally low, with evidence for population expansion being present in most basins. Habitat loss in southern basins could explain patterns of



**Table 3**

Genetic diversity values for the 30 populations of *Diplomystes* based on mitochondrial DNA. (N) Number of sequences, (S) number of haplotypes, (H) haplotype diversity, and ( $\pi$ ) nucleotide diversity.

Site no.	Locality	Basin	N	S	H	$\pi$
1	Las Cabras	Rapel	1	1	–	–
2	Puente Tinguiririca	Rapel	6	4	0.87	0.00139
3	Puente Negro	Rapel	6	5	0.93	0.00294
4	Los Queñes	Mataquito	12	6	0.82	0.00077
5	Puente Yacal abajo	Mataquito	11	6	0.84	0.00104
6	Pte. Claro	Maule	8	7	0.96	0.00096
7	El Pejerrey	Maule	11	7	0.82	0.00081
8	Vado Azul	Maule	2	2	1	0.00049
9	Estación 4	Itata	5	4	0.90	0.00545
10	IT-1	Itata	3	3	1	0.00693
11	Huepil	Itata	1	1	–	–
12	Puente Llacolen	Biobío	5	2	0.60	0.00030
13	El Pangal	Biobío	10	6	0.78	0.00485
12	Pte. Laja	Biobío	4	1	0	0
15	Rucue	Biobío	9	5	0.72	0.00542
16	Negrete	Biobío	11	8	0.89	0.00153
17	Pte. Villacura	Biobío	5	4	0.90	0.00158
18	Puente Villa Cautín	Imperial	3	3	1	0.00099
19	Puente Cautín	Imperial	11	8	0.93	0.00220
20	Puente Pitrunco N°2	Toltén	9	3	0.42	0.00165
21	Balsa Coipue	Toltén	9	8	0.97	0.00969
22	Puente Copihuelpe	Valdivia	9	4	0.69	0.00088
23	Calle-Calle 2	Valdivia	8	5	0.86	0.00080
24	Camping	Valdivia	5	3	0.70	0.00079
25	Balsa Enco	Valdivia	8	2	0.54	0.00053
26	Balsa Colonia	Baker	1	1	–	–
27	Balsa Baker	Baker	7	3	0.52	0.00099
28	Arriba Reserva Ullum	Colorado	10	5	0.76	0.00132
29	Bocatoma	Negro	1	1	–	–
30	Los Altares	Chubut	10	5	0.76	0.00150

**Table 4**

Genetic diversity values for *Diplomystes* calculated for each basin based on mitochondrial DNA. (N) Number of sequences, (S) number of haplotypes, (H) haplotype diversity, and ( $\pi$ ) nucleotide diversity.

Cuenca	N	S	H	$\pi$
Rapel	13	9	0.936	0.00208
Mataquito	23	8	0.802	0.00087
Maule	21	12	0.852	0.00082
Itata	9	7	0.944	0.00883
Biobío	44	16	0.779	0.00278
Imperial	14	10	0.923	0.00195
Toltén	18	10	0.810	0.00689
Valdivia	33	8	0.686	0.00074
Baker	8	3	0.607	0.00138
Colorado	10	5	0.756	0.00149
Negro	1	1	–	–
Chubut	10	5	0.756	0.00131

low diversity and signal of population expansion at southern latitudes. However, it cannot explain the same pattern in non-glaciated areas in northern basins. Other factors such as the ecology of *Diplomystes*, climate change, and volcanic activity might be involved in the low nucleotide diversity and population expansion in northern populations.

Particularly, it is noticeable that the biogeographical history of Patagonian fishes was usually visualized as the consequence of Quaternary glaciations on populations of marine related species like *P. trucha* (Ruzzante et al., 2006, 2008, 2011), *G. maculatus* (Zemlak et al., 2010), and *G. platei* (Ruzzante et al., 2008; Zemlak et al., 2008). The fate of the southward extensions of Neotropical ostriariophyan biota present under the warmer climatic conditions registered during the Tertiary (Cione et al., 2005; Cione and Báez, 2007) is a less explored approach (Cussac et al., 2009). For example, while the distribution area of *T. areolatus* lies almost completely out and northward of the estimated maximum extent of the major

ice sheet during the last glaciations, reaching its southernmost record at 42°22'S in Chiloe island (Unmack et al., 2009), *Hatcheria macraei* shows, in Lake Buenos Aires (46°32'S), a death temperature (31 °C) similar to that of a warm water fish, suggesting a 'physiologic relic' related to previous warm water adaptation (Gómez, 1990).

Other biological factors may also help explain the low genetic diversity in *Diplomystes*. The first is that diplomystids have naturally small populations which increases the probability of genetic fixation due to drift and reducing the likelihood of new alleles arising in populations by mutation. Second, examination of *D. camposensis* indicates low fecundity as they contain a small number of mature oocytes in ovary (300 oocytes) (Vila et al., 1996). Third, *Diplomystes* have a high degree of habitat specificity (Arratia, 1983, 1987; Habit, 2005; Habit et al., 2009), which reduces the amount of suitable habitat within a basin. In addition to these biological factors, the small size of Pacific drainages also contributes to low population sizes by physically constraining the amount of available habitat. Thus, naturally small population sizes found in *Diplomystes* would be expected to result in lower long-term levels of genetic diversity.

Climate changes and volcanic activity could have also impacted populations beyond the glacial margin. Ruzzante et al. (2008) suggested that a broad-scale causal factor, such as climatic change, could explain synchronous demographic changes in the freshwater fishes *Galaxias platei* and *Percichthys trucha*. Climate change could modify the volume and physical conditions of water, with potentially larger effects on sensitive species like *Diplomystes*. Volcanic activity has been documented in the Andes of Central Chile since the Plio-Pleistocene (Vergara, 1983; González-Ferrán, 1995) and products of this volcanic activity might also have impacted populations in several ways, such as causing water poisoning, changes in water temperature, and habitat reduction.

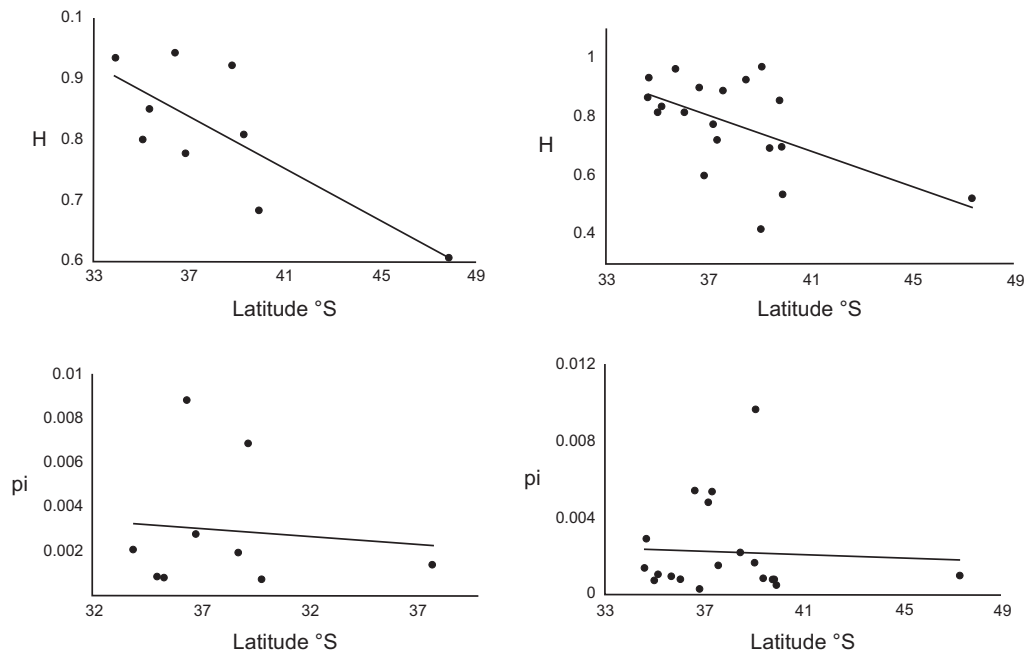
Although several factors might explain the observed patterns in *Diplomystes*, glaciations were probably one of the most important, especially in southernmost populations. This is especially clear south of Valdivia, where no populations of *Diplomystes* (with exception of the Baker and Manso basin populations further south) have been reported, consistent with the distribution of the ice sheet during the LGM. Other factors such as low population densities, climate change, and volcanism might have also contributed to reduce diversity in southern as well as northern populations.

#### 4.2. Phylogeographic patterns and drainage history

Similar to the observed patterns in previous phylogeographic studies on other freshwater fishes in the region (Ruzzante et al., 2006; Zemlak et al., 2008; Unmack et al., 2009, 2012), our results demonstrate the important role of the Andes in the evolution of hydrographic drainages and its impact on aquatic populations. All western Andes populations from Rapel Basin to Valdivia Basin are well differentiated from all other eastern Andes and Baker populations, forming distinct lineages that are reciprocally monophyletic. In addition to this, there were marked differences in terms of genetic diversification between the Atlantic and Pacific basins that suggest different patterns of connectivity on either side of the Andes. These differences are explained below.

#### 4.3. Atlantic connectivity

Similar to the patterns of genetic differentiation observed in *Percichthys trucha* and *Galaxias platei*, *Diplomystes* showed higher levels of genetic divergence among Pacific drainages than among Atlantic drainages. Phylogenetic gene trees, haplotype network, and AMOVA revealed that levels of genetic variation among some Pacific basins were as high as differentiation found among clades separated by



**Fig. 4.** Plots showing relationship between genetic diversity and latitude, calculated by basins (left) and populations (right). All relationships were not significant at the 0.05 level. Note, however, that the relationship between haplotype diversity and latitude was marginally not significant at both the basin level ( $R = -0.63$ ,  $p = 0.067$ ) and the population level ( $R = -0.43$ ,  $p = 0.059$ ).

**Table 5**

$F_{ST}$  values between basins calculated as pairwise differences. All comparisons were statistically significant at the 0.05 level. Negro basin was not included because only one individual was sampled.

Basins	1	2	3	4	5	6	7	8	9	10
1. Rapel										
2. Mataquito	0.06									
3. Maule		0.84								
4. Itata	0.40	0.53	0.47							
5. Biobío	0.87	0.90	0.90	0.78						
6. Imperial	0.91	0.94	0.94	0.78	0.87					
7. Toltén	0.79	0.85	0.85	0.67	0.80	0.43				
8. Valdivia	0.94	0.96	0.96	0.86	0.88	0.91	0.74			
9. Baker	0.93	0.96	0.96	0.79	0.90	0.92	0.80	0.96		
10. Chubut	0.93	0.96	0.96	0.81	0.89	0.92	0.80	0.95	0.45	
11. Colorado	0.94	0.96	0.96	0.82	0.90	0.93	0.82	0.96	0.80	0.74

**Table 6**

Results of the analysis of molecular variance (AMOVA) evaluating several biogeographical hypotheses. Results are expressed as the percentage of total variation.  $F_{ST}$  values are based on Tamura and Nei distances with a gamma correction (0.011). Values in parenthesis indicate the number of groups compared.

Groups	Among groups	Among populations within groups	Within populations
1. Chile vs. Argentina (2)	78	21.1	0.9
2. Chile vs. Argentina + Baker (2)	82.3	16.8	0.9
3. Basins (12)	96.6	1.2	2.2
4. Argentina + Baker vs. each Chilean basin (9)	96.6	1.2	2.2
5. Chile vs. each Argentinean basin and vs. Baker (5)	74.6	24.4	1
6. Areas of endemism (3)	45.5	50	4.5
7. Among Pacific drainages (9)	93	2.2	4.8

the Andes. These results strongly support the hypothesis that connectivity among Atlantic basins was higher than among Pacific basins. Two different events related to glacial cycles might explain higher connectivity among Atlantic basins. The first of them could

have occurred during the end of the last glaciation. The melting of ice released high levels of water and led to the formation of extensive palaeolakes. This could have caused widespread re-arrangements of tributaries and facilitated connectivity of drainages. Ruzzante et al. (2006) suggested this mechanism to explain the shallow haplotype phylogeny and low genetic structure observed in populations of *Percichthys trucha* from Atlantic basins. However, even though the existence of large paleolakes in the eastern flank of the Andes has been documented (Clapperton, 1993; Tatur et al., 2002; Turner et al., 2005), direct evidence about the possibility of these connections is still lacking. The second event that could facilitate connection and mixing among Atlantic basins is associated with sea level changes during glaciations and the formation of paleorivers across the continental shelf exposed by low sea levels (Ponce et al., 2011; Ruzzante et al., 2011). The role of paleorivers or confluence of adjacent drainage basins during periods of low sea-levels has frequently been proposed as a plausible mechanism to explain the common occurrence of freshwater fish lineages across adjacent, but currently isolated river systems (e.g., Hugueny et al., 2011; Chakona et al., 2013; Unmack et al., 2013).

Mixing among watersheds within the latitudinal range of the glaciers, but not further north, might explain greater divergence re-

ported in *Hatcheria* and slightly greater in *Percichthys* populations from the northernmost watershed, Rio Colorado, relative to populations of these species found further south (Ruzzante et al., 2006; Unmack et al., 2012). This same pattern might also be found in *Diplomystes* from Rio Colorado, which seem to show a slightly greater divergence than southern populations. However, considering the extremely low divergence among Atlantic-basin populations overall (<0.6%), it is also possible that the greater differentiation of *Diplomystes* from Colorado can be a product of a small sample size artifact given that only a single population was examined. Further sampling in basins from east of the Andes is needed to evaluate whether differentiation of Colorado basin populations indeed reflect longer isolation or a sampling artifact.

#### 4.4. Pacific connectivity

Although river rearrangements and sea level shifts could each explain mixing between Atlantic basins, these mechanisms seem unlikely to operate west of the Andes. Complex mountainous topography in the western flank of the Andes would not allow the formation of extensive paleolakes, so it is unlikely that this mechanism could have facilitated connectivity. Moreover, connections via sea level changes should have been less common west of the Andes because the narrower continental shelf prevents a major exposure of land during low sea levels (i.e., historic coastline was not much different from the current coastline, Fig. 1). In the absence of clear mechanisms that would have facilitated inter-basin connections, populations within basins or group of basins would have been isolated for longer periods of time than their Atlantic drainage counterparts. Hence, higher differentiation is predicted among populations from Pacific basins. High genetic structure in *Diplomystes* from west of the Andes, as indicated by phylogeny, haplotype network (very few shared haplotypes between river basins), and AMOVA analysis, strongly support this hypothesis. This pattern is consistent with those observed in other phylogeographic studies of codistributed freshwater fishes in this area, where high genetic structure between basins was also observed in Central and South-Central areas of the Chilean Province (Ruzzante et al., 2006; Unmack et al., 2009). The few other phylogeographic studies on freshwater fishes codistributed with *Diplomystes* in Central and South-Central areas of Chilean Province also show high genetic structure at the river basin level (Ruzzante et al., 2006; Unmack et al., 2009).

Although connections could have been less frequent between Pacific basins in Central and South-Central Chile, our results show that historic connections likely occurred between some drainages. This might be the case for the Rapel and Mataquito basins, which show the lowest genetic differentiation ( $F_{ST} = 0.042$ ) and share the two most abundant haplotypes from each of the two basins, suggesting recent gene flow. We did not find evidence in the literature about recent historical connections between these two basins. Nevertheless, gene flow would have to have occurred recently in the past to explain the low genetic structure observed. The same pattern was also observed for the catfish *Trichomycterus areolatus* (Unmack et al., 2009), whose populations from Rapel and Mataquito basins were all recovered as a single and shallow clade with low geographic structure. We suggest that connections between these two drainages could have been possible during or right after Pleistocene glaciations due to flooding or river capture in a topographically flat area called “*Depresion Intermedia*”. Increased river volumes as well as increased complexity in the drainage network due to glacier melting might have created opportunities for temporary connectivity between the Rapel and the Mataquito basins. Alternatively, contemporary human mediated connections (e.g., artificial channels built to aid agriculture) and/or translocations cannot be ruled out as an explanation for recent gene flow between

these basins. Although translocations seems a less likely explanation because *Diplomystes* are rare and not a commonly collected, and they are especially vulnerable when removed from their natural habitat as shown in a study that reported translocations of several native fish species within the Laja River (Biobio Basin) (Habit et al., 2002), dispersal through artificial channels could be a plausible possibility. There are a number of artificial channels that connect different basins for agricultural purposes, such as the Teno-Chimbarongo Channel connecting the Rapel and the Mataquito basins, and the Zañartu Channel, connecting the Biobio and Itata basins. In fact, *Diplomystes* has been collected from artificial channels in the Itata basin (Habit, 1994) suggesting that current inter-basin dispersal through artificial channels might be possible. Further research is needed to test these hypotheses and to better understand the potential consequences for biodiversity in these basins.

#### 4.5. Drainage reversals

South of latitude 42°S, geological evidence indicates that a major ice shield covered all Pacific drainages during LGM, including the Baker basin (Clapperton, 1993; Hulton et al., 2002). Populations from the Baker basin show low haplotype and nucleotide diversity and closely related haplotypes separated by few mutational steps, a pattern usually observed in populations originated by founder effect due to recent colonization. The close phylogenetic relationship between these populations and *Diplomystes* from east of the Andes (Fig. 2) indicates that colonization of the Baker basin occurred from Atlantic watersheds instead of from glacial refugia in the North. This trans-Andean dispersal can be well explained by drainage reversal, as suggested previously by other authors (Ruzzante et al., 2006; Zemlak et al., 2008; Unmack et al., 2012). After glacial retreat, natural ice dams disappeared and the big lakes that previously discharged into the Atlantic Ocean finally collapsed and discharged into the Pacific Ocean (Turner et al., 2005). Genetic patterns in *Galaxias maculatus* (Zemlak et al., 2008) and *Percichthys trucha* (Ruzzante et al., 2006) also support this mechanism.

#### 4.6. Areas of endemism

Our results show weak support for the hypothesis that populations are structured in areas of endemism. AMOVA revealed the lowest genetic variation explained for these groups relative to variation explained between drainage basins. Similarly, major phylogeographic breaks in our phylogenetic analysis do not match the boundaries between the areas of endemism defined by Dyer (2000). Although minor breaks occurred among almost every basin west of the Andes, two major phylogeographical breaks can be recognized. The first occurred between the Itata and Biobío basins, further south than the southern limit of Dyer's Central area of endemism, while the second major break occurred between Biobío and Imperial basins, further north than the northern limit of Dyer's Southern area of endemism. These phylogeographical breaks found in *Diplomystes* coincide with major breaks found in previous studies. Ruzzante et al. (2006) analyzed *Percichthys trucha* populations from two basins at the west of the Andes (Itata and Biobío) and several basins from east of the Andes. Interestingly, they found that the deepest phylogenetic divergence occurred between the Itata basin and all other basins, including Biobío, which is adjacent to the Itata basin. This strong divergence between Itata and Biobío basins is consistent with a major break in *Diplomystes*. Similarly, the second major break in *Diplomystes* also coincided with one of the major breaks observed in the catfish *Trichomycterus areolatus* (between clades D and E; Unmack et al., 2009). Therefore, even though explained variation is relatively high (45.5%) among the areas of endemism proposed by Dyer (2000), it is better explained by differentiation at the river basin level.

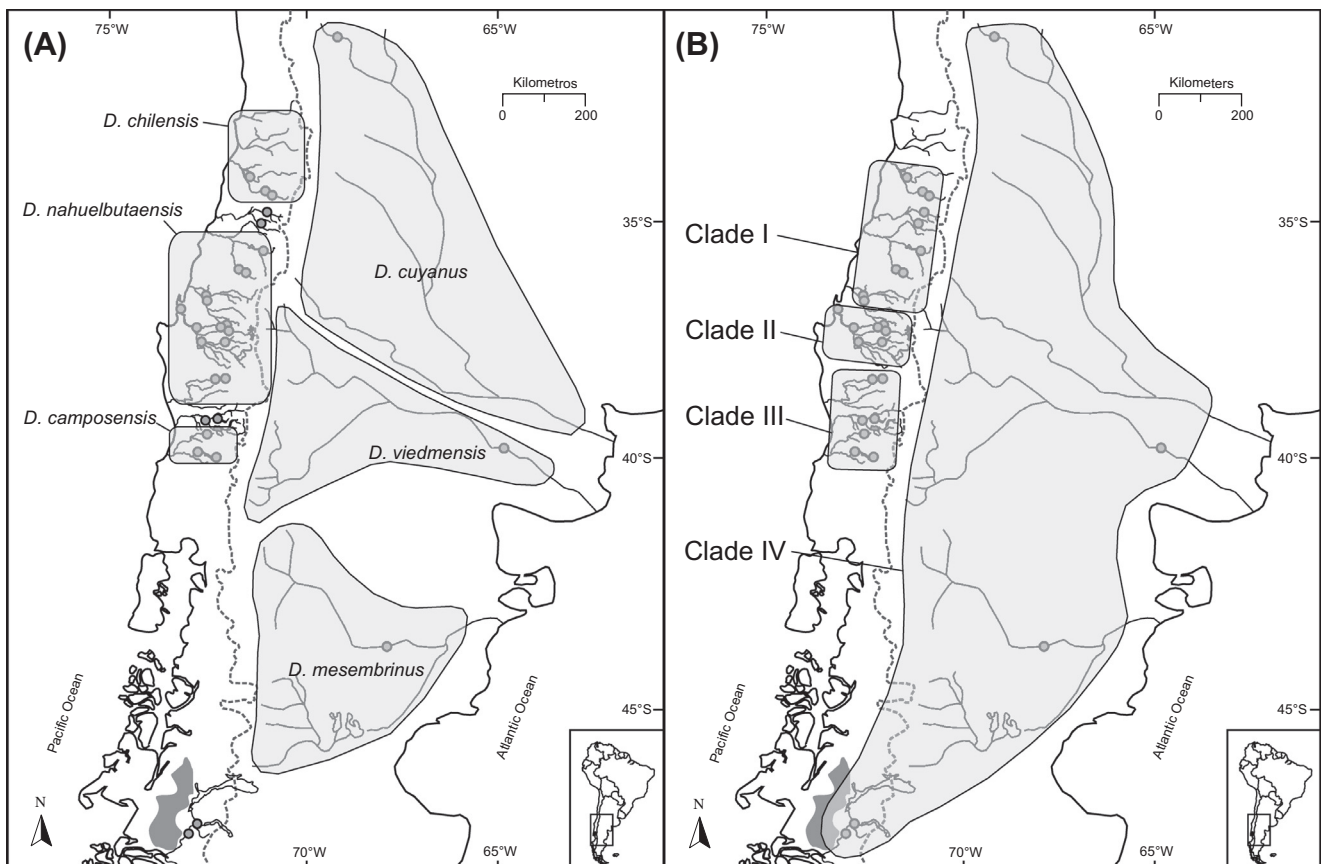
#### 4.7. Systematics

Despite its importance in the early diversification of Siluriforms, only few studies have addressed the taxonomy of Diplomystidae in detail. The most extensive taxonomic studies are those of Arratia (1987, 1992) and Azpelicueta (1994), both based on detailed osteological and morphological characters. From the analysis of samples from both sides of the Andes, Arratia (1987) recognized three species in Chile under the genus *Diplomystes* and only one species in Argentina containing three subspecies. The genus *Oliveichthys* was created to represent the Argentinean species. Azpelicueta (1994) focused only on samples from Argentina and as opposed to Arratia (1987), the three subspecies were considered as three valid species. Azpelicueta (1994) also returned these three species back to the genus *Diplomystes* because no major morphological differences were found to support the erection of a new genus. However, later authors have maintained the validity of *Oliveichthys* (e.g., Ferraris, 2007; Eschmeyer, 1990; Eschmeyer and Fong, 2013). These conflicting taxonomic relationships had not been investigated with molecular data until now. The only published genetic studies on any *Diplomystes* prior to our work were conducted on a single basin (Habit et al., 2009; Victoriano et al., 2012), but this is the first molecular study to examine the whole geographical distribution of Diplomystidae. Hence, our work offers an excellent opportunity to examine the systematic of this group in the light of new evidence.

Our results are not consistent with previous taxonomy in two ways. First, contrary to what we would have expected for six distinct species, we found relatively low overall genetic divergences (as measured from *p*-distance based on the mitochondrial dataset)

among all major lineages (less than 3% between the most distant lineages). Previous phylogeographic studies, based on populations within single freshwater fish species in the same region and using comparable mitochondrial markers, showed levels of population divergence even higher than between all the putative species of *Diplomystes* studied here. For instance, the catfish *Trichomycterus areolatus* (Unmack et al., 2009) presented 9.1% genetic divergence, while *Percichthys trucha* showed a 12% (Ruzzante et al., 2006). Furthermore, our results show that populations of *Diplomystes* from Argentina, which were proposed to represent samples from three different species (sensu Azpelicueta, 1994), showed only 0.6% of genetic divergence in our mitochondrial dataset (Cytb and CR combined). These levels of divergence are quite modest even compared to intraspecific divergence in other co-distributed freshwater fish species. Therefore, our results show no support for multiple species in Atlantic drainages, being more consistent with the taxonomic conclusions of Arratia (1987) for Atlantic diplomystids.

Second, based on the phylogenetic species concept and the geographic distribution of the main lineages found, our results suggest different species distributions reflected in differences in their species limits. These results support the Arratia (1987)'s taxonomic scheme regarding the number of species, but modify the geographical distribution of species limits (Fig. 5). Populations from the Rapel basin, which has been previously treated as *D. chilensis* (Dyer, 2000) and *D. cf. chilensis* (Arratia and Huaquín, 1995), cluster together with Mataquito, Maule and Itata populations in a closely related clade (Fig. 2, clade I). Note, however, that Maule and Itata basins have traditionally been considered part of the distribution of *D. nahuelbutaensis* along with Biobío and Imperial basins. Biobío populations, recognized as *D. nahuelbutaensis*, form the single clade



**Fig. 5.** Maps showing the differences between the distribution of the described species of *Diplomystes* (A) and the four major mitochondrial lineages found in this study (B) (see Fig. 3 for reference).



II. Only two haplotypes from Biobío and Itata basins are shared between clade I and II (see locality 11, Fig. 1). These apparently interchanged haplotypes from Biobío and Itata basins are from three localities that are very close geographically (localities 11, 13, and 15). This pattern seems unlikely to be product of ancestral polymorphism because population sizes in these basins are small relative to the probable old divergence between these basins, and ancestral polymorphism is more likely for large, recently separated populations than for small and old diverged ones. Instead, a most likely explanation is a recent interchange of individuals through artificial channels that connect both basins (e.g., Zañartu Channel, constructed in 1958). Indeed, diplomystids have been collected previously in artificial channels in the Itata basin (Habit, 1994). The impact of this probable secondary contact between different lineages/species will require further research.

Populations of *Diplomystes* from the Valdivia basin, which has been recognized traditionally as the only basin where the species *D. camposensis* is present, cluster together with populations from Tolten and Imperial basins, the latter being the type locality of *D. nahuelbutaensis*. Even though there is high genetic structure among Imperial, Tolten and Valdivia basins, some haplotypes are shared suggesting a more recent relationship. These basins might harbor populations of a single species with high population differentiation at the basin level or represent different, recently originated species. Assuming the former hypothesis, these populations should be considered part of the *D. nahuelbutaensis* distribution and the name *D. camposensis* would become a junior synonym of *D. nahuelbutaensis*. The multi-species hypothesis, on the other hand, is consistent with data in Arratia (1987) that considered as different species the populations from Imperial and Valdivia basins. Consequently, populations from the Tolten basin, would be either a divergent population or a new sister species of *D. nahuelbutaensis*.

East of the Andes, all three Argentinean species cluster together with populations from the Baker basin in the shallow clade IV, suggesting either a recent separation of these species or that they all are populations of a single species. In fact, both this clade and clade II (Biobío), are the least diverse clades, but clade IV encompasses four basins whereas clade II a single one.

On the basis of our phylogenetic results, we suggest the existence of at least four species of Diplomystidae; however, further detailed morphological and molecular studies are needed to fully test this hypothesis. Species distributions would be as follows: a first species (*D. cf. chilensis*) from Rapel to Itata basins, a second species *Diplomystes* sp. (that would need to be described) in the Biobío basin, a third species, *D. nahuelbutaensis*, from Imperial to Valdivia basin, and a fourth species, *D. viedmensis* from east of the Andes and Baker basin.

We recognize that it is also possible that the northern species (*D. cf. chilensis*) might represent a distinct species (i.e., different from what was described as *D. chilensis* from Maipo and Aconcagua basins) given that sequences from populations from Maipo and Aconcagua basins (type localities) are not available for comparisons. This needs to be studied in more detail.

*Diplomystes* from Biobío basin seems to be diagnosable from all other diplomystids based on new collected data on internal morphology (M. Pinna pers. com. 2012), which would support our hypothesis. This species would need a new name given that *D. nahuelbutaensis* should be reserved, at least, for populations from the Imperial basin (its type locality). Further studies are needed to determine whether populations from Tolten and Valdivia basins should be treated as different species from *D. nahuelbutaensis* or as an extension of its distribution.

Our results do not support the distinction of three different species in the east flank of the Andes (Azpelicueta, 1994), being more consistent with the scheme of a single species (*D. viedmensis*) (Arratia, 1987).

#### 4.9. Conservation

Most species of *Diplomystes* are categorized under some conservation status. Currently, all described species from Chile are categorized as at risk of extinction by the Chilean government entity CONAMA (2008), while in Argentina *D. viedmensis* and *D. mesembrinus* are categorized as 'rare' by Bello and Ubeda (1998). According to Arratia (1983) and Campos et al. (1998), the main factors affecting the conservation of species in Chile are fragmentation, habitat reduction and pollution, and the introduction of exotic species. Due to the delicate conservation status of *Diplomystes* species, the genetic data presented in this study provides helpful information for identifying areas/populations that require special attention. High inter-basin genetic structure and low intra-basin genetic variability in most basins suggest the need for conservation efforts at the level of each basin in order to protect as much phylogenetic diversity as possible. Populations from Itata and Tolten basins are particularly special because of their high genetic diversity compared to populations from other basins. They require special attention and further research in order to properly quantify possible patterns of genetic diversity within each basin. Populations from Rapel and Mataquito basins are poorly known and may require special attention. These basins are close to cities with the highest human populations and urban growth (INE, 2005). Finally, further studies on basic aspects of the biology and ecology of *Diplomystes*, along with detailed intra-basin population genetic studies are needed in order to implement appropriate conservation strategies in this unique group of catfish.

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#### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2014.01.015>.

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