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Species tree analyses and speciation-based species delimitation support new species in the relict catfish family Diplomystidae and provide insights on recent glacial history in Patagonia

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ABSTRACT

Diplomystidae is an early-diverged family of freshwater catfish endemic to southern South America. We have recently collected five juvenile specimens belonging to this family from the Bueno River Basin, a basin which the only previous record was a single juvenile specimen collected in 1996. This finding confirms the distribution of the family further South in northern Patagonia, but poses new questions about the origin of this population in an area with a strong glacial history. We used phylogenetic analyses to evaluate three different hypotheses that could explain the origin of this population in the basin. First, the population could have originated in Atlantic basins (East of the Andes) and dispersed to the Bueno Basin after the Last Glacial Maximum (LGM) via river reversals, as it has been proposed for other population of *Diplomystes* as well as for other freshwater species from Patagonia. Second, the population could have originated in the geographically close Valdivia Basin (West of the Andes) and dispersed south to its current location in the Bueno Basin. Third, regardless of its geographic origin (West or East of the Andes), the Bueno Basin population could have a longer history in the basin, surviving *in situ* through the LGM. In addition, we conducted species delimitation analyses using a recently developed method that uses a protracted model of speciation. Our goal was to test the species status of the Bueno Basin population along with another controversial population in Central Chile (Biobío Basin), which appeared highly divergent in previous studies with mtDNA. The phylogenetic analyses showed that the population from the Bueno Basin is more related to Atlantic than to Pacific lineages, although with a deep divergence that predated the LGM, supporting *in situ* survival rather than postglacial dispersal. In addition, these analyses also showed that the species *D. nahuelbutaensis* is polyphyletic, supporting the need for a taxonomic reevaluation. The species delimitation analyses supported two new species which are described using molecular diagnostic characters: *Diplomystes arratiae* sp. nov. from the Biobío, Carampangue, and Laraquete basins, maintaining *D. nahuelbutaensis* valid only for the Imperial Basin, and *Diplomystes habitae* sp. nov. from the Bueno Basin. This study greatly increases the number of species within both the family Diplomystidae and Patagonia, and contributes substantially to the knowledge of the evolution of southern South American freshwater biodiversity during its glacial history. Given the important contribution to the phylogenetic diversity of the family, we recommend a high conservation priority for both new species. Finally, this study highlights an exemplary scenario where species descriptions based only on DNA data are particularly valuable, bringing additional elements to the ongoing debate on DNA-based taxonomy.

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1. Introduction

Diplomystidae is an ancient family of freshwater catfish endemic to rivers from southern South America. Given the relict nature of the family, its conservation value is of great interest. Recently, new Diplomystid populations were discovered in small coastal basins of South Chile (Muñoz-Ramírez et al., 2020) shedding light on how little we still know about freshwater biodiversity from this region and suggesting that new findings of these rare catfish could still be possible in poorly surveyed basins. Here we report and analyze a striking new finding of Diplomystids in the Andean basin of the Bueno River that extends the West Andes distribution of the family towards an area that was strongly impacted by the Pleistocene glacial cycles (Fig. 1). Although a previous record exists, represented by a single individual found dead in 1996 and stored in a private collection (Muñoz-Ramírez et al., 2010), no other samples have ever been recorded even after many surveys conducted across the Bueno Basin (e.g. Soto and Arismendi, 2005). Nevertheless, and besides its rarity, this finding poses striking questions about the biogeographic origin of this population, with potential hypotheses including drainage reversals, river captures, and glacial refugia.

The Family Diplomystidae is the earliest-branching lineage in the order Siluriformes, and its position as the sister group of all other catfish groups has been well supported by morphological (Arratia, 1987; dePinna, 1991; Lundberg and Baskin, 1969) and molecular-based phylogenetic analyses (Rivera-Rivera and Montoya-Burgos, 2018; Zhang et al., 2021). Sullivan et al., (2006) placed the family as the sister group of Loricarioidei (one of the two other main clades within Siluriformes) based on phylogenetic analyses of two nuclear genes. However, subsequent phylogenetic analyses accounting for heterogeneity in evolutionary rates among lineages supported Diplomystidae as the sister group to all other catfish (Rivera-Rivera and Montoya-Burgos, 2018). Currently, the family encompasses seven recognized species, four species endemic to Chile, two species endemic to Argentina, and one species shared between Chile and Argentina (Fig. 2). In Chile, the family has

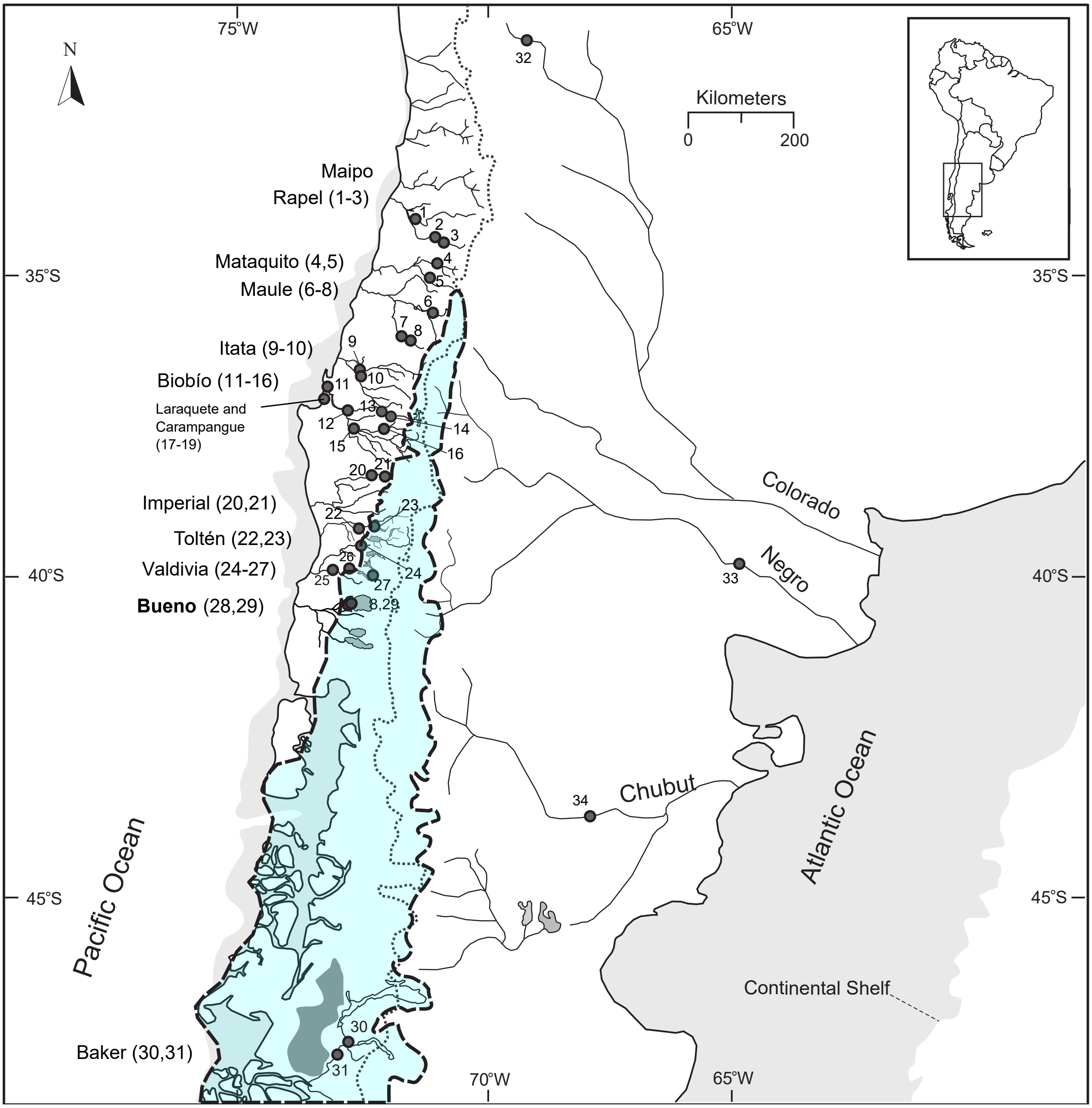

Fig. 1. Distribution map depicting sample locations and basins where the family Diplomystidae is present. Dashed line and light blue shading depict the ice sheet extension at the Last Glacial Maximum. For further details on sampling sites and individuals used for analyses see Supplementary Material Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

been known from all Andean basins from the Maipo Basin to the Valdivia Basin, and after a distributional gap of 850 km, it has also been reported in the Baker Basin. The northernmost species *Diplomystes chilensis* (Molina, 1782), endemic to the Maipo basin, is currently assumed extinct (Arratia and Quezada-Romegialli, 2017; Muñoz-Ramírez et al., 2014). The recently described *D. incognitus* Arratia & Romeglialli 2017 is present in the Rapel, Mataquito, Maule, and Itata basins (Arratia and Quezada-Romegialli, 2017). *Diplomystes nahuelbutaensis* Arratia 1987 has been reported in the Biobío and Imperial basins, and it has recently been also recorded in two small coastal basins (Laraquete and Carampangue) (Muñoz-Ramírez et al., 2020). *Diplomystes camposensis* Arratia 1987 is found in the Toltén and Valdivia basins. In Argentina, *Olivaichthys cuyanus* Azpelicueta 1994 is present in the Colorado Basin, *O. viedmensis* Mac Donagh 1931 is present in the Negro Basin, and *O. mesembrinus* is present in the Chubut Basin. The population from the Baker Basin in Chile is genetically close to the Argentinean *Olivaichthys mesembrinus* (Muñoz-Ramírez et al., 2014). Although the family has been recently re-examined (Arratia and Quezada-Romegialli, 2017), questions still remain about the species status of some populations. For instance, populations from the Biobío and Imperial basins have substantial genetic divergence, suggesting potential cryptic species (Muñoz-Ramírez et al., 2014). However, the scarcity of mature individuals available, crucial for detailed morphological analyses, has prevented further taxonomic discussion, maintaining these populations under the same nominal species, *D. nahuelbuaensis* (Arratia and Quezada-Romegialli, 2017). Unfortunately, the high ontogenetic variability in the genus has hampered the use of juvenile individuals to distinguish species by morphology (Arratia and Quezada-Romegialli, 2017). The newly found population in the Bueno Basin has not been morphologically identified at the species level because all individuals available are small juveniles. However, its geographic proximity to the Valdivia Basin may suggest they could be closely related to *D. camposensis*.

During the Pleistocene Glaciations, southern South America was deeply impacted by the advance of an extensive ice sheet (Hulton et al., 2002; Sugden et al., 2005) driving changes in the distribution and genetic configuration of the aquatic fauna. The uplift of the Andes and the Pleistocene glacial cycles are generally considered as the two most important historical events that have shaped patterns of lineage divergence and diversity in Patagonian freshwater species (Ruzzante et al., 2006, 2008; Unmack et al., 2009; Xu et al., 2009; Zemlak et al., 2008). However, it is not always clear how these events influenced diversification. Andean uplift should have been a key major process in the separation of Atlantic and Pacific basins producing vicariance and deep divergences within several related taxa with trans-Andean distributions (e.g. Muñoz-Ramírez et al., 2014; Ruzzante et al., 2006), whereas other processes like river capture and drainage reversals could have been more important locally, driving dispersal events within taxa (e.g. ; Ruzzante et al., 2020; Zemlak et al., 2008). Glacial cycles have also induced changes in sea levels impacting coastline position and potentially connecting and isolating geographically close river basins (Barber et al., 2011). Furthermore, glacial cycles played an important role in the reduction and extinction of many local populations near or within areas of direct glacier impact, causing important population reductions, bottlenecks, and loss of genetic diversity (González-Wevar et al., 2015; Muñoz-Ramírez et al., 2016; Victoriano et al., 2012). Glacial refugia in northern areas could have allowed survival and provided a source for postglacial colonization, whereas less known cryptic refugia (i.e. those within the glacial margins) may have allowed some populations to survive in southern areas near the coast promoting population divergence (e.g. Xu et al., 2009). Some species were better than others at coping with these historical events, highlighting the role of key life-history traits for species survival. For example, the strict freshwater

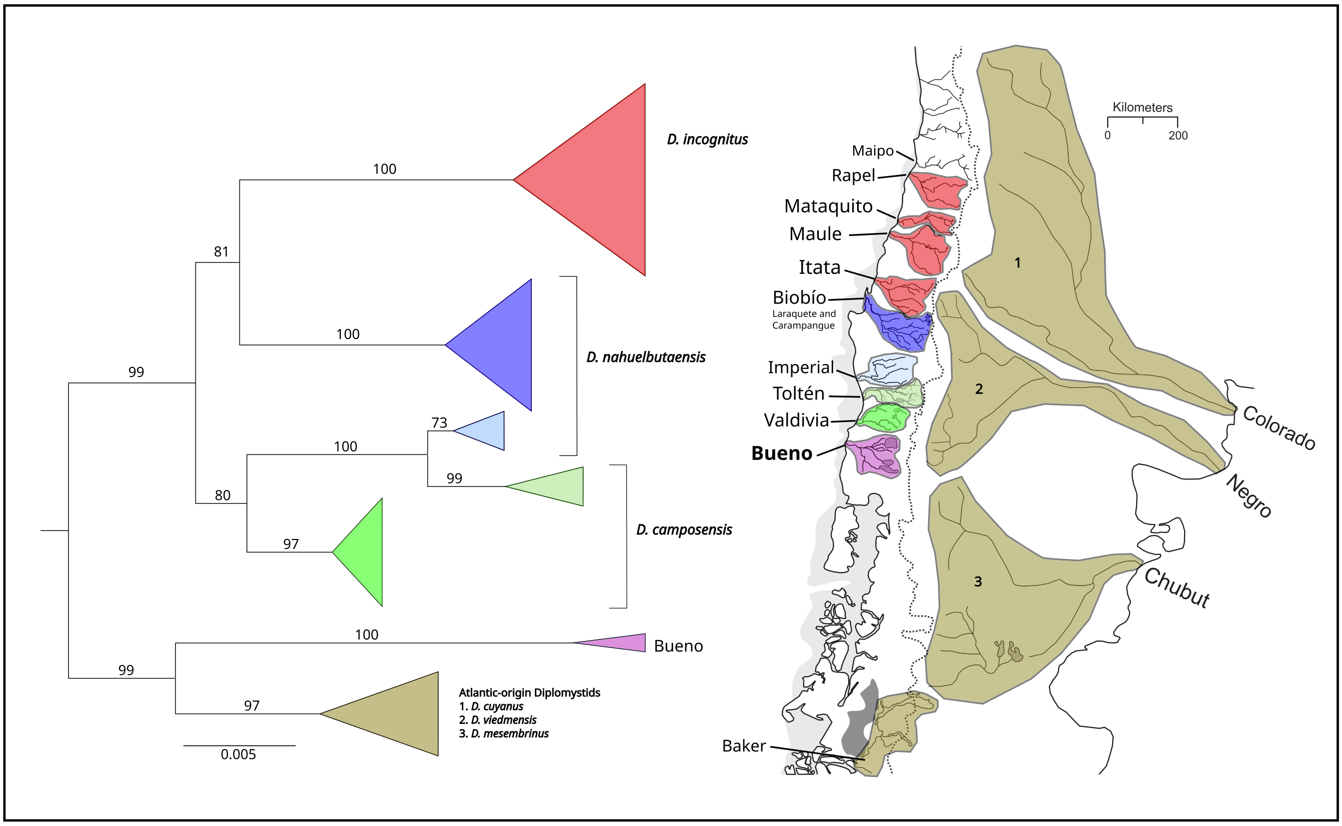

Fig. 2. Distribution of Diplomystid species across basins and their phylogenetic relationships based on a single locus (mtDNA cytB + CR), mid-point rooted Maximum Likelihood analysis. Colors of collapsed clades in the gene tree match the basins where they are present in the map. Note the polyphyletic nature of *D. nahuelbutaensis* based on the mtDNA tree. A fully expanded version of this gene tree is available in Supplementary figure S2.

fish *Galaxias platei* suffered an important demographic reduction that is consistent with a bottleneck with subsequent population expansion after the Last Glacial Maximum (LGM), whereas its congeneric species *G. maculatus*, with diadromous and landlocked populations, maintained high genetic diversity (Victoriano et al., 2020; Astorga et al., 2022; Tymoshuk et al., 2022).

The geographic distribution of the family Diplomystidae reflects the strong influence of Pleistocene glaciations on its evolution (Muñoz-Ramírez et al., 2014). For instance, the family is absent from rivers strongly impacted by the ice shield during the LGM (Figs. 1 and 2). Furthermore, the population from the Baker River (Pacific basin), genetically closer to Diplomystids from Atlantic basins, strongly supports a trans Andean dispersal via drainage reversal caused by ice-dam collapse (Benito and Thorndycraft, 2020; Thorndycraft et al., 2019). These drainage reversals could have been relevant for freshwater dispersal from basins that previously flowed towards the Atlantic Ocean to basins that currently flow towards the Pacific Ocean. Evidence from phylogeographic studies with a number of freshwater taxa supports this hypothesis (Muñoz-Ramírez et al., 2014; Ruzzante et al., 2006, 2020; Unmack et al., 2012; Zemlak et al., 2008).

Phylogenetic relationships between populations can provide a means to understand population and species history along with the history of the basins they inhabit by providing the timing and pattern of population diversification. Given all the potential processes impacting the evolution of the southern South American landscape and drainage morphology, here we use a dated population tree estimate to test three hypotheses that could explain the origin and presence of Diplomystids in the Bueno Basin. First, populations from the Bueno Basin could have recolonized from the adjacent Valdivia Basin through river captures or river rearrangements soon after glaciations. This could have been possible, for instance, during periods of high discharge as the ice sheet melted. Second, the catfish may have moved from Atlantic basins into the Bueno Basin via drainage reversal after the LGM. Third, the population in the Bueno Basin could have survived through the LGM *in situ* in some local glacial refugium downstream. In addition, given the taxonomic uncertainty of some populations, including those from the Biobío Basin and the newly found Bueno Basin, we used a recently developed speciation-based species delimitation analysis based on a protracted model of speciation to test whether these populations represent different new species. DNA-only species descriptions are not commonly used as the primary or sole source of data for describing species (but see Halt et al., 2009; Jörger and Schrödl, 2013). Nevertheless, when morphological data cannot be easily obtained, a DNA-only description could be justifiable (Cook et al., 2010; Jörger and Schrödl, 2013). Given the difficulties to collecting *Diplomystes* and because the only material available at the moment are juveniles, species supported by the species delimitation analysis are described using molecular-based diagnoses. Finally, using phylogenetic diversity estimations, we assess the contribution of each basin to the phylogenetic diversity of the family to discuss the conservation value of the newly-found species.

2. Material and methods

2.1. Sampling

A total of eight individuals from the Bueno Basin were collected during the summer 2019 (n = 3) and 2021 (n = 5) from two different sections of the Bueno River (−40.2528, −72.6178 and −40.2561, –72.5942, respectively) near the outlet of Ranco Lake (Fig. 1). Specimens from 2019 were released alive after collecting a small piece of the adipose fin by clipping. Specimens from 2021 were kept as vouchers. All the vouchers show a long and continuous lateral line typical of the genus *Diplomystes*, so they were assigned to this genus following Arratia and Quezada-Romegialli (2017). Additionally, three individuals from the coastal basins Laraquete and Carampangue (Muñoz-Ramírez et al., 2020), genetically close to populations from the Biobío Basin, were included to increase data from the Biobío lineage for the species delimitation analysis and were considered for all purposes as part of the Biobío Basin due to their close geographical and genetic proximity.

2.2. Laboratory protocols and molecular data

A small tissue sample from the adipose fin or muscle from each specimen was used for DNA extraction, which was conducted using the DNeasy Tissue Kit (QIAGEN Inc., Chatsworth CA) following de manufacturer’s protocol. All individuals were amplified for two mitochondrial DNA regions and two nuclear genes following Muñoz-Ramírez et al. (2014): a fragment of 1137 bp of the *cytochrome b* gene (*cytB*), a fragment of 943 bp of the control region (*CR*), a fragment of 1497 bp of different portions of the growth hormone gene (*GH*) (exons three through five and introns three and four), and a fragment of 872 bp of the *S7* gene (intron one). These newly generated data were complemented with published data from Muñoz-Ramírez et al. (2014, 2016, 2020) to conduct all analyses. GenBank accession numbers for the newly generated sequences are OR215010-OR215017 (*cytB*), OR215018-OR215024 (CR), OR215034-OR215042 (*S7*), and OR215025-OR215033 (*GH*). Additional details about samples used in analyses and a complete list of GenBank accession numbers can be found in the Supplementary Material Table S1.

2.3. Phylogenetic estimation

The phylogenetic position of the Bueno Basin population was estimated in two ways. First, a Maximum Likelihood genealogy was estimated using RAxML-NG v. 1.1.0 (Kozlov et al., 2019) with mtDNA regions *cytB* and *CR* concatenated. Each mtDNA region was treated as a separate partition with its own model of molecular evolution. Best-fit models of molecular evolution were chosen by Modeltest-NG (Darriba et al., 2020) using the Bayesian information criterion (BIC). The RAxML analysis was estimated with 1000 nonparametric bootstrap replicates, followed by a search for the best-scoring ML tree.

Second, we estimated a species tree (or population tree in our case) using the coalescent method StarBeast3 (Douglas et al., 2022) implemented as part of the software BEAST2 v2.6.6.0 (Bouckaert et al., 2014) using both the mtDNA and nuclear data (three independent loci). The *cytB* and *CR* gene regions were linked as one single locus (mtDNA), but analyzed as two separate partitions for the site model estimations. Each nuclear locus, *GH* and *S7*, was analyzed as a separate locus. A total of 22 individuals were assigned into populations represented by Andean basins. The analysis was run specifying models of molecular evolution based on results from Modeltest-NG (Darriba et al., 2020), with a Yule prior for the population tree. The strict clock model was selected over the relaxed clock model via the Bayes Factor method (Kass and Raftery, 1995) after obtaining the marginal likelihoods by the Path Sampling method (Baele et al., 2012). To estimate dates for each node we used a calibration point for the divergence between the Chubut and the Baker populations based on geological evidence for the reversal of the Chubut Basin after ice-dam collapse between 12.6 and 11.7 ka (Benito and Thorndycraft, 2020). A normal distribution with mean 0.01215 Ma and a standard deviation of 2.5E-4 Ma, with non-fixed monophyly for the Chubut-Baker clade, was used as prior. Two independent MCMC chains were run for 500 million generations, sampling every 50,000 generations to produce 10,000 sampling values each to check for convergence to the same optimum. Parameter estimates were checked for convergence in Tracer v1.4 (Rambaut and Drummond, 2007), discarding the first 20 % of the samplings as burn-in. The population tree was finally summarized using the TreeAnnotator application, removing the first 20 % of trees as burn-in and selecting the Maximum clade credibility tree with the median heights.

2.4. Species delimitation and DNA diagnosis

To test whether the Bueno Basin and the Biobío Basin represent parts of known species or new species, we use a recently developed speciation-based approach called DELINEATE (Sukumaran et al., 2021). Classical approaches to species delimitation based on the Multispecies Coalescent (MSC) identify disruptions in Wright-Fisher panmixia due to gene flow barriers, and assume that these disruptions are explained by species boundaries (Yang & Rannala, 2010). As has been noted (Sukumaran and Knowles 2017), however, if there is within-species lineage structuring, then, as long as there is sufficient power available with the data sampled, these approaches will result in conflation of population units with species units, resulting in spurious species boundaries as distinct populations are mis-characterized as distinct species. In contrast, while DELINEATE relies on the Multispecies Coalescent (MSC) as well, it only uses it to first diagnose population units. It then infers the actual species boundaries using an explicit probabilistic speciation process model that organizes these population units into higher-level species units. The DELINEATE approach accounts for the possibility that some structure does not involve speciation events by modeling the formation of population lineages and their subsequent development into independent species as separate processes. DELINEATE uses information about the current taxonomy of the taxa as understood by the investigator to calibrate the model. Specifically, the investigator provides species identities for a subset of the population lineages being considered. These "known" species identities for this subset of population are used to infer a tempo of speciation, i.e., the rate at which an independent population lineage evolves into distinct species, for the entire system. With this, the probabilities of different species delimitation configurations can be calculated and ranked, with the species delimitation of the highest probability constituting the maximum likelihood estimate species delimitation. This estimate of course includes the species assignments for the subset of population lineages as provided by the investigator, but it will also include species assignments for the remaining lineages, i.e., the population lineages of uncertain, unknown, or undetermined species affinities. Each of these population lineages of unknown species affinities will be assigned a species identity. Each of the populations of unknown species affinities may end up being categorized as a population of a species previously identified by the investigator or, alternatively, as a population of "new" unnamed species, previously undisclosed or undeclared by the investigator.

We use the tree inferred by StarBeast3 as the input tree of population lineages required for DELINEATE. For the taxonomic information required, we considered two different schemes. One scheme considered the currently accepted taxonomic view of the family, with three extant species for the Pacific basins and three species for the Atlantic basins (hereafter the Azpelicueta scheme; Azpelicueta, 1994). The second scheme reflected a more conservative taxonomic view that considers all species from Atlantic basins as a single one (hereafter the Arratia scheme; Arratia, 1987). Although the latter is not the prevailing view for the Atlantic taxonomic diversity, we used it to test the robustness of results under a more conservative scenario that would make splitting more difficult (i.e. by decreasing the speciation rate parameter).

Given the uncertainty about the taxonomic status of the Biobío Basin population (Muñoz-Ramírez et al., 2014), only the population from the Imperial Basin was assigned to *Diplomystes nahuelbutaensis* because the Holotype of this species is from the Imperial Basin, whereas the status of the Biobío Basin population was left to be tested by the analysis. The Baker Basin population is included as part of the Chubut Basin, and therefore under the same taxonomic status of the Chubut Basin, after Muñoz-Ramírez et al., (2014).

Once the results of the species delimitation analyses were available, we used the molecular data to describe the new species supported. Species diagnoses were written to be state-specific and contrastive to unambiguously comply with the International Code of Zoological Nomenclature as proposed by the commissioners of the International Commission on Zoological Nomenclature (Rheindt et al., 2023). Molecular diagnostic characters were obtained using the nucDIAG function from the R-package SPIDER (Brown et al., 2012). All gene regions were used, including the *cytB* and *CR* sequence alignments and the nuclear gene alignments, although alignment portions with gaps or missing data were ignored for searching diagnostic nucleotides (but were counted for the relative position of diagnostic nucleotides in the alignment). For example, a portion of 90 bp (positions 33–122) in the *CR* alignment was not considered because it contained large portions of gaps in most individuals. Files with the sequence alignments and an R-script with the relevant functions can be found at Dryad (https://doi.org/10.5061/dryad.hqbzkh1nb). Additionally, to obtain a comparison of the genetic distance between species, pairwise mean genetic distances were calculated using both the Kimura 2-parameter distance and the p-distance on the *cytB* gene in the program MEGA 11 (Tamura et al., 2021).

2.5. Estimating conservation value using phylogenetic diversity

The conservation value of each basin was measured in regard to the contribution of the lineage or species present at a given basin to the overall phylogenetic diversity (PD) of the entire family. This was obtained by calculating the proportion of PD that is lost when a given basin population is removed, repeating this procedure for each basin. Due to the small size and close geographical and genetic proximity to the Biobío Basin, Laraquete and Carampangue basins (that originate in the Coastal Range) were excluded from the analysis, so comparisons were only made between Andean Basins (basins that originate in the Andes Range). PD calculations were conducted using the caper R-package (Orme et al., 2013) and the population tree obtained with StarBeast3.

3. Results

3.1. Phylogenetic results

The estimated Maximum Likelihood mtDNA genealogy presented virtually the same topology seen in previous studies (Muñoz-Ramírez et al., 2014, 2020), except for the new inclusion of the Bueno Basin. As for the phylogenetic position of the Bueno Basin samples, they all formed a single monophyletic clade with high bootstrap support that was more closely related to Diplomystids from Atlantic basins than to any other Diplomystid clade, yet strongly diverged.

The multilocus Bayesian population tree resulting from the starBeast3 analysis (hereafter population tree) was broadly consistent with the mtDNA genealogy. It showed that the Bueno population was phylogenetically closer to Atlantic basins (including Baker Basin) than to Pacific basins, despite its strong divergence, forming a clade with a high posterior probability. The separation between these lineages (Bueno vs Atlantic basins) was estimated at 202.3 kya (95 % HPD = 72.9–344.1 kya), well before the LGM. The recently described *D. incognitus* (Rapel, Mataquito, Maule, and Itata basins) was monophyletic and the sister lineage to the Biobío Basin, whereas *Diplomystes camposensis* (Toltén and Valdivia basins) was also found monophyletic and sister to the Imperial basin. The monophyly of *D. camposensis* in the population tree differed from the Maximum Likelihood mtDNA genealogy that showed the Valdivia basin as sister to a clade formed by the Toltén and the Imperial basins likely due to gene tree heterogeneity in the later, an aspect that is accounted for by the multispecies coalescent model in the population tree. The closer relationship between the populations of the Biobío Basin (including Laraquete and Carampangue basins) and *D. incognitus* shows that the species *D. nahuelbutaensis* (Biobío and Imperial basins) is polyphyletic. The divergence between the Imperial Basin and *C. camposensis* (Toltén and Valdivia basins) was 37.3 kya (95 % HPD = 1.3–80.3 kya), whereas the divergence between the Biobío lineage and *D. incognitus* (Rapel, Mataquito, Maule, and Itata) was 124.5 kya (95 % HPD = 44.5–214.3 kya). The MRCA for all populations was estimated at 336.7 kya (95 % HPD = 186–496 kya).

3.2. Species delimitation

The DELINEATE analyses showed strong support for both Bueno and Biobío lineages as new separate species from all other species in the family, regardless of the taxonomic scheme used for analyses. The Arratia scheme DELINEATE analysis had 15 lineages, with 11 lineages assigned to four known species, and the remaining four lineages with species affinities inferred. Within the constraints of the phylogeny and the known affinities, there were 137 possible partitions, with each partition corresponding to an alternate species assignment model. The partition with the highest constrained probability (i.e., the maximum likelihood estimate of the species delimitation model, with a probability of 0.235), placed three of the lineages of unknown species affinities ("Laraquete", "Biobío", "Carampangue") into one new species and the other ("Bueno") into a separate species. A total of 36 partitions were included in the 95 % confidence interval. The Azpelicueta scheme DELINEATE analysis in turn had 227 possible partitions that were compatible with the constraints. The maximum likelihood estimate of the species delimitation model converged with the previous analysis, as noted above, with a probability of 0.289. Under the Azpelicueta taxonomic view (Azpelicueta, 1994), the probability that the Biobío and the Bueno lineages correspond each to a new species were 0.974 and 0.969, respectively. Under the more conservative taxonomic view (Arratia, 1987), the probability that Biobío and Bueno lineages were new species were 0.731 and 0.686, respectively.

The resulting taxonomic scheme (i.e. distribution of species across basins) is depicted in Fig. 3. In addition to the DELINEATE analysis, we found that Diplomystids from the Bueno and Biobío basins have several important molecular differences that make them distinct to all other known Diplomystid species (Table 1). Individuals from the Bueno Basin exhibit the longest *CR* sequences ranging from 933 to 973 bp, while all other Diplomystids have sequences ranging from 883 to 933 bp in length. Furthermore, these individuals have eleven fixed nucleotide differences (nine in mtDNA genes + two in nuclear genes) and represent the only population with a fixed aminoacid difference in the *cytB* gene. The Biobío lineage, including samples from the small Laraquete and Carampangue basins, was also clearly distinct with several distinguishing characteristics described in detail in Table 1.

3.3. Description of the new species

Here we provide a molecular diagnosis to distinguish the two new proposed species from all other species within the family Diplomystidae. Table 1 provides a summary of the traits and character states that are diagnostic for the two new species contrasted to those from all other Diplomystids.

3.3.1. *Diplomystes arratiae sp. nov.*

Synonyms:

Diplomystes papillosus: Oliver, C. (1949): Bol. Soc. Biol. Concepción vol. 24 (55).

Diplomystes nahuelbutaensis: Muñoz-Ramírez et al. (2014). Mol. Phylogenet. Evol., vol. 73(2014) 146–160. Page 148, Table 1, samples from the Biobío Basin.

Diplomystes nahuelbutaensis: Muñoz-Ramírez et al. (2020). Neotrop. Ichththyol., vol. 18(1). Page 7, Fig. 3A, lineage from the Biobío Basin.

Diplomystes nahuelbutaensis: Arratia (1987). Bonner Zoologische Monographien 24. Figs. 12 and 16.

Diplomystes nahuelbutaensis: Arratia and Quezada-Romegialli (2017). PeerJ.2991. Figs. 3B, 8A, 11B, 12B, 15C, 17A, 17B.

Type Material: One individual from the Lia River, Carampangue Basin (Car_01) and one from the Laraquete River (also known as Las Cruces River), Laraquete Basin (Lar01) are stored at the Museo Nacional de Historia Natural, Santiago, Chile, under codes MNHN-ICT 7701 (Holotype) and MNHN-ICT 7700 (Paratype), respectively. Other material used are those published in Muñoz-Ramírez et al. (2014). A full list

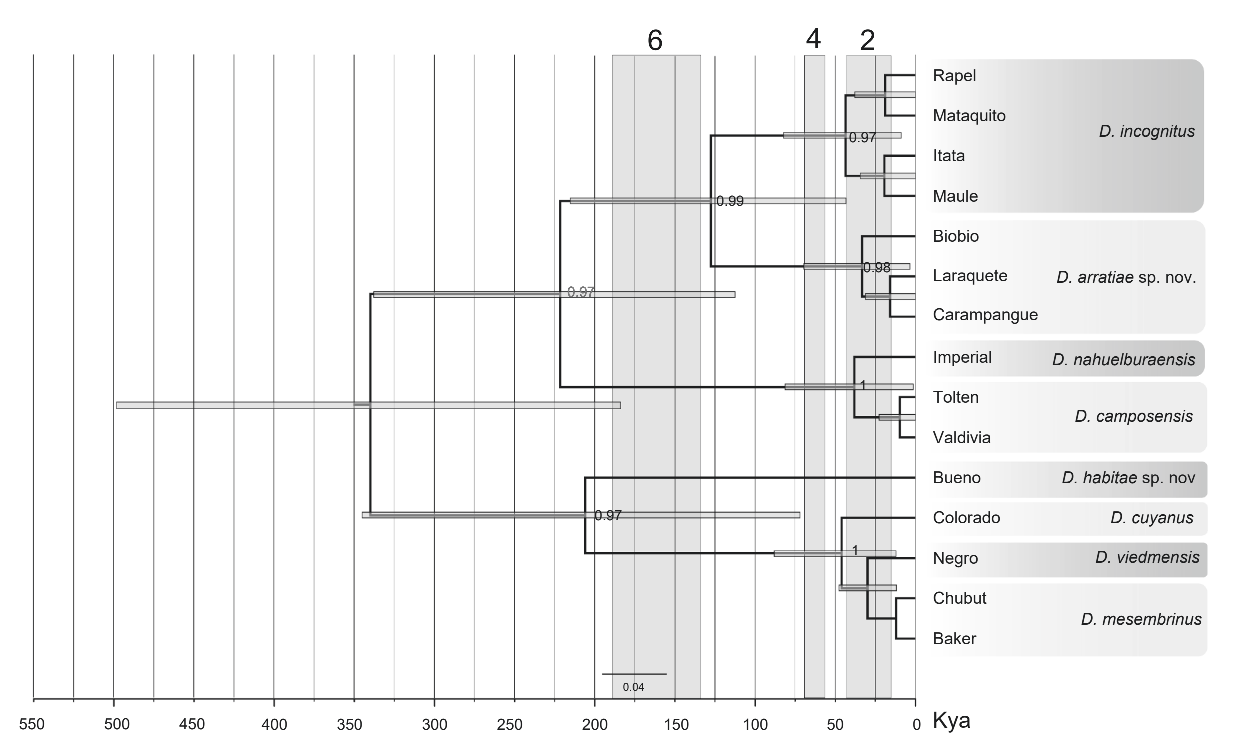

Fig. 3. Dated multilocus population tree obtained with StarBeast3. Number at nodes are the posterior probabilities. Shaded areas 2, 4, and 6 represent last glaciation events OIS-2, OIS-4, and OIS-6, respectively, following the Oxigen Isotope Stages in Singer et al. (2004).

Table 1
Variation in molecular characters between main clades of Diplomystidae. Diagnostic nucleotides were not searched for in positions of the alignment presenting gaps. The Atlantic clade contains all three species from the Atlantic basins Colorado, Negro, and Chubut plus the population from the Baker Basin. Positions for the diagnostic nucleotides are given in relation to the position in the alignment, not in the isolated sequence. At each position, two nucleotide bases are given within parenthesis indicating the nucleotide base that is unique to the species (first element) and the nucleotide base or bases present in all other species (second element). The last column shows the 41th aminoacid in the translated *cytB* gene.

Clade	*CR* Length	*cytB*	*CR*	*GH*	*S7*	*cytB* Aminoacid 41
D. incognitus	883–903	426(C/T), 690(G/A), 813(G/A), 846(T/C), 859(T/C), 885 (T/C), 1089(C/T)	388(T/A)	–	–	M
D. arratiae sp. nov.	883–884	387(C/T), 483(G/A), 552(G/A),918(C/A), 1065(C/T)	387(T/A), 545(C/G), 914(T/C)	–	–	M
D. nahuelbutaensis	883	–	–	–	–	M
D. camposensis	883–903	–	–	–	201(A/G) 327 (A/T)	M
D. habitae sp. nov.	933–973	121(G/A), 456(G/A), 471(T/C) 582(A/G), 798(C/T), 861 (G/A), 915(A/C)	27(T/(C or A)), 913(G/A)	304(A/G)	83(T/C)	V
Atlantic clade	892–933	243(G/A), 321(A/(T or C), 630(T/C)	205(C/T)	–	–	M

of individuals with lab codes and GenBank accession numbers are given in Table S1 in the Supplementary material. Holotype: MNHN-ICT 7701.

Etimology: The name of this species is dedicated to Gloria Arratia for her invaluable contributions to ichthyology in general and to the family Diplomystidae in particular.

Diagnosis: *Diplomystes arratiae* sp. nov. can be distinguished from all other species by presenting any of the following unique molecular features (diagnostic nucleotides are given in parenthesis): 1) mtDNA *cytB* diagnostic nucleotide differences at positions 387(C), 483(G), 552(G), 918(C), 1065(C); 2) mtDNA *CR* nucleotide differences at positions 387 (T), 545(C), and 914(T).

Distribution: The species is present in the Biobío Basin and in the coastal basins of the Carampangue and Laraquete rivers.

Taxonomic Remarks: All species within Diplomystidae are so far allopatric (Arratia and Quezada-Romegialli, 2017). This implies that the geographic location of collected specimens, in this case the basin, is informative for species identification.

ZooBank registration: urn:lsid:zoobank.org:act:69F8DEFD-2315-4B69-83FC-EF922EA665A8.

3.3.2. Diplomystes habitae sp. nov.

Type Material: Five individuals collected in February 9, 2021 stored in the Museo Nacional de Historia Natural, Santiago, Chile, under codes MNHN-ICT 7702–7706. Other three individuals that provided DNA sequences were released alive immediately after collecting them so no vouchers are available. DNA samples from all eight individuals included in this study are stored at the Laboratory of the first author of this study under Lab codes Bue_01, Bue_02, Bue_03, D1Bue, D2Bue, D3Bue, D4Bue, and D5Bue. Holotype: MNHN-ICT 7705. Paratypes: MNHN-ICT 7702–7704, and 7706.

Etimology: The name is dedicated to Evelyn Habit for her continuous contributions to the conservation and understanding of Chilean native freshwater fish.

Diagnosis: *Diplomystes habitae* sp. nov. can be distinguished from all other species of Diplomystidae by the presence of any of the following unique molecular features (diagnostic nucleotides are given in parenthesis): 1) mtDNA *cytB* diagnostic nucleotide differences at positions 121 (G), 456(G), 471(T), 582(A), 798(C), 861(G), and 915(A); 2) mtDNA *CR* nucleotide differences at positions 27(T) and 913(G); 3) *GH* nucleotide differences at position 304(A), 4) gene *S7* nucleotide difference at position 83(T), 5) *CR* sequence length ranging from 933 to 973 bp, and 6) a valine at position 41 when translating the *cytB* gene to aminoacids.

Distribution: The species is currently known only from the Bueno River in the Bueno Basin (Fig. 1).

Taxonomic Remarks: The five individuals kept as vouchers from the Bueno Basin show a long and continuous lateral line typical of the genus *Diplomystes*, so they were assigned to this genus following Arratia and Quezada-Romegialli (2017). The inclusion of this species to the genus *Diplomystes* makes the genus paraphyletic relative to *Olivaichthys*. Therefore, the genus *Diplomystes* should be used instead of *Olivaichthys* for species from Atlantic basins and the Baker Basin.

ZooBank registration: urn:lsid:zoobank.org:act:9AEB96F9-62D8-476B-980C-662FBA8611B1.

Regarding mean genetic distances between species, the two new species were amongst the most diverged lineages (Table 2). For instance, considering the Kimura 2-parameter mean distance, the distance between *D. arratiae* sp. nov. and its sister species *D. incognitus* was 0.228 (2.28 %), whereas the distance between *D. habitae* sp. nov. and *D. viedmensis* was 0.155 (1.55 %). These distances were substantially higher than those between other sister species. For instance, the distance between *D. viedmensis* and *D. mesembrinus* was 0.00132 (0.13 %) and the distance between *D. camposensis* and *D. nahuelbutaensis* was 0.0115 (1.15 %).

3.4. Phylogenetic diversity

Phylogenetic diversity (PD) provided by the Bueno and Biobío basins were the two that most contributed to the PD of the entire family Diplomystidae (i.e highest and second highest, respectively) as these were the two basins that caused the greatest reduction in PD when removed (Fig. 4). Bueno Basin contributed with a 13.1 %, whereas Biobío contributed 8.7 %; all other basins contributed within the range of 0.7 % to 4 % to the PD of the family.

4. Discussion

4.1. Survival through the LGM

The general influence of Pleistocene glaciations, and the LGM in particular, on the distribution and genetic configuration of the freshwater biota from southern South America has long been recognized (Astorga et al., 2022; Ruzzante et al., 2006, 2020; Vera-Escalona et al., 2015; Xu et al., 2009; Zemlak et al., 2008). Drainage reversals, river capture and glacial refugia are among the known events that could have shaped the evolutionary history and distribution of Patagonian species. Our phylogenetic results support the hypothesis that Diplomystids from the Bueno basin survived the last glaciation *in situ* as they diverged from closest relatives in Atlantic basins long before the LGM (c.a. 202 kya). Indeed, divergence time estimates suggest that the species from the Bueno Basin may have survived several of the last glaciations including OIS-2, OIS-4, and OIS-6, as all of these events occurred within the last 200 ky ago (Rabassa and Clapperton, 1990). Given that the geological evidence suggests that the glacial ice sheet in the LGM covered most part of the Bueno Basin upstream (Clapperton, 1993), refugia should have been located downstream in rivers and tributaries closest to the coast, highlighting the importance of coastal and lower basin tributaries in the

Table 2

Mean genetic distances between species for the *cytB* gene alignment. Values above the diagonal were calculated based on the Kimura 2-parameter model, whereas the values below the diagonal are P-distances.

K2P\P-distance	1	2	3	4	5	6	7	8
1. *D. incognitus*	–	0.0228	0.02415	0.02323	0.03162	0.0321	0.03265	0.0333
2. *D. arratiae* sp. nov.	0.02331	–	0.02046	0.01991	0.02743	0.02846	0.02846	0.02883
3. *D. nahuelbutaensis*	0.02473	0.02086	–	0.01137	0.0269	0.02802	0.02802	0.02997
4. *D. camposensis*	0.02379	0.02031	0.0115	–	0.02438	0.02508	0.02533	0.02616
5. *D. mesembrinus*	0.03263	0.02816	0.02761	0.02498	–	0.00611	0.00132	0.01528
6. *D. cuyanus*	0.03313	0.02925	0.02879	0.02572	0.00615	–	0.00704	0.01568
7. *D. viedmensis*	0.03372	0.02925	0.02879	0.02599	0.00132	0.00709	–	0.01621
8. *D. habitae* sp. nov.	0.03442	0.02965	0.03085	0.02685	0.01549	0.01591	0.01645	–

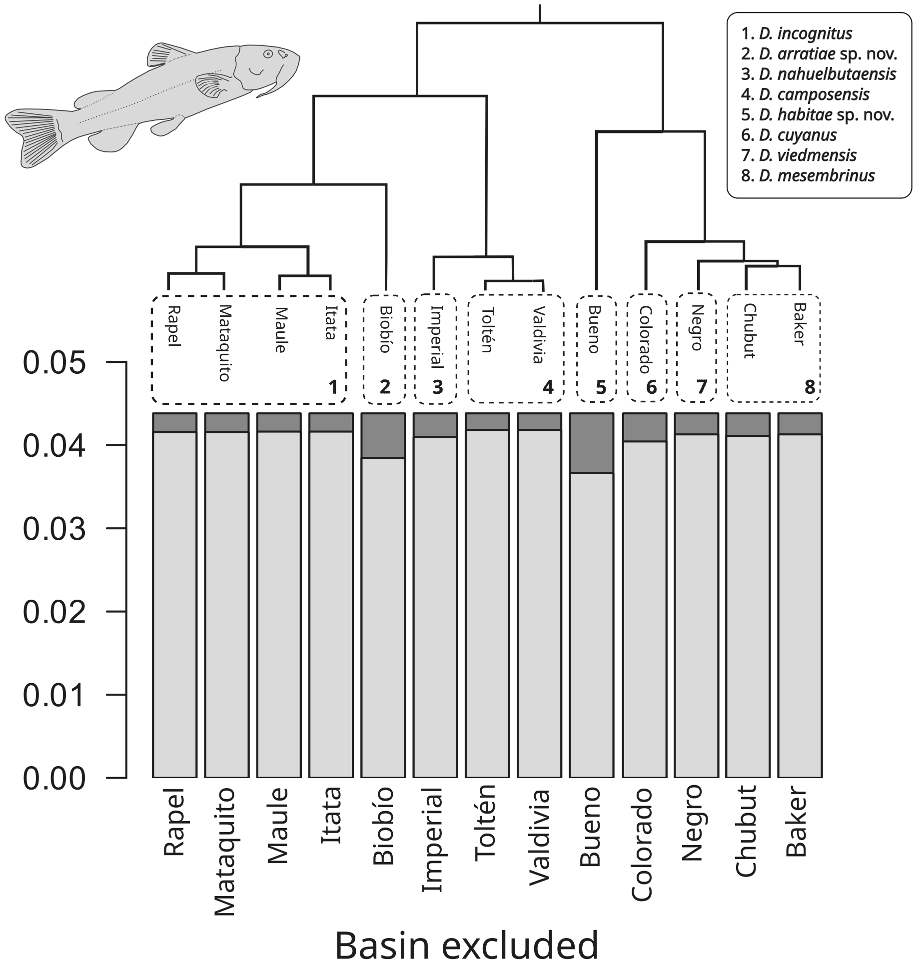

Fig. 4. Contribution of different basins to the phylogenetic diversity (PD) of the catfish family Diplomystidae. The contribution of each basin was calculated as the portion of PD that is lost (dark grey area in the bar plot) when a given basin is excluded from the PD calculation of the entire family. The light grey areas in the barplot show the PD that is left when a given basin is excluded. Bueno and Biobío are the basins that most contribute to the PD of the family. The population tree is given at the top for reference and dashed rectangles show how basins are grouped into species after the species delimitation analyses. Coastal basins were not included in this plot.

long-term survival of Diplomystids and other freshwater species in Patagonia.

Other freshwater taxa from the same area have also shown important levels of genetic divergence between the Valdivia and Bueno basins supporting some degree of isolation (Crandall et al., 2002; Ruzzante et al., 2020; Unmack et al., 2009; but see Unmack et al., 2012). For example, populations from the crab *Aegla abtao* differed 2.6 % between these basins (Crandall et al., 2002), whereas populations of *Trichomycterus areolatus* differed ca. 4.3 % (Unmack et al., 2009). Populations of the freshwater *Percichthys trucha* were also clearly differentiated between these basins based on microsatellites (Ruzzante et al., 2020), although this differentiation was lower and only evident after four levels of hierarchical Structure analysis. These examples suggest that the Bueno Basin could have remained relatively isolated from other basins during the last glaciations. Indeed, in the case of Diplomystids from Bueno, the deep divergence shown in both the population tree and the mtDNA genealogy strongly reject north–south inter-basin migrations in *Diplomystes* from northern Patagonia. Evidence for inter-basin migration has been provided for other Diplomystid species between rivers from Central Chile, but these have been facilitated by artificial canals (Muñoz-Ramírez et al., 2015). It is possible that some north-south inter-basin connections could have been possible between some other basins in southern Chile. For instance, the Imperial, Toltén, and Valdivia basins show low divergence in the population tree and have a few shared mtDNA haplotypes. However, further research is needed to understand whether natural (e.g. river capture) or human-mediated (e.g. artificial canals) mechanisms are responsible for the low divergence between these basins.

4.2. Speciation-based delimitation and its value for taxonomy in freshwater systems

River basins are similar to islands for strict freshwater species and as such, species that cannot disperse between basins are expected to develop genetic divergence over time. For these species, equating genetic divergence with species limits would be a mistake because some of these populations will not develop full reproductive isolation until a sufficient amount of time has passed. Species delimitation methods that use the multispecies coalescent (MSC) are sensitive to detect genetic structure as evidence of species limits. Therefore, testing species limits with the multispecies coalescent for strict freshwater species, where inter-basin structure is expected between populations of the same species, can be challenging and may inevitably cause overspliting, unless some aspects of the speciation process can be accounted for. In this study we used a recently developed approach (Sukumaran et al., 2021) that builds on the MSC model and incorporates some biological realism to the problem of species delimitation by modeling the speciation process as a protracted process. By doing this, we did not equate all genetic structure present between populations to species. Instead, we used existing knowledge of species limits to inform the testing process.

The species delimitation procedure provided strong evidence for two new species of Diplomystidae, which we described using molecular diagnostic characters. *Diplomystes habitae* sp. nov., from the Bueno Basin, is the sister species to all Diplomystids from Atlantic basins, whereas *D. arratiae* sp. nov., from the Biobío, Carampangue, and Laraquete basins, is the sister species to the recently described *D. incognitus*. Although the Biobío lineage was suggested in Muñoz-Ramírez et al. (2014) as a separate species from *D. nahuelbutaensis* (Imperial Basin), subsequent work (Arratia and Quezada-Romegialli, 2017) have maintained the population from Biobío as part of *D. nahuelbutaensis* due to the morphological similarities between these populations and the lack of sufficient material (adults) to conduct further analyses and comparisons. Therefore, based on morphological similarity these species could be considered as cryptic. However, *D. arratiae* sp. nov. presents important molecular differences compared to the population from the Imperial Basin to which it was previously considered conspecific (Table 1). Furthermore, its phylogenetic position, closer to *D. incognitus* than to the population of the Imperial Basin (*D. nahuelbutaensis* sensu stricto), strongly support its status as a different species. Similarly, the new *D. habitae* sp. nov. presents some remarkable molecular differences. For instance, before the present study, none Diplomystidae sequenced for the mtDNA gene *cytB* ever presented phylogenetically informative variation in the translated aminoacid sequence (e.g. mostly monomorphic aminoacid sequence) (Muñoz-Ramírez et al., 2014, 2016, 2020). The fact that *D. habitae* sp. nov. present at least one phylogenetically informative amino acid difference supports the uniqueness and deep divergence of this species.

Although species delimitation methods are routinely being used for the discovery of new species, the description of the new entities based only on molecular data is still a debated issue (Renner, 2016; Seberg et al., 2003; Tautz et al., 2002). We acknowledge that species descriptions should ideally use multiple sources of evidence (e.g. morphology, DNA, ecology) to describe new species. However, under some scenarios, it is challenging to obtain multiple sources of variation either because there is little morphological variation (as is the case for cryptic species), or the only available specimens are immature individuals that lack diagnostic characters. For such cases, DNA sequence information can contribute valuable diagnostic characters and could serve as the backbone of a taxonomic description (Jörger and Schrödl, 2013). Diplomystids are well known for their subtle morphological variation between species, most of it only being observable in adults, while juveniles present unmatched ontogenic variation (Arratia and Quezada-Romegialli, 2017). Under this scenario, the use of DNA markers is a valuable tool both to delimit species and to provide diagnostic characters for species identification (Renner, 2016). Our Diplomystid specimens from the Bueno Basin, as well as most *Diplomystes* that are occasionally collected in fish surveys (e.g. Muñoz-Ramírez et al., 2010; Beltrán-Concha et al., 2012) are juveniles, making diagnostic molecular characters of paramount relevance in the context of species descriptions.

The results of the speciation-based species delimitation test, along with the deep genetic divergence of the new species described here strongly support their validity as new species. Therefore, the desire of obtaining adults for a morphological diagnosis should not preclude the task of describing and naming these species, especially if the efforts to sample additional *Diplomystes* individuals can be extenuating and the success of those efforts are not guaranteed. An example of how difficult the sampling of *Diplomystes* can be is the survey study reported in the Bueno Basin by Soto and Arismendi (2005), where 47 rivers were sampled with none *Diplomystes* being reported. Nevertheless, in an ever-improving effort to refine the taxonomic knowledge of the family future studies could improve our species descriptions by adding morphological descriptions and diagnoses if adult specimens become available.

4.3. Conservation remarks

Species represent different amounts of evolutionary history, reflecting the tempo and mode of divergence across the Tree of Life (Vane-Wright et al., 1991). The extinction of a species in an early diverged, monotypic or species-poor clade should therefore result in a greater loss of biodiversity than that of a young species with many close relatives (Mace et al., 2003). Phylogenetic diversity (PD) is a measure of diversity that accounts for the evolutionary history of lineages (Faith, 1994), assigning higher diversity, and thus greater conservation value, to areas harboring older lineages or species. The Bueno and Biobío basins were the two basins that most contributed to the PD of the entire family Diplomystidae (i.e. highest and second highest, respectively). This is likely due to both their microendemism and a deeper branching history (i.e. they separated earlier from their sister species). If Diplomystids from these basins went extinct, a large portion of the family's PD (ca. 22 %) would be lost.

Currently, the major threats for freshwater fish in Patagonia are the presence of invasive species such as salmonids and didymo, pollution by inefficient wastewater depuration (from salmon farming, dairy industry, and urban centres) and the alteration of river networks via increased dam construction for hydroelectric power production (Habit and Cussac, 2016). These stressors are predicted to increase in importance, especially dam construction due to ever-increasing power demands (Díaz et al., 2019). In addition to these general threats, low fecundity (Vila et al., 1996), high habitat specificity (Habit et al., 2009, Oyanedel et al., 2018) and low genetic diversity (Muñoz-Ramírez et al., 2014, 2016) are all biological characteristics of Diplomystids that can exacerbate their vulnerability and make them particularly sensitive to these stressors. We highly encourage activities to promote and support the conservation of these species and urge stakeholders to work in developing an integrated basin management strategy to decrease negative activities and prioritize education and conservation.

CRediT authorship contribution statement

Carlos. P. Muñoz-Ramírez: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Nicole Colin:** Conceptualization, Data curation, Resources, Writing – review & editing. **Cristian B.**

Canales-Aguirre: Data curation, Resources, Writing – review & editing. **Aliro Manosalva:** Data curation, Writing – review & editing. **Ruby López-Rodríguez:** Data curation, Writing – review & editing. **Jeet Sukumaran:** Methodology, Writing – review & editing. **Konrad Górski:** Conceptualization, Data curation, Funding acquisition, Investigation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All sequences used are available at GenBank with accession numbers detailed in the supplementary Table S1. Sequence alignments used for analyses are available at Dryad (https://doi.org/10.5061/dryad.hqbzkh1nb).

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Appendix A. Supplementary data

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