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Evolution of trans-Andean endemic fishes of the genus *Cheirodon* (Teleostei: Characidae) are associated with chromosomal rearrangements

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Abstract

Background: Among Neotropical fishes, the family Characidae is highly diverse and speciose and its taxonomy is not completely resolved. In Chile, the family is represented by five species, all within the genus *Cheirodon*, of which *C. pisciculus, C. galusdae, C. kiliani*, and *C. australe* are endemic, while *C. interruptus* is introduced. We compared chromosomal markers in order to appreciate the taxonomy and evolution of these trans-Andean fishes.

Results: The specimens were photographed in stereomicroscope to observe the ventral protrusive teeth and cusps for morphological analysis and species identification. All of the species analysed had equally diploid chromosome number 2n = 50, with karyotypes dominated by high number of acrocentric chromosomes as compared to those of other members of Cheirodontinae. The distribution of heterochromatin was inconspicuous and was similar in all the species. The number of active NORs (nucleolus organizer regions) was polymorphic, with the greater number of them in *C. kiliani* and *C. galusdae*. The location of 5S and 18S rDNA ranged in number and position, showing two sites in different chromosomes. The fluorescent in situ hybridization with telomeric probe did not reveal interstitial sites in all analysed species.

Conclusions: The comparative analysis of karyotypes and morphological markers revealed a biogeographic pattern of distribution, with the species that occur in the southern region forming one group and those in central and northern Chile forming another.

Keywords: Andean cordillera, Endemism, Freshwater fishes, Chromosome, Sea level, South America

Background

Although South America is typically megadiverse in biodiversity, the hydrogeography of Chile and its natural isolation due to the Andean cordillera makes an exception. These patterns have influenced the evolutionary history of its fish fauna, which is highly endemic and inconspicuous in morphology and coloration what makes them difficult to distinguish [28]. Among the diverse fishes of the Neotropical region, the family Characidae is one of the most complex groups in terms of its taxonomy [13]. In Chile, the family is represented by a single genus, namely *Cheirodon* [6, 27]. Its species are morphologically similar, which makes it difficult to distinguish them [21].

Five species of *Cheirodon* occur in Chile, they are distributed allopatrically in a North to South direction: *C. pisciculus* (Girard, 1855), *C. galusdae* [4], *C. kiliani* [3], and *C. australe* (Campos, 1992) are endemic to Chile [3], whereas *C. interruptus* (Jenyns, 1842) is native to the Atlantic basins of Argentina, Uruguay, and Brazil (Malabarba 2003). It may have been introduced to Chile in the 1960s along with the larvae of Pejerrey, *Odontesthes bonariensis*

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(Valenciennes, 1835) [1, 3]. In fact, the first records of *C. interruptus* in Chile were from Peñuelas Lake, an artificial lake where *O. bonariensis* was likely first reared [20].

The Chilean *Cheirodon* species have already been the subject of morphological and molecular analyses [3, 14, 21], to define their phylogenetic relationships. These analyses tended to group geographically distant species, such as *Ch. australe* and *Ch. interruptus* (the introduced species) together [3, 14, 21]. In addition, some of the morphological characters used in those analyses can vary inter- and intra-specifically, which may lead to erroneous identification of *Cheirodon* populations [13]. Furthermore, the DNA sequence analyses used in Chilean *Cheirodon* did not include *Ch. pisciculus* nor *Ch. galusdae*, respectively, [14], so the phylogenetic relationships between the trans-Andean species remain unclear.

Due to the difficulties in sampling as well as their small size making difficult to obtain chromosomes, there is no cytogenetic information about the *Cheirodon* except the 2n and karyotypes of the Cheirodontinae genera *Odontostilbe* and *Serrapinnus* only [22, 25, 30]. These data may be used to generate cytotaxonomic information and detect evolutionary relationships to better understand the diversity of the genus *Cheirodon* [2]. Accordingly, in order to infer the taxonomy and evolution of the trans-Andean Characidae, in the present work we elucidated the conventional and molecular cytogenetic characteristics of its five species and contrast them with available morphological, molecular and cytogenetic data for the group.

Methods

Biological materials and sampling areas

A total of 15 individuals were collected for each one of the following species in different areas: *Ch. interruptus* (Estero Marga-Marga, Viña del Mar, V region, Chile, coordinates 19H 0263800.90 UTM E–6342166.09 UTM S), *Ch. pisciculus* (Río Angostura, Hospital, Metropolitan region, Chile, coordinates 19H 0338122.17 UTM E–6250729.69 UTM S), *Ch. galusdae* (Río Andalién, Concepción, VIII região, Chile, coordinates 18H 0682055.00 UTM E–5925386.00 UTM S), *Ch. kiliani* (Rio Calle-Calle, Antilhue, XIV region, Chile, coordinates 18H 0674363.00 UTM E–5592270.00 UTM S) and *Ch. australe* (Lagoa La Poza, desembocadura Lago Llanquihue, Puerto Varas, X região, Chile, coordinates 18G 0679651.18 UTM E–5428179.56 UTM S). The capture of the specimens was authorised by the Subsecretaría de Pesca y Acuicultura del Gobierno de Chile (SUBPESCA) and by the Servicio Nacional de Pesca y Acuicultura del Gobierno de Chile (SERNAPESCA), Authorization No. 740.

Representative individuals of different morphological types (two males and two females for each specie) were anesthetised, labelled, and preserved in absolute ethanol for taxonomic identification. The specimens were photographed using a Leica M205C Stereomicroscope to observe the ventral protrusive teeth and cusps. The analysed material was deposited in the Department of Zoology of the Universidade Federal de Rio Grande do Sul, Brazil, under vouchers UFRGS 22295 (*Ch. interruptus*), UFRGS 22296 (*Ch. galusdae*), UFRGS 22297 (*Ch. pisciculus*), UFRGS 22298 (*Ch. kiliani*), and UFRGS 22299 and UFRGS 22300 (*Ch. australe*).

Chromosome preparations

Chromosome preparations were obtained from fin tissue regenerated for three days for animals acclimated at 20 °C in aquarium. The regeneration occurs in situ, therefore, does not require special incubation conditions (20 °C), more than those that do the fish to remain activefollowing the protocol of [29]. This technique was employed here due to the small size of the animals (< 3.0 cm). To obtain the number, size, and morphology of chromosomes, the slides were stained with Giemsa diluted in 5% phosphate buffer ($KH_2 PO_4 + Na_2 HPO_4$) pH 6.8 for 10 min and then washed in running water and air dried. To visualize heterochromatic regions, we used the C-band technique by Sumner [23], adapted by Lui et al. [12]. The nucleolus organizer regions (Ag-NORs) were visualised according to [8].

Fluorescence in situ hybridisation (FISH) was utilised to detect chromosomal location of rDNA (18S and 5S) sites and telomeric sequences, according to the protocol of Pinkel et al. [18], under high stringency (2.5 ng/μL probe, 50% formamide, 2× SSC, and 10% dextran sulphate). The 18S rDNA probe was amplified from the DNA of *Prochilodus argenteus* (Spix & Agassiz, 1829) [7], using the primers NS1 5′-GTAGT CATATGCTTGTCTC-3′ and NS8 5′-TCCGCAGGTTC ACCTACGGA-3′ [31]. The 5S probe was obtained from DNA amplification of *Leporinus elongatus* (Valenciennes, 1850), using the primers A 5′-TACGCCCGATCTCGTCCGATC-3′ and B 5′-GCTG GTATGGCCGTAGC-3′ [15]. The telomere probe (TTAGGG) n was amplified using the primers (TTAG GG)$_5$ and (CCCTAA)$_5$, in the absence of a DNA template, according to [9]. The chromosome mapping of the 18S probe was achieved using the Kit Biotin Nick Translation (Roche), and the 5S and telomeric mappings were performed using Kit Dig Nick Translation (Roche), following the manufacturer's recommendations. Signal detection was performed with digoxigenin conjugated to rhodamine (18S probe) and streptavidin conjugated to FITC (5S and Telomeric probes) (Roche Applied Science).

All analyses used mitotic chromosomes subjected to sequential treatment to optimize the use of the chromosome material. The treatments were applied in order of increasing invasiveness: Giemsa stain, double-color FISH with 18S and 5S rDNA probes, C-bands and silver staining of nucleoli organizing regions (Ag-NORs).

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Karyotype analysis

Conventional chromosome preparations were observed under optical microscope, Olympus® BX41. FISH metaphases were analysed using an epifluorescence microscope with appropriate filters. Images were captured using a camera, CCD DP71 12 mp, with the software DP controller (Olympus). At least 10 specimens, five from males and five from females, were analysed for each species. Adobe Photoshop v. CC (2015) software was used to karyotype arrange and Easy Idio v. 1.0 software was used to construct ideograms. Chromosomes were classified following Levan et al. [11] and karyotypes organised in decreasing order of size.

Results

The adaptation of the technique used by [29] produced high-quality metaphases from regenerated fin tissue of different species of *Cheirodon*. The representatives of each species were returned to their localities of origin, except those deposited in museum. All species under study possessed the diploid chromosome number 2n = 50 and karyotypes varying subtly in proportion among metacentric (m), submetacentric (sm), subtelomeric (st) and acrocentric (a) chromosomes (Table 1, Fig. 1).

Specie-specific variation was observed for FISH marker patterns of the 18S and 5S rDNA sites, which were located into different acrocentric chromosomes or in synteny on the same chromosome. The 18S site patterns was associated with the cytotaxonomic groupings.

The 18S signals were observed in the long arms (q) and the 5S in the short arms (p), except in the pairs 16, 21, and 25 in *Ch. austral*, in which the 5S region was located in the q arm (Fig. 2). Intraspecific variation was observed too with presence or absence of markers in one or two chromosomes in the same pair, especially in *Ch. australe*, the most polymorphic specie for these markers (Fig. 2, Fig. 4).

In addition, the Ag-NORs enabled us to detect the activity level of the NORs. The highest levels of relative activity were found in *C. galusdae* and *C. kiliani* (Fig. 2). In these species, the location of the 18S rDNA is polymorphic with 2 pairs of chromosomes carrying these sequences, one of them being syntenic with 5S rDNA (Fig. 2c and d).

FISH with the (TTAGGG) n probe revealed only telomeric positive sites (Fig. 3a). The C-band pattern was very poor and similar in terms of quantity and distribution in karyotypes of different species (Fig. 3b). Heteromorphic sex chromosomes were not identified in the studied species at the level of analysis applied.

The ideograms allowed us to summarise and compare the general similarities and differences in the karyotypes of the five species as compared to their geographical distribution. The ideograms (Fig. 4) allowed us to summarise and compare the general similarities and differences in the karyotypes of the five species as compared to their geographical distribution. Moreover, these data are complementing with the shape of teeth and number of ventral procurrent rays, morphological characters used for the identification of the species.
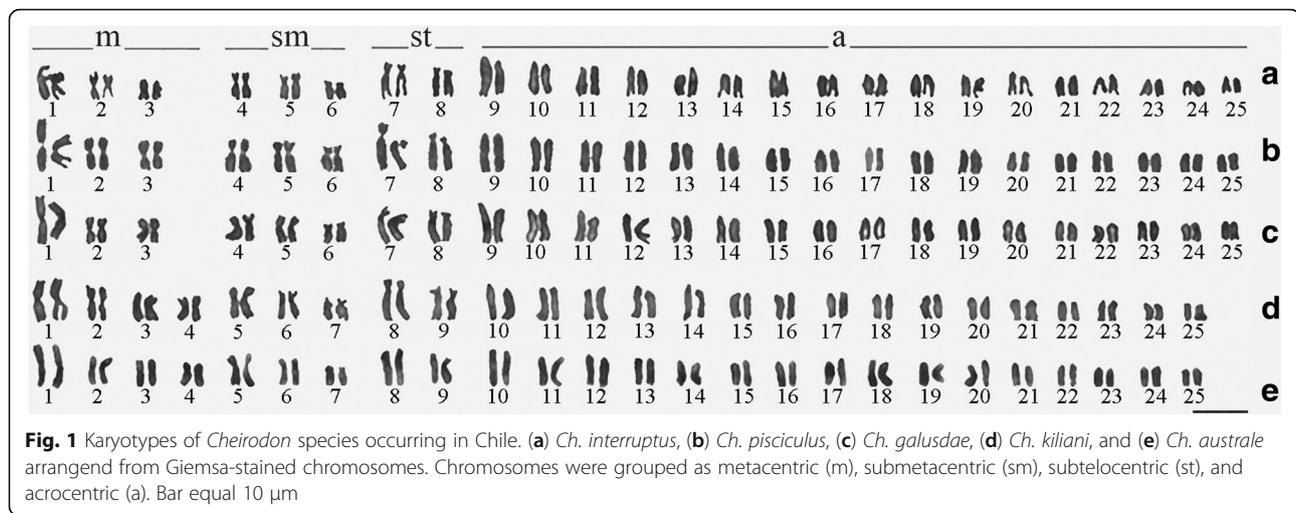
Discussion

The karyotype of the five species under study had invariably the same 2n = 50, dominated by acrocentric chromosomes. Karyotypes were probably differentiated from other Characidae due to multiple inversions and/or chromosomal translocations. This very similar pattern (karyotype structures) is consistent with the monophyletic status hypothesized for the Chilean *Cheirodon* [3, 4, 13, 14]. This finding supports the comparative and evolutionary analyses of the Chilean *Cheirodon*, as well as phylogenetically related groups that occur on the other side of the Andean cordillera.

The karyotype macrostructure revealed differences between particular species. This differentiation was accompanied by their endemism in regions or particular watersheds. An ancient period of reproductive isolation compatible with the hypothesis of fluctuation as was suggested to explain the phylogeography of a widespread Chilean catfish *Trichomycterus areolatus* [26] may explain the observed patterns,. Much of the genetic

Table 1 Chromosomal markers in species of genus *Cheirodon*

Species	2n, NF and Karyotype	18S	5S	Sinteny p: 5S/q: 18S	Ag-NORs[+]
C. interruptus	2n = 50, NF = 66 6 m + 6sm + 4st + 34a	q:13, 17, 21	p: 13, 14, 18, 19, 24	13	q:13, 17, 21
C. pisciculus	2n = 50, NF = 66 6 m + 6sm + 4st + 34a	q:10, 11, 13, 15	p: 11, 17, 19, 21	11	q:11, 13, 15
C. galusdae	2n = 50, NF = 66 6 m + 6sm + 4st + 34a	q:11, 13	p: 11, 15, 16, 17, 21	11	q:11, 13
C. kiliani	2n = 50, NF = 68 8 m + 6sm + 4st + 32a	q:12, 14	p: 13, 14, 17, 20, 21, 22	14	q:12, 14
C. australe	2n = 50, NF = 68 8 m + 6sm + 4st + 32a	q:12, 13, 14, 15, 18	p: 10, 11, 13, 14, 15, 17, 21, 25 and q: 16, 21 and 25	13	q:10,12, 13, 14

2n = chromosome number, NF = number or chromosome arms, *q* = long arm, *p* = short arm, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric

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Fig. 1 Karyotypes of *Cheirodon* species occurring in Chile. (**a**) *Ch. interruptus*, (**b**) *Ch. pisciculus*, (**c**) *Ch. galusdae*, (**d**) *Ch. kiliani*, and (**e**) *Ch. australe* arrangend from Giemsa-stained chromosomes. Chromosomes were grouped as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a). Bar equal 10 μm

variation found in the endemic fishes of the Atlantic forest along the Brazilian coast may be explained by the population structure promoted by the ancient drainage systems of the Pleistocene [24]. Groups of fishes such as the *Characidium* genus underwent allopatric speciation. In these species, sex chromosome differentiation was associated with the hydrographic basins in which they evolved [19].

Chilean *Cheirodon* form two different groups according to their karyotype macrostructure, corresponding also with the groups based in their dental cusps which were analysed by [3].. A slight variation in the karyotypes is associated with the presence and/or absence of p arms in one of the pairs of small chromosomes. In fact, *Ch. australe* and *Ch. kiliani*, which are found in the south, share the same karyotypes composed of 8 m + 6sm + 4st + 32a and the same FN = 68. However, *Ch. galusdae*, *Ch. pisciculus*, and the introduced species *Ch. interruptus*, which are distributed in the north, possess the same karyotypes composed 6 m + 6sm + 4st + 34a chromosomes and FN = 66. This differences may be attributed to pericentric inversions, given that the 2n is the same. Nevertheless, there are no remnants of telomeric sequences evidenced by FISH in the pericentromeric regions of their karyotypes. The data support the hypothesis that these chromosomal rearregements are ancient events associated with the speciation of *Cheirodon*, which probably occurred prior to the formation of the Andean cordillera during the Tertiary Period and was likely influenced by sea-level fluctuation.
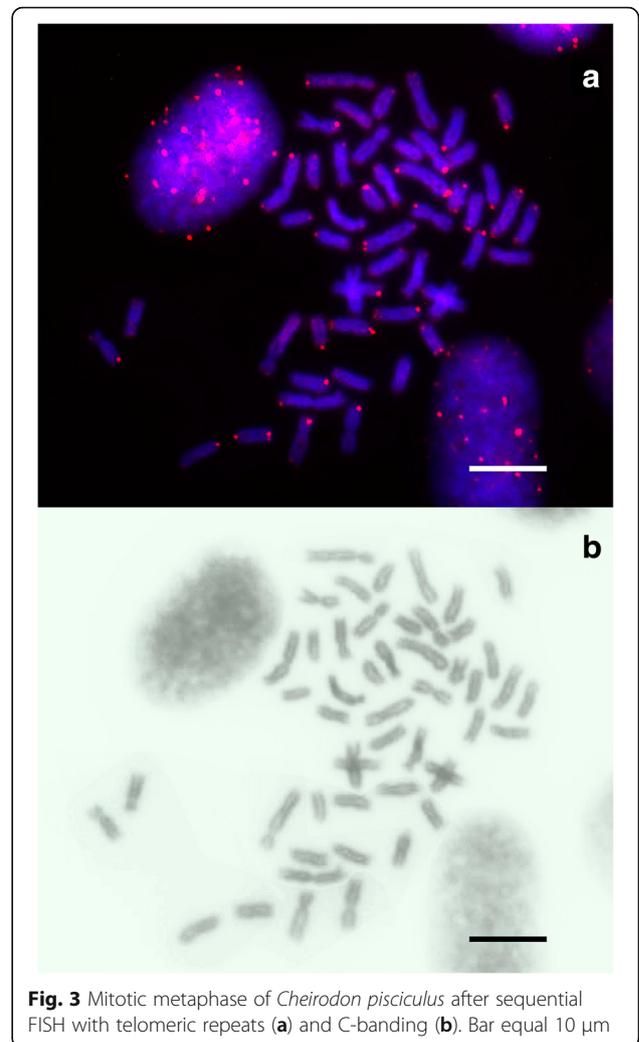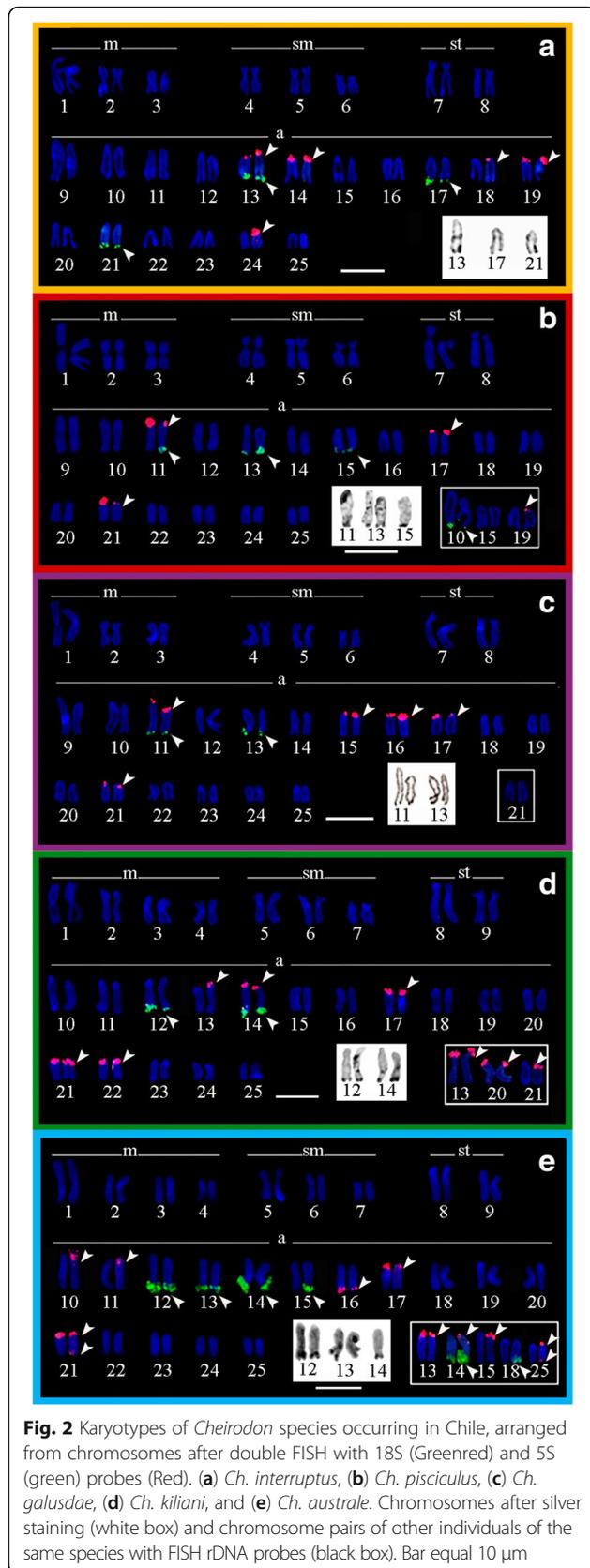
These patterns, revealed by the cytogenetic analysis of all species of *Cheirodon* occurring in Chile, suggest that differences were associated with the geographic distribution of species. Morphological [3] and molecular [14] studies on *Cheirodon* tended to group species as *Ch. australe* and *Ch. interruptus* together,

even though they are found in different geographic regions. We consider that this incongruence between the cytogenetic, morphological and molecular data reflects a lack of morphological characters for each of these species, being all of them differentiated mainly by the combination of the same characters, perhaps, by local adaptations [21]. In addition, the absence of chilean *Cheirodon* species in previous phylogenetic analyses makes it difficult to reach correct conclusions [14, 21].

The variations and polymorphisms that our data indicated, especially the activity and localisation of major ribosomal cluster (18S rDNA) and 5S rDNA chromosome mapping, suggest that all these populations demonstrate a dynamic variation in karyotype microstructure. Many teleost fishes (72% of species) possess just one chromosome pair bearing NORs as reviewed by Gornung [5]. Thus, the rDNA polymorphisms here observed, suggest that karyotype diversification in the Chilean *Cheirodon* species was produced by more than pericentric inversions; probably transpositions of ribosomal regions between non-homologous chromosomes also occurred. Since inversions and translocations change the order of the loci in the chromosomes, these rearrangements can reduce the genetic flow between populations by reducing fertility in 50% of the gametes of the heterozygous hybrids [10].
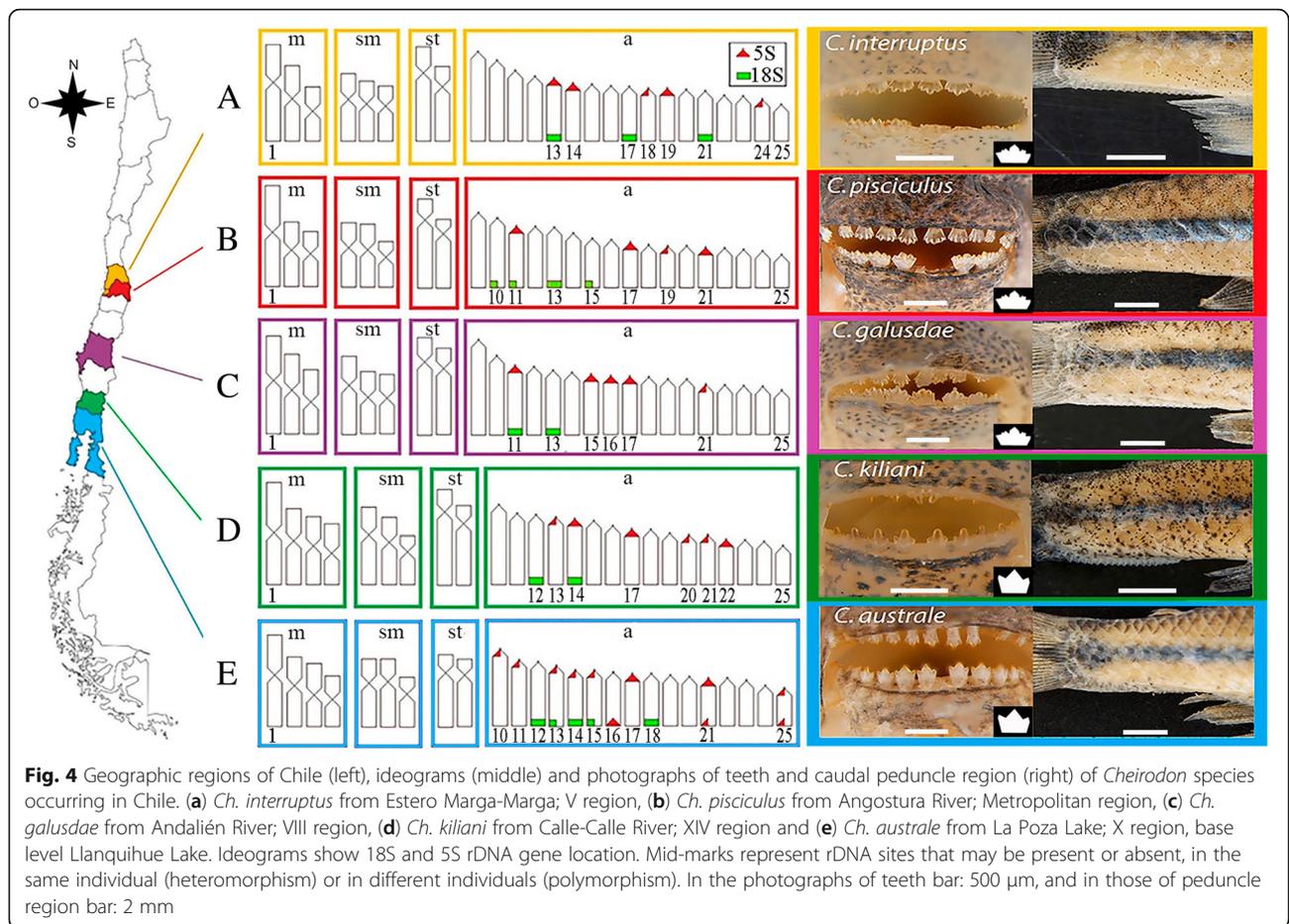
Similar karyotypes and some chromosomal homologies were found between species of *Cheirodon* and other genera of Cheirodontinae, such as *Serrapinus* and *Odontostilbe* [13]. For example, the first pair of large metacentric chromosomes present in karyotypes of five species of Chilean *Cheirodon* appears to be a characid chromosome marker [16].

Alternatively, the greatest karyotype differences among *Serrapinus*, *Odontostilbe*, and *Cheirodon* refers

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Fig. 2 Karyotypes of *Cheirodon* species occurring in Chile, arranged from chromosomes after double FISH with 18S (Greenred) and 5S (green) probes (Red). (**a**) *Ch. interruptus*, (**b**) *Ch. pisciculus*, (**c**) *Ch. galusdae*, (**d**) *Ch. kiliani*, and (**e**) *Ch. australe*. Chromosomes after silver staining (white box) and chromosome pairs of other individuals of the same species with FISH rDNA probes (black box). Bar equal 10 μm

Fig. 3 Mitotic metaphase of *Cheirodon pisciculus* after sequential FISH with telomeric repeats (**a**) and C-banding (**b**). Bar equal 10 μm

to number and size of a chromosomes, 2n. The karyotypes of the *Cheirodon* species analysed have 2n = 50 and FN = 66 to 68. In contrast, *Odontostilbe* and *Serrapinus* have a 2n = 52 and FN = 79 to 104 for *Serrapinus* and FN = 89 to 101 for *Odontostilbe* [17, 22, 25, 30]. Therefore, the karyotypes of species described in this study, with large number of acrocentric chromosomes varying in relative size, seems to be a particular pattern for the representatives of the genus *Cheirodon*.

However, karyotypes of *Ch. australe* and *Ch. kiliani* had one more metacentric chromosome pair and fewer acrocentric chromosomes than that of the other species of the Chilean *Cheirodon*. This evidence suggests that karyotypes of *Cheirodon* occurring in the north could be derived from those of southern species by pericentric inversion of a smaller pair of metacentric chromosomes (probably No. four), generating a small acrocentric pair (Fig. 5). This hypothesis is consistent with the observations of [3], who also proposed that the northern species
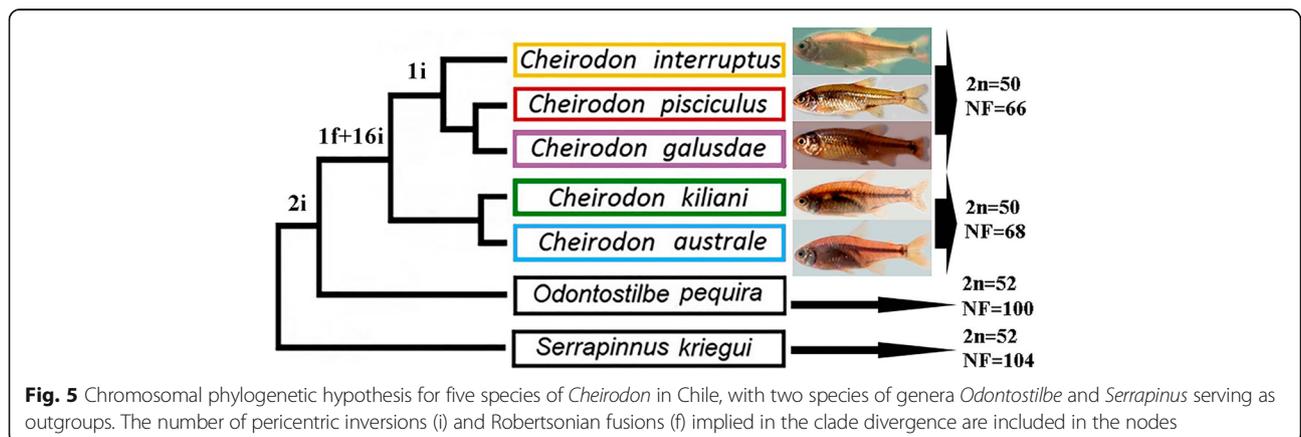
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Fig. 4 Geographic regions of Chile (left), ideograms (middle) and photographs of teeth and caudal peduncle region (right) of *Cheirodon* species occurring in Chile. (**a**) *Ch. interruptus* from Estero Marga-Marga; V region, (**b**) *Ch. pisciculus* from Angostura River; Metropolitan region, (**c**) *Ch. galusdae* from Andalién River; VIII region, (**d**) *Ch. kiliani* from Calle-Calle River; XIV region and (**e**) *Ch. australe* from La Poza Lake; X region, base level Llanquihue Lake. Ideograms show 18S and 5S rDNA gene location. Mid-marks represent rDNA sites that may be present or absent, in the same individual (heteromorphism) or in different individuals (polymorphism). In the photographs of teeth bar: 500 μm, and in those of peduncle region bar: 2 mm

of *Cheirodon* were derived from the southern species based on a comparison of their dental cusps.

Conclusions

In conclusion, the five species studied have very similar karyotypes, including the introduced species from the east side of the Andean cordillera, *Ch.*

interruptus. This finding is consistent with new theories about the recent, rapid emergence of the Andean cordillera which, after slow initial growth over a period of tens of millions of years, then underwent fast changes over periods of one to four million years of 1.5 km or more new emergence [34]. Thus, the isolation caused by the Andean cordillera could be relatively recent or even incomplete with regard to

Fig. 5 Chromosomal phylogenetic hypothesis for five species of *Cheirodon* in Chile, with two species of genera *Odontostilbe* and *Serrapinus* serving as outgroups. The number of pericentric inversions (i) and Robertsonian fusions (f) implied in the clade divergence are included in the nodes

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some watersheds, especially in the southern Patagonia region. Sea-level fluctuation may have been more effective for reproductive isolation among Chilean *Cheirodon*. These two events mentioned have contributed to allopatric speciation revealed by differentkaryotypeof these species.

Availability of data and materials
The biological material was deposited in the Department of Zoology of the Universidade Federal de Rio Grande do Sul, Brazil, under vouchers UFRGS 22295 (*Ch. interruptus*), UFRGS 22296 (*Ch. galusdae*), UFRGS 22297 (*Ch. pisciculus*), UFRGS 22298 (*Ch. kiliani*), and UFRGS 22299 and UFRGS 22300 (*Ch. australe*).

Authors' contributions
Conceived and designed the experiments: RFA, OMF, LRM. Performed the experiments: MAS, JC. Analyzed the data: MAS, MHS, MCA. Contributed reagents/materials/analysis tools: RFA, LW. Wrote the paper: MAS. Reviewing the manuscript: RFA, OMF, LRM. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The work was authorized by the *Subsecreataria de Pesca y Acuicultura del Gobierno de Chile* (SUBPESCA) and by the *Servicio Nacional de Pesca y Acuicultura del Gobierno de Chile* (SERNAPESCA), Authorization No. 740. All procedures were made according to international protocols for animal testing and authorized by the Ethic Committee in Animal Experimentation (protocol number 4509/08) of *Universidade Estadual de Ponta Grossa*. The fish individuals were transported to the laboratory and anaesthetized with clove oil before processing.

Consent for publication
We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. Thus, all authors agree to the submission and publication of this manuscript in *Revista Chilena de Historia Natural*.

Competing interests
The authors have declared that no competing interests exist.

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