Evolution and diversification of the Southern Chilean genus Ceroglossus (Coleoptera, Carabidae) during the Pleistocene glaciations

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Abstract. The Quaternary glaciations affected the evolution and current distribution of taxa in southern Chile, including the emblematic and endemic genus Ceroglossus Solier. This genus of ground beetles has fascinated a great number of entomologists due to their conspicuous colour patterns, but despite this attention their taxonomy has remained controversial, with a large amount of taxa described based on unclear or variable morphological characters. In this work, we carry out a phylogenetic analysis of the 10 described species of Ceroglossus, based on mitochondrial and nuclear markers, and perform a species delimitation analysis using different algorithms, to clarify the backbone of their evolutionary tree and to lay the basis for a comprehensive and integrative taxonomic revision of this genus. Our results show that Ceroglossus is divided in five main lineages, composed of 13 putative phylogenetic species. The five lineages diverged before the Quaternary, while the divergence of the species within each lineage occurred during the glaciations and followed a parallel pattern among the different lineages. Additionally, we carry out an evolutionary interpretation of the revised available chromosomal data to shed light on the mechanisms that promoted their diversification.

Introduction

Glacial cycles during the Pleistocene played a fundamental role in shaping the diversity and distribution of numerous groups of organisms (Sommer & Nadachowski, 2006; Abellán et al., 2011; Torres-Mantelet et al., 2020). Although their effects have been extensively studied on taxa in the Northern hemisphere (Hewitt, 2004; Cardoso & Vogler, 2005; Hirata et al., 2017; Carneiro de Melo Moura et al., 2019; Korbut et al., 2019), they have been less researched in the affected areas of the Southern hemisphere (López-López et al., 2016; Shepherd et al., 2017; Pardo-Gandarillas et al., 2018; Ansari et al., 2019), such as southern Chile (Harrison, 2004). The climatic changes associated with these events, in addition to the continuous advances and retreats of the ice caps, have shaped the evolution and current distribution and diversity of most taxa living in southern Chile (Trovant et al., 2015; Gutiérrez-Tapia & Palma, 2016; Muñoz-Mendoza et al., 2017; Alfar et al., 2018). These studies have assessed the impact of glaciations on Southern Chilean fauna, but there are still many pending questions. Conditions suitable for the formation of ice caps have existed in this area since ∼14 million years ago (Mercer, 1983; Harrison, 2004), but the greatest effect on the organisms living in this area was produced by the repeated glacial cycles during the last half of the Pleistocene (Harrison, 2004).

Realizing how past climatic events have shaped the history of different taxa is important not only to understand their evolution or their current patterns of distribution, but also to predict how they would respond to future environmental changes. This is
especially important in groups with controversial or poorly understood taxonomy, such as Collembola or tiger beetles from remote areas (Katz et al., 2015; López-López et al., 2016), which may hinder the development of management strategies to preserve and protect them. For example, molecular techniques have been used to assess the status of endangered tiger beetles (Diogo et al., 1999; Herrera-Russert, 2018; Aunins et al., 2020), Japanese giant water bugs (Suzuki et al., 2020) or bumblebees (Simanonok et al., 2021).

The outstanding genus Ceroglossus Solier (Coleoptera: Carabidae) has long been a group of interest for amateur and professional entomologists due to their conspicuous color patterns and big size. The genus is considered part of the small but distinct tribe Ceroglossini, closely related to the more diverse tribes Carabini and Cychrini, although most recent works place them as sister to Pamborini (Toussaint & Gillett, 2018), having diverged from them in the Cretaceous (around 110 Mya). Ceroglossus are omnivorous beetles commonly known as ‘peorros’ (‘flatulents’) due to the foul-smelling liquid they expel as a defensive strategy. This genus is distributed in southern areas of Chile, between Curicó (~35°S) and Tierra del Fuego (~55°S). The presence of Ceroglossus is linked to mature forests of Nothofagus, and they are highly affected by alterations of this habitat (Briones & Jerez, 2007; Henríquez et al., 2009; Cerda et al., 2015).

There are currently ten species described in this genus: C. archoides Ratay and Godoeau, C. buqueti (Laporte), C. chilensis (Eschscholtz), C. darwini (Hope), C. guerinii Géhin, C. magellanicus Géhin, C. morpheus Ratay, C. ochsenii (Germain), C. speciosus Géhin and C. suturalis Fabricius. In contrast to this relatively low number of species, nearly 100 subspecies have been described (Jaffrézic & Ratay, 2006; Jiroux, 2006; Ratay & Jaffrézic, 2009; Ratay & Godoeau, 2010; Ratay, 2009, 2011, 2012a,b, 2013a,b, 2017). The characters used to discriminate these taxa include the elytral texture, the colour and shape of the pronotum, the shape of the head, the contour of the pronotum and the elytra, the presence of carinae on the antennal segments, or the elytral sculptures. However, these characters are often misleading and even variable within some of these taxa (Ratay, 2013a).

Ceroglossus shows a wide range of colour polymorphism within each species, making difficult the identification of the different groups (Okamoto et al., 2001). These studies also found that despite large polymorphism, colour is a feature closely related to geography and not representative of the species. This means that ecological and/or environmental factors are responsible for colour diversity (Kratochwil, 2019). Similarities in coloration among sympatric taxa deviate from a random distribution in this genus (Muñoz-Ramírez et al., 2016). Jiroux (2006) believes that the advantages derived for Ceroglossus from this interspecific convergence are related to Müllerian mimicry. Ceroglossus constitute an important link in the trophic chain of the Chilean primary forest, being predated by diverse birds like the chucao (Scolorchilus rubecula) and the huét-huét (Pteroptochos tarnii), in which the vision is the most developed sense and is essential for prey capture. Their preys can defend themselves by adopting camouflage patterns or, on the contrary, presenting bright colours as a warning imitating nonedible toxic prey. However, the absence of a harmful organism similar to Ceroglossus, plus the fact that all Ceroglossus species possess the defensive strategy of squirting a liquid of an unpleasant and irritating odour, suggests that Müllerian mimicry is not the most likely explanation for the observed colour patterns. Alternative and still doubtful hypotheses include sexual selection or similarity with the substrate (Muñoz-Ramírez et al., 2016). Therefore, the evolutionary significance of the colour polymorphism in Ceroglossus still remains an open question, and the availability of a phylogenetic framework for the genus will help to interpret the similarities in coloration in the appropriate context.

Jiroux (1996) proposed four groups of species in Ceroglossus based on morphological characters: buqueti, chilensis, darwini and suturalis. Okamoto et al. (2001) carried out a phylogeny of these groups based on the nad5 mitochondrial fragment, recovering the four groups as monophyletic. According to the molecular results by Okamoto et al. (2001), the origin of this genus dates back to only 25–30 Mya. Afterwards, two phylogenies based on the mitochondrial coxl fragment (Muñoz-Ramírez et al., 2016; Muñoz-Ramírez, 2015) also recovered C. darwini, C. chilensis, C. buqueti and C. suturalis as monophyletic groups. The last study (Muñoz-Ramírez, 2015) also concluded that the described species C. guerini and C. ochsenii were not supported by the molecular data.

One of the mechanisms that promote speciation are chromosomal rearrangements (White, 1978; Coyne & Orr, 1998; Brown & O’Neill, 2010), by reducing the reproductive fitness of the hybrid offspring of individuals with different (original and rearranged) karyotypes (Searle, 1998). This leads to the subsequent isolation of the respective variants, which finally results in their separation as independent species. This process has been shown to be important for the diversification of different groups of organisms (Kawakami et al., 2011; de Vos et al., 2020), including beetles (Gómez-Zurita et al., 2004; López-López et al., 2013; Xavier et al., 2018).

The aim of this paper is to perform a molecular analysis (including mitochondrial and nuclear sequences) of a large sample of beetles of the genus Ceroglossus, in order to: i) reconstruct a phylogeny of the genus, ii) test its taxonomic diversity, iii) check for cryptic or undescribed taxa, and iv) establish the basis for future taxonomic-focused papers that could accommodate the species and subspecies described to date. We intend to shed light on the taxonomy of this group using species delimitation methods that provide further evidence for putative undescribed taxa in the genus. Furthermore, the estimation of the divergence time according to a molecular clock previously proposed for Coleoptera will allow us to discuss the evolutionary dynamics of the genus in the context of the Pleistocene glaciations and the geographical barriers in southern Chile. Additionally, an evolutionary interpretation of the available chromosomal data, based on the phylogeny obtained, will shed light on the possible role of karyotypic changes for the diversification and evolution of this group of beetles.

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Material and methods

A total of 508 individuals of *Ceroglossus* were collected during several years in Southern Chile (Table S1), using pitfall traps with wine vinegar as lure. Preliminary identification of specimens based on external morphological characters was putatively made in the field to label the samples. Due to the variability of the characters used to classify *Ceroglossus* taxa, this identification was not considered definitive and was not taken as a proper taxonomic identification in the absence of molecular results. For instance, some specimens labelled as ‘dar var’ (*darwini varIan*is) because they were identified as *C. darwini* in the field, were revealed to be *C. magellanicus* according to the molecular results.

The specimens were stored in 100% ethanol and kept at −20°C until the analyses. DNA extraction was performed from a leg of each specimen using an Invisorb Spin Tissue Mini kit (Stratec Molecular GMBH, Berlin, Germany), following the protocol specified by the manufacturer.

A fragment of the subunit 5 of the mitochondrial NADH dehydrogenase region (*nad5*) was amplified using the Kapa polymerase (Kapa Biosystems, Wilmington, MA, U.S.A.) and the primers described by Su et al. (1996): V1.06–1 (5′-CCGTCTCTGCTTTTAGTCCA-3′) and AO-3 (5′-ATATTCAATTTCAACCTTGTCA-3′). Additionally, we sequenced the Internal transcribed spacer region (ITS1) of the ribosomal DNA, using the primers 18sf1 (5′-TACACCGCGCCGTACCTA-3′) and 5p8sb1d (5′-ATGTGCGTTCRAATGTCGATGTTCA-3′) (Ji et al., 2003). PCRs were composed of 50 cycles, each starting with 1 min at 94°C and ending with 2 min at 72°C. The annealing steps were 1 min at 50°C for the *nad5* fragment and 1 min 40s at 57°C for the ITS1. PCR products were visualized on an agarose gel and sequenced either at Macrogen (Amsterdam, Netherlands) or Secugen (Madrid, Spain). In total, we got *nad5* sequences from 447 individuals, whereas the ITS1 was sequenced from 148 specimens. The sequences are available in GenBank (MW296380-MW296826 for the *nad5* sequences, MW315564-MW315711 for the ITS1 sequences).

The sequences were visually inspected and corrected in Geneious (http://www.geneious.com) and aligned in the same program using the MUSCLE algorithm (Edgar, 2004). We created two alignments, one for each fragment. We added the 63 *nad5* sequences obtained by Okamoto et al. (2001), and we included sequences from 17 taxa from the related genera *Carabus* Linnaeus and *Calosoma* Weber as outgroups. For the ITS1 matrix, a 521 bp indel that was present in only some sequences was removed, as it did not include any phylogenetically informative position and could potentially generate an unreliable tree.

Phylogenetic inference was carried out in IQTREE 2.1.2 (Minh et al., 2020) and BEAST 1.10.4 (Drummond et al., 2012). In IQTREE, maximum likelihood analyses were carried out for both matrices, searching for the most appropriate model (Kalyaanamoorthy et al., 2017) and including an ultrafast bootstrap with 10 000 replicates (Hoang et al., 2018). For the BEAST analyses, the matrices were imported into jModelTest (Darriba et al., 2012) in order to determine the optimal nucleotide substitution model for each gene. Independent analyses using different clock and tree models were implemented for each matrix. For the *nad5* fragment, we compared (i) Yule and Constant Coallescent tree models, and (ii) different clocks (strict, relaxed with exponential distribution, and relaxed with lognormal distribution). In all cases, strict and relaxed clocks were tested. The clocks were based on the rates calculated by Andújar et al. (2012) for the *nad5* fragment in their study on the subfamily Carabinae (normal distribution with mean 0.0159 subst/site-My, with standard deviation 0.001 subst/site-My, with minimum and maximum values equal to 0.0102 and 0.0223 subst/site-My), in which they calibrated the rate using fossil evidence and biogeographical events. For ITS1, we compared (i) a strict and a relaxed clock, and (ii) a Yule and a Constant Coallescent tree model. In this case, in the absence of a reliable rate available for this fragment, the clock was calibrated using a rate of 1 subs/site-My, producing a tree where the branch lengths were relative and did not correspond to absolute values in million years. We carried out an additional analysis using a concatenated matrix formed by both fragments, but the results were discarded as it was not possible to recover a tree without artefacts and acceptable support, possibly due to the amount of missing data.

The analyses ran for 100 million generations, sampling each 1000 steps. The trace files were imported into TRACER (available at http://tree.bio.ed.ac.uk) to check convergence. In the same program, we compared the clock and tree models by marginal likelihoods (log Bayes factors) estimated using the smoothed harmonic mean estimator (Newton & Raftery, 1994; Suchard et al., 2001) and using 1000 bootstrap replicates to select the best combination. The final trees were obtained from TREEANNOTATOR (distributed with BEAST) after removing the first 1000 trees.

Species delimitation analyses were performed on the resulting trees using different approaches: single- and multi-threshold GMYC (Pons, 2006; Fujisawa & Baraclough, 2013), bPTP (Zhang et al., 2013) and ABGD (Puillandre et al., 2012). A series of Consensus Clusters were obtained by comparing the results of these algorithms according to the methodology proposed by López-López et al. (2016) based on the work of Casteln et al. (2016). This method aims to correct the tendency of the species delimitation algorithms to over-split the dataset, producing a number of putative taxa that is usually higher than the real number of species. In order to produce the Consensus Clusters, we compared the results of the different species delimitation algorithms and always selected the most conservative option (for example, if a group of individuals is divided into two taxa by the GMYC but only one by the bPTP, we consider them as a single group). Then, we also consider factors including the shared geographic distribution or the absence of morphological differences between reciprocally monophyletic clusters to further reduce the number of groups. The resulting Consensus Clusters are composed of individuals that have been grouped together by at least one species delimitation algorithm and are geographically and morphologically coherent, and thus can be considered as Operational Taxonomical Units (OTUs).
Additionally, we built a phylogeographic network for each main clade and each fragment, using POPART (available in http://popart.otago.ac.nz), colouring the haplotypes according to the geographical location.

**Results**

**DNA sequences**

The 703 bp *nad5* fragment was sequenced from 510 individuals, corresponding to 320 haplotypes. The ITS1 fragment had a variable length, including a 400 bp insertion found in a few samples. The total length of the ITS1 matrix, after removing this insertion, was 1089 bp. In both cases, the optimal nucleotide substitution model was GTR+$\Gamma$

Surprisingly, the ITS1 sequences did not match with the ITS1 sequences from other carabids when a BLAST search was performed, but with unspecific sequences from other organisms. Nevertheless, they can be aligned with ITS1 from carabids, showing correspondence in several regions, but also a high number of indels and different nucleotides in the central portion of the sequence. Thus, we concluded that these sequences correspond to a copy that may have originated from a duplication of the ITS1 region in the ancestor of *Ceroglossus*, and that has a higher affinity for our primers than the real ITS1. Nevertheless, subsequent analyses suggested that this pseudo-ITS1 had a coherent phylogenetic signal, and thus we decided to use these sequences for the phylogenetic analyses. Even though that means that the outgroup sequences are paralogs of our sequences, we decided to keep them as they were partially homologous and could provide a reference point to place the root of the tree. Indeed, a preliminary analysis without these outgroups produced the same topology in the ingroup.

The Bayes factor comparisons showed that a relaxed clock model with exponential distribution and a Constant Coalescent tree model performed better than their alternatives for the *nad5* fragment. Similarly, the optimal model combination for the ITS1 fragment was a relaxed clock with a Yule model, but this analysis produced a topology in which the taxa were mixed in taxonomically incoherent groups; thus, we decided to use the second ranked combination, composed by the same clock model and a Constant Coalescent tree model.

**Phylogenetic analyses**

In all trees (Figs 1, 2), the samples group into five clades in both trees, corresponding to five morphological species: *C. darwini*, *C. magellanicus*, *C. suturalis*, *C. buqueti* and *C. chilensis*. Nevertheless, their relationships change depending on the fragment that was analysed, and the algorithm used. The only exception is that *C. suturalis* is shown as the first taxon that diverged from the others (except in the ITS1 BEAST tree), and that *C. darwini* is always closely related to *C. magellanicus*.

The calibrated *nad5* tree (Fig. 1) shows that the genus *Ceroglossus* originated 12.45 million years ago, *C. darwini* and *C. magellanicus* diverged 5.42 Mya, whereas *C. buqueti* and *C. chilensis* separated 7.44 Mya. The clades *C. darwini* + *C. magellanicus* and *C. buqueti* + *C. chilensis* diverged 10.47 Mya. The number of branches within each clade is sparse before 2 Mya, but a large increase in the branching rate can be observed from this point to present, throughout the Pleistocene.

**Species delimitation**

The species delimitation methods produced similar results (Fig. 1) except the GMYC with a multiple threshold, which over split each clade into multiple entities. The Consensus Clusters delimited according to López-López et al. (2016) coincide with separate groups in the phylogeographic networks (Figs 3, 4).
putative taxonomic identity of the specimens corresponding to each Consensus Cluster is given in Table 1.

The C. buqueti clade includes five Consensus Clusters that may correspond to different phylogenetic species or subspecies, given that they have a strong genetic structure and different geographic distribution in spite of the absence of geographical barriers and an apparent continuity of populations (Fig. 3). Within this group, the taxon that diverged first (Cluster B5) is distributed at the northern end of their range, and the most derived taxa can be found from there towards the southern area. If these Clusters are confirmed as separate species by future studies, Cluster B3 would correspond to the original C. buqueti, whereas Clusters B1 and B2 would correspond to the subspecies C. b. chiloensis and C. b. sybarita elevated to independent species. The reduced number of samples in Clusters B4 and B5 makes it impossible to reliably propose a putative taxonomic identity for them, which would require more exhaustive sampling.

The three Consensus Clusters found in C. chilensis would also correspond to three phylogenetic species or subspecies distributed along a north–south axis (Fig. 4). In this case, the first divergence separated the most meridional species (Cluster C3), which experienced an expansion towards the south. If future studies confirm the identity of the three C. chilensis Clusters as separate species, Cluster C2 would correspond to the original C. chilensis, whereas Clusters C1 and C3 would correspond to the subspecies C. c. kraatzianus and C. c. solieri elevated to species.

The C. suturalis clade also divides into three Consensus Clusters that do not entirely correspond to the described species based on morphology (Fig. 4). C. morpheus, C. ochsenii and C. guerini are included with other C. suturalis in Cluster S1. In a previous study, Muñoz-Ramírez (2015) also found that these traditional species did not form monophyletic groups. These results suggest that C. morpheus, C. ochsenii and C. guerini would actually be subspecies of C. suturalis, which would correspond to the Cluster S1. On the other hand, the specimens identified as the subspecies C. s. olivaceus make up the Cluster S2, suggesting that it may correspond to a separate species, as supported by preliminary morphometric analyses and morphological differences. Cluster S3 is formed by a single sequence of C. ancudanus from the work by Okamoto et al. (2001). This isolated and rare species has not been found

Fig. 2. Phylogenetic trees obtained from the Maximum Likelihood analyses of the ITS1 matrix (left) and the nad5 matrix (centre), and from the Bayesian Inference analysis of the ITS1 matrix (right). Support values in the nodes correspond to ultrafast bootstrap (left and centre) and posterior probability (right). For clarity, support values have been omitted from the nodes within each species group. Branch lengths are proportional to the rate of substitution in the Maximum Likelihood analyses (left, centre), and to a relative molecular clock (not to scale) in the Bayesian Inference analysis (right). [Colour figure can be viewed at wileyonlinelibrary.com].

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Fig. 3. Phylogeographic haplotype network for the *C. buqueti* clade. The colours correspond to localities, marked on the map on the right. The localities or areas commented in the text are marked on the map. The Consensus Clusters are highlighted in the background, and their distribution is marked in the map. [Colour figure can be viewed at wileyonlinelibrary.com].
Fig. 4. Phylogeographic haplotype networks for the *C. chilensis* (upper left), *C. suturalis* (upper right), *C. darwini* (lower left) and *C. magellanicus* (lower right) clades. The colors correspond to localities, marked on the map on the right. The localities or areas commented in the text are marked on the map. The Consensus Clusters are highlighted in the background, and their distribution is marked in the map. The haplotypes that include samples identified as some subspecies that are commented on the text are marked with symbols on the *C. suturalis* and *C. darwini* subfigures. [Colour figure can be viewed at wileyonlinelibrary.com].
during several visits to its locality, which may be a sign of its extinction. Collecting and sequencing more individuals is necessary to corroborate this result.

Within the C. darwini clade, the species C. darwini, C. archoides and C. speciosus, easily distinguished by the morphomethometrical analysis of the pronotum contour (data not shown) and by morphological characters (Rataj & Godeau, 2010), are included in a single Consensus Cluster. Neither C. archoides or C. speciosus, nor any of the subspecies of C. darwini, constitute monophyletic groups (Fig. 4). The species C. speciosus has been proposed to be, according to its morphology (Laurant Rataj, personal observation), the result of a hybridization between populations of C. darwini and C. archoides that came into contact. Considering that the four C. speciosus specimens from Punta Estaquilla have been misidentified and correspond to C. darwini, C. speciosus would form a clade in both the phylogenetic tree and the haplotype network. In that case, this taxon (marked as subclade Ds in the phylogeographic network, Fig. 4) would represent an independent lineage that evolved independently in parallel to the C. darwini + C. archoides subclade and that only survives in some localities on the northern part of the Chiloe Island.

The C. magellanicus clade is composed of only one Consensus Cluster, in which no morphological subspecies could be recovered as monophyletic (Fig. 4). Nevertheless, a slight correspondence between the groups separated in the phylogeographic network and the geographic distribution can be observed.

Discussion

Phylogeny and diversity of Ceroglossus

Our results corroborate the existence of the four previously defined species groups or clades: C. darwini + C. magellanicus, C. suturalis, C. chilensis and C. buqueti. Nevertheless, the separation and phylogenetic distinctiveness of C. darwini and C. magellanicus suggest that they can be considered as different clades. These five Ceroglossus species groups diverged during the last half of the Miocene, between 12 and 5 million years ago (Fig. 1).

The phylogeny outlined in this work is a first step for re-examining the taxonomy of Ceroglossus. However, a more comprehensive taxonomic study, including all the subspecies and carefully examining as many characters as possible, is necessary to provide a correct and formal taxonomic revision of this genus. Whereas the phylogeny based on fragments analysed in this work do not reliably solve the relationships between the five species groups, the phylogeographic analysis based on the nad5 fragment provides enough information to discern the evolutionary history of each clade and show their parallelisms and how the past climatic and environmental changes have shaped out their current diversity and distribution.

Remarkably, some taxa, widely considered as discrete species, are revealed as sublineages within other species. For example, C. speciosus and the recently described species C. archoides (Rataj & Godeau, 2010) are included within C. darwini. Additionally, C. morpheus, C. ochsenii and C. guerini are included within C. suturalis. The molecular results obtained in this work suggest that the morphological characters used for discriminating these taxa should be revised. The discriminatory characters must be chosen carefully in Ceroglossus, as the high phenotypic variability in this genus is more dependent on the locality than the phylogenetic lineage (Muñoz-Ramírez et al., 2016).

Nevertheless, the species delimitation algorithms suggest that the taxonomic diversity of Ceroglossus may be higher than it was previously thought. Although these methods usually overestimate the number of species, in this case the divisions are coherent even taking the conservative alternative provided by the Consensus Clusters. These divisions are coherent with morphology and geographic distribution. This evidence supports that each of the 13 Consensus Cluster may correspond to separate species (Table 1), according to the phylogenetic species concept (Cracraft, 1980), in which a species is a group of organisms that share at least one derived character and descend from a common ancestor. These can also be considered as independent Operational Taxonomical Units (OTUs). Alternatively, they may correspond to populations that have become isolated during the migratory and evolutionary history of this genus and are still undergoing a process of speciation. These results indicate that a comprehensive revision of this genus is necessary to assess their systematics.

Patterns of distribution across the Ceroglossus clades

The five clades of Ceroglossus show a similar phylogeographic pattern. The Consensus Clusters within each clade are distributed latitudinally along their area of distribution (Figs 3, 4). In some cases, such as in C. buqueti, the most derived taxa correspond to the most meridional clusters (B1 and B2). This may indicate a gradual expansion towards the south following the retreat of the ice layers, or an extinction of the most thermophile taxa in southern areas during the glacial maxima. In other cases, such as in C. chilensis, the first divergence separated the most meridional species (C3), which experienced a similar expansion towards the south. The separation of the other two C. chilensis taxa may have occurred either by a colonization towards the north by the most thermophile individuals (C1), or by allopatric speciation due to an ecological barrier.

Remarkably, the most southern cluster within each clade (B2, C3, S2) is distributed in Chiloe island and the southern continental areas (Aysén and Magallanes regions), but no connection is observed with the northern areas. This suggests that there is some impassable barrier for Ceroglossus between Chaitén and Puyuhuapi, whereas they can (or could) move freely from Chiloé to the continent, either to the northern area or to the southern.

On the other hand, the northern and thermophile C. magellanicus did not show any expansion towards the southern regions. This may indicate that this lineage has a lesser dispersal capacity than other Ceroglossus, and that their populations have remained stable. This may be related to their distribution along the northern area, where the glaciations had less effect.

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Table 1. OTUs delimited by the species delimitation methods used in this work (Fig. 1), indicating the clade in which they are included, the preliminary identification of the specimens on the field based on the morphological characters, and a putative taxonomic identity for each identified OTU (which would require proper redescriptions in a comprehensive taxonomic study) based on our results and the principle of priority of the International Code of Zoological Nomenclature.

| Clade | OTU | Tentative identification on the field, based on morphology | Putative identity according to our results* |
|-------|-----|----------------------------------------------------------|--------------------------------------------|
| C. buqueti | B1 | C. b. breuningi<br>C. b. calvus x snizeki x lafquen<br>C. b. chiloensis<br>C. b. lafquen<br>C. b. regalis<br>C. b. refictus<br>C. b. snizeki<br>C. b. violaceocupreus | C. chiloensis |
| | | C. b. chiloensis<br>C. b. snizeki<br>C. b. violaceocupreus | C. sybarita |
| | B2 | C. b. calvus<br>C. b. chiloensis<br>C. b. magdaleenaensis<br>C. b. sybarita | C. buqueti |
| | | C. b. andestus<br>C. b. arriagadai<br>C. b. buqueti<br>C. b. cherquencoensis<br>C. b. chiloensis<br>C. b. leopardalinus<br>C. b. lorenzi<br>C. b. regalis<br>C. b. solieri<br>C. b. subnitens x cherquencoensis<br>C. b. cherquencoensis<br>C. b. olibitus | C. sp. 1 |
| | B4 | C. b. andestus<br>C. b. cherquencoensis x subnitens<br>C. b. olibitus | C. sp. 2 |
| | B5 | C. b. andestus<br>C. b. cherquencoensis x subnitens<br>C. b. olibitus | C. sp. 2 |
| | | C. buqueti<br>C. b. subnitens x cherquencoensis<br>C. b. cherquencoensis<br>C. b. olibitus | C. sp. 1 |
| C. chiloensis | C1 | C. c. villarenensis<br>C. c. jaffrezici<br>C. c. rataji<br>C. c. gloriosus<br>C. c. solieri<br>C. c. kraitzianus<br>C. c. villariensis x gloriosus<br>C. c. imperialis x jaffrezici | C. kraitzianus |
| | C2 | C. c. fallaciom<br>C. c. cyanicollis | C. chilensis |
| | C3 | C. c. meridionalis<br>C. c. ketih<br>C. c. solieri<br>C. c. kraitzianus<br>C. c. kraitzianus<br>C. c. villariensis x gloriosus<br>C. c. imperialis x jaffrezici | C. solieri |
| C. suturalis | S1 | C. suturalis (various ssp.)<br>C. morpheus<br>C. ochsenii<br>C. guerini<br>C. s. olivaceus<br>C. s. ancanadanus | C. suturalis<br>C. olivaceus<br>C. ancanadan<br>C. darwini<br>C. darwini<br>C. archoides<br>C. speciosus<br>C. magellanicus (various ssp.)<br>C. magellanicus (various ssp.)<br>C. magellanicus | C. magellanicus<br>C. magellanicus<br>C. magellanicus<br>C. magellanicus<br>C. magellanicus<br>C. magellanicus<br>C. magellanicus |

*These names are provisional and should not be taken as official until a proper taxonomic study is carried out. Our study does not include enough data to reliably propose a name for the OTUs B4 and B5.
According to morphology-based taxonomy, *C. archoides* is distributed in two localities separated by ∼70 km, one on each side of the Río Bueno. This river has also been proposed as an effective barrier (at least in the western part) between *C. darwini* (southern side) and *C. magellanicus* (northern side). Nevertheless, our results group the samples identified as *C. archoides* within *C. darwini*. Additionally, some individuals that were preliminarily identified as *C. magellanicus*, collected in localities north of Río Bueno, are placed within the *C. darwini* clade, and vice versa. This result indicates that Río Bueno could not have been an impassable barrier (as it seems today) for *Ceroglossus*, or at least it has allowed occasional crossings in the past that may have produced some degree of introgression. Alternatively, the isolated populations included in the *C. archoides* subclade may be the remnants of an ancestral population with a wider distribution.

**Climatic cycles and *Ceroglossus***

The five species groups diverged between 12 and 5 million years ago (Fig. 1), during and after the end of the last major Andean uplift (Folguera & Ramos, 2011). The creation of new niches due to the environmental and climatic changes produced by this geological event (Strecker et al., 2007; Sepulchre et al., 2009) may have contributed to their separation.

Each Cluster or OTU underwent a diversification during the period ranging from 2 million years ago to the present. This period is characterized by the climatic fluctuations known as the Pleistocene glaciations. This phenomenon caused continuous advances and withdrawals of ice layers, which are known to have driven diversification within numerous animal taxa. Our results imply that this could have been the main promoter of the generation of lineages within each *Ceroglossus* species. In fact, the effects of the glaciations in this genus are noticeable nowadays, as *Ceroglossus* is still absent from some areas that were covered by ice during the Last Glacial Maximum (LGM) where the soil has not yet completely regenerated after the ice retraction. This is mainly caused by the larval requirement of a deep and well-developed soil layer (Cerda et al., 2015).

During the glacial maxima, the ice covered the Andes, the eastern slopes of the Coast Range, and the Central Valley that spans between them. *Ceroglossus* was probably relegated to the western slopes of the Coast Range, from which they recolonized the Central Valley and the Andes. Some lineages may have been isolated in refuges, such as *C. archoides* and the velvety subspecies of *C. buqueti*. Actually, some records of fossil *Ceroglossus* in areas affected by the glaciations may correspond to these relict populations (Ashworth & Markgraf, 1989).

**Recolonization of southern areas after the last glaciation**

Only one of the putative species in each clade, except in *C. magellanicus*, is distributed along the southern continental area (Clusters B2, C3, S2 and D). This area was completely covered by ice during the last glacial maximum (Denton et al., 1999; Harrison, 2004), which is absolutely inhospitable for these beetles and implies that this colonization occurred after this event. The phylogeographic networks suggest that the southern populations of *Ceroglossus* originated from colonizations from Chiloé Island, but not through the continent. It is possible that a disappeared land bridge connecting the southern part of Chiloé with the continent favoured the crossing, whereas a geographical or ecological barrier prevented the movement along the continent. This hypothetical land bridge also explains the distribution and phylogeography of other animal taxa (Moreno et al., 1994; Brieva & Formas, 2001). The expansion of these clades is recent and originated from a populational bottleneck. This is evidenced by the star shape observed in the haplotype networks within these clades, with a central haplotype shared with a large number of individuals, corresponding to the original haplotype that colonized the southern areas, surrounded by a variable number of less frequent haplotypes originated from it (Figs 3, 4).

**Chromosomal evolution in *Ceroglossus***

It is possible to provide some insights into the karyotypic evolution of *Ceroglossus* if we plot the cytotypic data previously published for the genus *Ceroglossus* (Galián et al., 1996) in the context of the phylogeny obtained in this work (Fig. 5).

According to this evidence, the ancestral *Ceroglossus* karyotype (2n = 28+XY) arose by a chromosomal fission from the typical Carabinae formula (2n = 26+XY), which is conserved in the related genera. The ancestral *Ceroglossus* karyotype is conserved in *C. darwini*, *C. magellanicus* and the *C. suturalis* clade (represented in this tree by *C. guerini*), but experienced multiple rearrangements in the *C. buqueti* + *C. chilensis* lineage leading to a 2n = 40+XY formula in *C. buqueti*. This increase in the chromosomes number may be related to the presence of repetitive DNA (Galián et al., 1996).

Additionally, a further rearrangement in *C. chilensis* resulted in the creation of a trivalent, corresponding to a multiple sex chromosome system similar to that observed in other Carabidae (Serrano, 1980) or Cicindelidae (López-López et al., 2013). This rearrangement involved an autosome and a heterosome (Fig. 3), either by a fusion of the ancestral Y chromosome and an autosome, or a rearrangement of the X chromosome with an autosome.

The presence of highly distinct karyotypes in the *C. buqueti* + *C. chilensis* lineage suggest that the chromosomal rearrangements may have played an important role in the evolution of these species, generating populations with rearranged karyotypes that become reproductively isolated from others and lead to the origin of new lineages by stasipatric speciation (White, 1978). The increase of the chromosome number allows the creation of more allelic combinations, which can provide a higher environmental plasticity and adaptation capability to these taxa. This could explain the higher diversity observed in the *C. buqueti* clade.
Conclusions

Our results indicate that the diversity of Ceroglossus is higher than expected. The species delimitation methods, based on molecular data, suggest that there may be up to 13 putative lineages that could be considered as candidates to subspecies or phylogenetic species in this genus (Table 1), which would need to be corroborated with a taxonomic revision including more morphological, morphometric, and molecular data.

The results also reveal that the climatic fluctuations during the Pleistocene glaciations were a major influence in the intraspecific diversification in this group of beetles. In fact, the distribution of Ceroglossus is still affected by the last glaciation. Present and future climatic changes are expected to produce a huge impact on the species of this genus, whose natural habitat, also sensible to climatic fluctuations, is heavily endangered mainly due to deforestation, compromising the future of Ceroglossus and other taxa associated to this fragile environment.

Author contributions

The study was conceived by all the authors. VA, LR and JG collected the samples in the field. ALL and VA processed the specimens and carried out the phylogenetic and phylgeogetic analyses. JG carried out the cytogenetic analyses. All the authors collaborated in writing and commenting the original manuscript and its consecutive revisions.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. List of specimens used in this work, including the Cluster/OTU in which they were placed by the combination of the species delimitation algorithms, the preliminary morphological identification made in the field, and the locality and year in which they were collected. The samples from the work by Okamoto et al. (2001) are represented by their GenBank accession codes and shaded in orange.

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Data availability statement

The data that support the findings of this study are available in GenBank at [https://www.ncbi.nlm.nih.gov/nucleotide/](https://www.ncbi.nlm.nih.gov/nucleotide/), reference numbers MW296380-MW296826 and MW315564-MW315711, and in the supplementary material of this article.

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