Molecular diversity of two freshwater anomuran crab species in Southern Chile (Decapoda: Anomura: Aeglidae) compared to associated morphometric differences

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ABSTRACT

The family Aeglidae consists of a single genus (*Aegla*) of anomuran crabs restricted to the Neotropical region of South America. Much of the present day distribution of freshwater species in Southern Chile has been impacted by Pleistocene glacial cycles. The melting of ice sheets created elaborate lake and river systems throughout this area and played an important role in the speciation of *Aegla*. In this study, we sampled one river (*A. cholchol*) and one lake (*A. rostrata*) phylogenetically closely related species of *Aegla*, to examine the molecular divergence across three genetic loci (EF1 intron, 16S, COI) and how genetic variation differs between the two habitats. We estimate the relative timing of divergence using Bayesian molecular dating methods and the associated molecular data. We then examine how the molecular differences are associated with morphometric differences among these species and how that relates to divergence time.

1 INTRODUCTION

The family Aeglidae consists of a single genus (*Aegla*) of anomuran crabs restricted to the Neotropical region of South America. Aeglid crabs are morphologically distinct, sharing several physical characters including branchial morphology and carapace structure (Martin & Abele 1988). They are the only taxon of the Anomura found entirely in freshwater habitats, making them ecologically unique. These crabs occupy a variety of habitats such as streams, lakes, and caves, from depths that plunge 320 m (Jara 1977) to heights that reach 3,500 m (Bond-Buckup & Buckup 1994).

Much of the present day distribution of freshwater species in Southern Chile has been impacted by Pleistocene glacial cycles (e.g., Xu et al. 2009). The melting of ice sheets created elaborate lake and river systems throughout this area and played an important role in the speciation of *Aegla*. There are approximately 19 species of aeglid crabs in Chile, 16 of which are endemic to the country, and many of which may be in grave danger due to the deterioration of the Chilean stream environments (Pérez-Losada et al. 2002a). Past studies have suggested conservation strategies to protect these fragile species, and a recent assessment of the Chilean species lists three as critically endangered, two as extinct in the

wild, one as near threatened, and six as vulnerable (Pérez-Losada et al. 2002a). In particular, *A. cholchol* has undergone a population reduction of at least 30% (reported in 2002, Pérez-Losada et al. 2002a), and studies have shown their habitats are subject to contamination, alteration, and pollution from agricultural runoff, cattle farms, urbanization, and farming. The implication for conservation efforts within aeglid crabs, in combination with their morphological and ecological uniqueness, emphasizes the need for increased attention and research on the group.

Aegla cholchol is a river species found within the Chol-Chol, Cautín and Quepe rivers; all tributaries of the Imperial River which led into the Pacific Ocean. *Aegla cholchol* is morphologically similar to *A. rostrata* but differs in pereopodal characters and attributes of the carapace (Jara & Palacios 1999). *Aegla rostrata* is found primarily in lakes (Riñihue, Panguipulli and Calafquén) but occasionally in river environments (Calle Calle and Huanehue) (Jara 1977). Studies suggest the current distribution of *A. rostrata* is the result of melting ice sheets following the Pleistocene glaciation (Jara 1977).

In this study, we examined the genetic relationships and divergence times of a pair of closely related (Pérez-Losada et al. 2002) river (*A. cholchol*) and lake species (*A. rostrata*) in Southern Chile, and correlate species diversification and origins with the Last Glacial Maximum in the region. It is presumed that river basins located near or at the Coastal Range served as refugial areas for the freshwater fauna during glacial maxima, subsequently serving as source areas for the colonization of pre-andean lakes once they became deglaciated (see Jara 1977, 1989). Furthermore, previous research suggests there is a tendency towards a reduction in rostrum length, width of the forehead and ornamentation as an adaptation to river or stream environments (Ringuelet 1949). On the contrary, the reverse tendency can be considered when riverine populations of *Aegla* are considered as ancestors (plesiomorph) to the lacustrine populations, so that a magnified ornamentation conveys a derived (apomorph) condition (see Jara 1986, 1989). Using combination of morphometrics and genetics, we compare a river species and lake species to test hypotheses associated with the morphological adaptations between ecotypes (lacustrine vs. riverine) and provide possible explanations for the morphological trends suggested by the morphological and genetic data.

2 MATERIALS AND METHODS

2.1 Taxon and Locality Sampling

Freshwater crabs of *A. cholchol* and *A. rostrata* were sampled from two localities (one river and one lake) across Southern Chile from February through December 2009 (Fig. 1). Individuals of *A. cholchol* were collected from the Quepe River (38º15’06’’S, 72º41’31’’W) approximately 45 meters above sea level, and individuals of *A. rostrata* were collected from Lake Panguipulli (39º39’06’’S, 72º18’ 77’’W), approximately 130 meters above sea level. Lake Panguipulli is a pre-Andean lake interconnected by a series of short rivers with at least two other, i.e., Calafquen and Riñihue lakes, which are part of a seven lakes chain in the upper Valdivia River basin, associated with the temperate rain forests of Southern Chile. Studies looking at the lake’s bathymetric profile and morphometric parameters have concluded it to be of glacial origins (Campos et al. 1981). The Quepe River originates in Lake Quepe, located in the Araucanía Region of Southern Chile. This river joins the Cautín River 112 km downstream, eventually forming the Imperial River

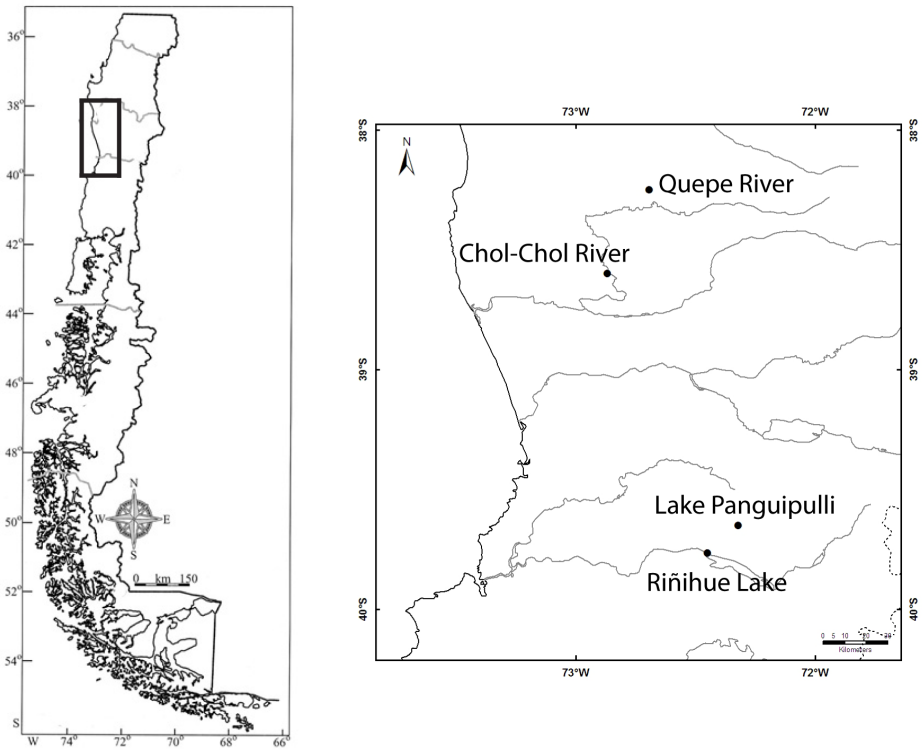

Figure 1. Sampling localities of *Aegla* in northern Chile.

where the Cautín and Chol-Chol rivers merge. All crabs were collected under rocks with dip nets in the river (*A. cholchol*) or by placing bag nets baited with fish heads at 45 m depth on the lake's sandy bottom (*A. rostrata*). Specimens were preserved in ethanol and deposited in the Monte L. Bean Life Science Museum at Brigham Young University.

To broaden our sampling efforts across the geographic distribution of these two species, GenBank sequences of *A. rostrata* and *A. cholchol* were incorporated in the molecular analyses (Appendix 1). These included four individuals of *A. cholchol* collected from the Chol-Chol river (38º36'S, 72º52'W) and two individuals of *A. rostrata* collected from *Riñihue* Lake (39º46'S, 72º27'W) (Fig. 1; Pérez-Losada et al. 2004).

We selected two species of *Aegla* (*A. abtao* and *A. alacalufi*) to include in the molecular analysis based on recent genetic studies and available sequence data (Pérez-Losada et al. 2002b, Pérez-Losada et al. 2004). All outgroups sequences were obtained from GenBank (Appendix 1). All individuals used in the molecular analysis (excluding GenBank taxa) were included in the morphometric analysis.

2.2 DNA Extraction, PCR and Sequencing

Total genomic DNA was extracted from the gills or muscle using the Qiagen DNeasy® Blood and Tissue Kit (Cat. No. 69582). Targeted gene regions were amplified by means of the polymerase chain reaction (PCR) using one or more sets of primers: 16S, large

ribosomal subunit (~450 bps, Pérez-Losada et al. 2002b); COI, cytochrome oxidase c subunit I (~870 bps, Xu et al. 2009); EF1, elongation factor 1 intron (~350 bps, Xu et al. 2009).

Reactions were performed in 25 µl volumes containing 0.5 µM forward and reverse primer for each gene, 200 µM each dNTP, PCR buffer, magnesium chloride, 1 unit HotMasterTaq polymerase (5 PRIME), and 30-100 ng extracted DNA. The thermal cycling profile conformed to the following parameters: Initial denaturation for 1 min at 94ºC followed by 30-40 cycles of 1 min at 94ºC, 1 min at 48-50ºC (depending on gene region), 1 min at 72ºC and a final extension of 10 min at 72ºC. PCR products were purified using filters (PrepEase^{TM} PCR Purification 96-well Plate Kit, USB Corporation) and sequenced with ABI BigDye® terminator mix (Applied Biosystems, Foster City, CA, USA). An Applied Biosystems 9800 Fast Thermal Cycler (Applied Biosystems, Foster City, CA, USA) was used in PCR and cycle sequencing reactions, and sequencing products were run (forward and reverse) on an ABI 3730xl DNA Analyzer 96-capillary automated sequencer.

2.3 Phylogenetic Analyses

Sequences were assembled, cleaned, and edited using the computer program Sequencher 4.7 (GeneCodes, Ann Arbor, MI, USA). Alignments were created using MUSCLE (multiple sequence comparison by log-expectation) or MAFFT, which have been found to be more accurate and faster than other alignment algorithms (Edgar 2004, Katoh et al. 2005). Alignments were concatenated into two separate datasets, one consisting of mitochondrial (mt) genes (16S, COI; 1328 basepairs), and a second consisting of all 3 gene regions (16S, COI, EF1 intron; 1694 basepairs).

The model of evolution that best fit the individual datasets (16S, COI, EF1) was determined by MODELTEST 3.7 (Posada & Crandall 1998). The Maximum Likelihood (ML) analysis was conducted using RAxML (Randomized Axelerated Maximum Likelihood) (Stamatakis et al. 2005, Stamatakis et al. 2007, Stamatakis et al. 2008) with computations performed on the computer cluster of the Cyberinfrastructure for Phylogenetic Research Project (CIPRES) at the San Diego Supercomputer Center. Likelihood settings followed the General Time Reversible Model (GTR) with a gamma distribution and invariable sites and RAxML estimated all free parameters following a partitioned dataset. Confidence in the resulting topology was assessed using non-parametric bootstrap estimates (Felsenstein 1985) with 1000 pseudoreplicates and values > 50% are presented on the resulting phylogeny.

The Bayesian (BA) analysis was conducted in MrBayes v3.1.2b4 (Huelsenbeck & Ronquist 2001) on the Marylou5 Dell PowerEdge M610 computing cluster at Brigham Young University. Three independent BA analyses (each consisting of four chains) were performed using parameters selected by MODELTEST. All Markov chain Monte Carlo (MCMC) algorithms ran for 10,000,000 generations, sampling one tree every 1000 generations. To ensure that independent analyses converged on similar values, we graphically compared all likelihood parameters and scores (means and variances) using the program Tracer v1.4 (Rambaut & Drummond 2007). Observation of the likelihood (-LnL) scores and split frequencies allowed us to determine burn-ins (~1,000,000 generations) and stationary distributions for the data. Once the values reached a plateau, a 50% majority-rule consensus tree was obtained from the remaining saved trees. Posterior probabilities (p*P*) for clades were compared for congruence and post-burn-in-trees were combined

between individual runs. Values > 0.5 are presented on the BA phylogram (presented as percentages).

2.4 Divergence Time Analyses

To estimate the relative timing of divergence between *A. cholchol* and *A. rostrata* we used Bayesian molecular dating methods implemented in BEAST v1.5.2 (Bayesian evolutionary analysis by sampling trees) (Drummond & Rambaut 2007). We chose to use a strict molecular clock, calibrated by specifying a substitution rate, to determine the rates among branches. Using a set substitution rate, we can estimate the divergence dates for any particular clade. A recent study, examining the aeglid species *A. alacalufi*, estimated the substitution rate for mtDNA to be ~0.118 substitutions per site per million years (Xu et al. 2009). Since their study was based on a closely related species and similar gene set (16S, COI, COII), we applied this rate to our mitochondrial dataset (16S, COI). To ensure that our data were clocklike, we computed the log likelihood values (-ln L) for trees with and without the molecular clock enforced (in PAUP*, Swofford 2002), and a likelihood ratio test (LTR) was calculated to test the null hypothesis that the data followed a molecular clock. Three independent runs with MCMC chain length of 10 million were performed, sampling every 1000 generations. To ensure that analyses converged on similar values, we graphically compared all likelihood parameters and scores using the program Tracer v1.4 (Rambaut & Drummond 2007). Estimates of the mean divergence times with 95% highest posterior density regions (HPD) and posterior probabilities (represented as percentages) are noted on the chronogram.

2.5 Geometric Morphometric Analyses

Data were recorded for 104 individuals of *A. cholchol* (65 males and 39 females) and 82 individuals of *A. rostrata* (46 males and 36 females). A subset of individuals from the morphometric study was included in the phylogenetic analysis. Digital pictures of all specimens were taken with a Nikon Coolpix PS100 camera. The crabs were placed in a Petri dish with ethanol and positioned over a grid for scaling and numbering purposes. Distances were standardized between the specimens and the camera. Twelve primary landmarks were chosen to capture the fundamental shape of the carapace (Fig. 2). To reduce redundancy due to the symmetrical nature of the carapace, landmarks were only placed on the right side of the crab. The landmarks were digitized using the tpsDig2 program v2.12 and tpsUtil v1.44 (Rohlf 2004a, b).

The configurations of the specimens were superimposed to remove nonshape variation through Generalization Procrustes Analysis (GPA, Rohlf & Slice 1990, Rohlf 1999) in the tpsRelw program v1.46 (Rohlf 2005). Nonshape variation included translation, rotation and scaling. The GPA process worked in three steps to align the landmarks involved with each specimen (Buchanan & Collard 2010). First, the set of landmark coordinates was centered at the origin, or centroid, and scaled all configurations to the unit centroid size (centroid size is a measurement of overall size of a specimen computed as the square root of the sum of the squared distances from all the landmarks to the centroid). Secondly, the consensus configuration was determined. Lastly, each landmark configuration was rotated to minimize the sum-of-squared residuals.

We used the tpsRelw program to compute relative warps. The relative warps were generated from the weight matrix, which is derived from the differences during

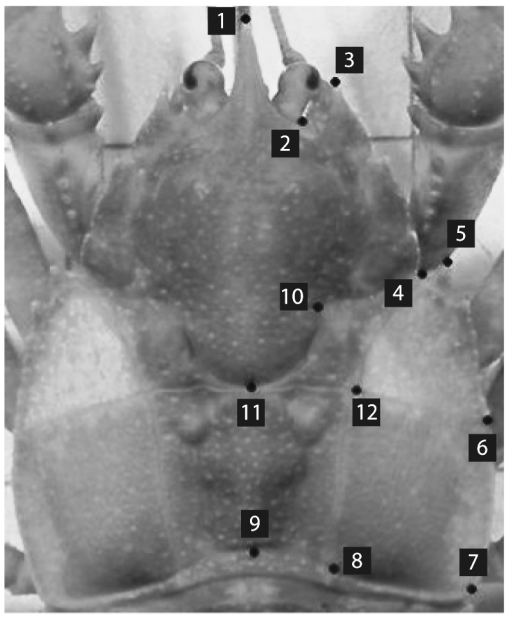

Figure 2. Description of locations and landmarks for geometric morphometric analysis: 1 Tip of the rostrum; 2 Orbital spine; 3 Tip of the anterolateral spine; 4 Union between the third hepatic lobe and the epibranchial tooth; 5 Tip of the epibranchial tooth; 6 Union between the branchial line and the posterior of the linea aeglica lateralis; 7 Posterior vertices of the cephalothorax; 8 Posterior extreme of the dorsal longitudinal linea; 9 Centre-posterior extremes of the cephalothorax; 10 Cervical groove; 11 Midpoint of the transverse dorsal linea; 12 Extreme of the bar line.

superimposition. The relative warps first described the major patterns of shape variation within and between the groups. Using the extremes of the relative warps, hypothetical specimens were created and compared.

A MANOVA (multivariate analysis of variance) was run on shape variables and centroid size using SAS statistical analysis software. This tested size and shape variation within and between species to determine if differences in and between species were statistically significant.

3 RESULTS

3.1 Phylogenetic Analyses

In total, we included 85 sequences for 16S and COI, and 77 sequences for EF1 (Appendix 1). Missing data were designated as a "?" in the alignment. Three individuals (KACa1963, KACa1981, KACa2002) were excluded from the combined (nuclear/mitochondrial) analysis because of missing data. The optimal models of evolution selected in MODELTEST were the Hasegawa, Kishino and Yano (HKY) model with invariant sites (16S), Tamura-Nei (TrN) model with gamma-distributed among-site rate heterogeneity and invariant sites (COI), and Kimura (K81uf) model with invariant sites (EF1 intron).
Individual gene trees for all three genes showed very similar patterns, although the nuclear tree (EF1 intron) was less resolved. Topologies derived from the ML and BA analyses on the concatenated dataset were strongly congruent. Here, we present the BA phylogram (Fig. 3).
Our study recovered the river species, *A. cholchol* to be a paraphyletic group divided into several clades (Fig. 3). Phylogenetic relationships were not correlated with locality as individuals collected from the Chol-Chol River and Quepe River did not form reciprocally monophyletic groups. The lake species, *A. rostrata* was recovered as a monophyletic

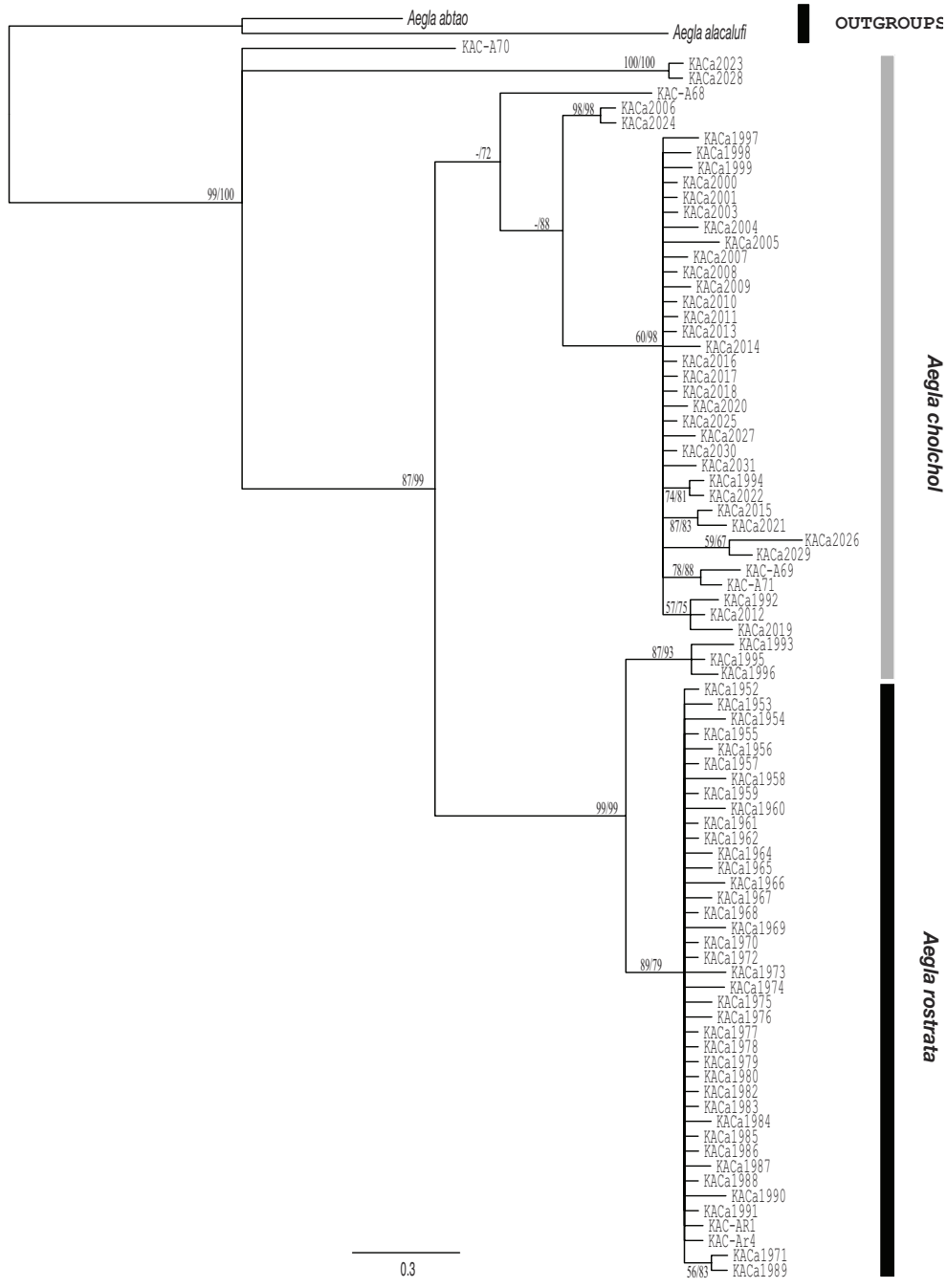

Figure 3. Bayesian (BA) phylogram for *Aegla cholchol* (n = 43), *Aegla rostrata* (n = 40) and outgroups (n = 2) based on a 16S (mtDNA), COI (mtDNA), and EF1 intron (nDNA) concatenated dataset. ML bootstrap values and BAY posterior probabilities are represented as percentages and noted above or below the branches (ML/BA). Values < 50% are not shown.

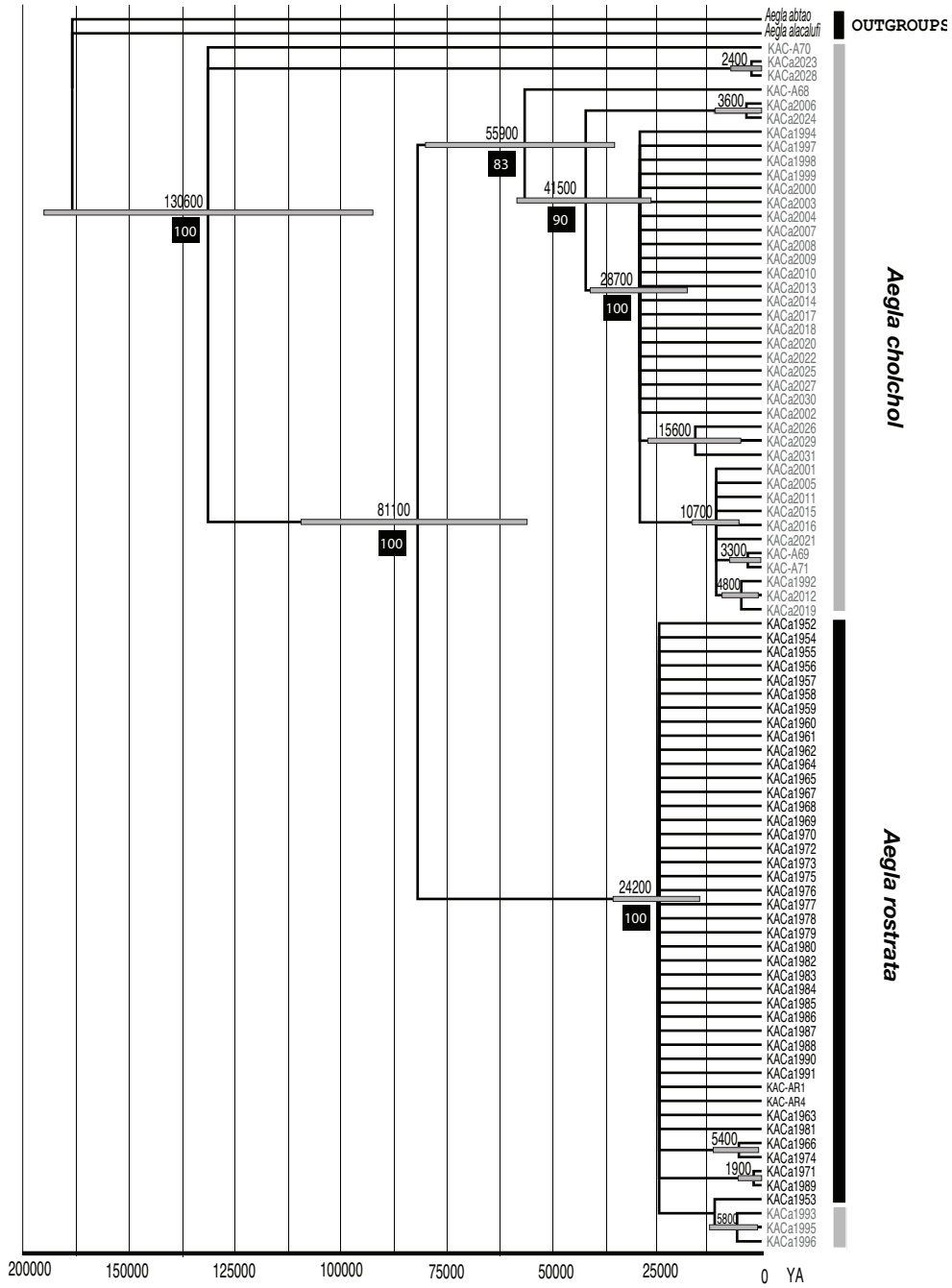

Figure 4. Divergence time chronogram based on a 16S (mtDNA) and COI (mtDNA) dataset. Vertical bars (black or gray) indicate individuals of *Aegla cholchol*, *Aegla rostrata,* or outgroup taxa. Divergence time estimates (YA) are noted adjacent to their respective nodes. Horizontal gray bars represent the 95% highest posterior density regions (HPD). Black boxes contain Bayesian posterior probabilities represented by percentages. Values < 50% are not shown.

group. As seen in *A. cholchol*, populations from different localities (Lake Panguipulli and Riñihue Lake) did not form genetically distinct clades (Fig. 3).

The combined (16S, COI, EF1 intron) and mitochondrial (16S, COI) phylogenetic analyses (Fig. 3, 4) generated congruent topologies with the exception of three individuals (KACa1993, KACa1995, KACa1996). In the mitochondrial phylogeny these individuals grouped within the *A. rostrata* clade (Fig. 4), however with the addition of the nuclear gene they formed a sister group to *A. rostrata* (Fig. 3). Individual genes trees showed similar trends; 16S and COI trees retained these samples within *A. rostrata*, while EF1 intron placed them outside *A. rostrata*. A possible explanation for this finding could be incomplete lineage sorting (see discussion).

3.2 Divergence Time Analyses

In total, we ran three separate divergent time analyses under a strict molecular clock starting from different random seed values to ensure the program converged on similar mean ages and highest posterior density regions (HPD). The time to most common recent ancestor (TMCRA) between *A. rostrata* and *A. cholchol* was estimated at 130,600 years ago (ya) (169,300-91,700) with the two lineages diverging around 81,100 (108,600-55,300) ya (Fig. 4). Following this split there was a radiation within *A. cholchol*, giving rise to three major clades in our phylogeny (Fig. 4). Results suggest the *A. rostrata* lineage radiated around 24,200 (35,000-14,600), which corresponds to the Last Glacial Maximum in southern South America (25,000-23,000).

We did explore the option of implementing a relaxed molecular clock and ran multiple trees under this assumption. We set a prior on the mean rate based on previous data (0.118 substitutions/site per million years) under a lognormal distribution (also implemented different distributions) and compared the results to our strict molecular clock analysis. We found 1) all of the mean nodes ages of the strict clock were nested inside the 95% highest posterior density (HPD) regions of the relaxed molecular clock analysis 2) the 95% HPD regions of the strict clock were much tighter when compared to the relaxed model 3) the relaxed molecular clock analyses computed unrealistically high mean rates of evolution for the mitochondrial dataset (0.7-0.4 substitutions per site per million years) depending on the prior distribution we implemented (normal vs. uniform vs. lognormal). Lastly, to ensure that our data was clocklike, we computed the log likelihood values (-ln L) for trees with and without the molecular clock enforced (in PAUP*) and a likelihood ratio test failed to reject the null hypothesis that the data followed a molecular clock ($P>0.25$).

3.3 Morphometric Analyses

Interspecific Variation

Analysis of the shape variation revealed a clear separation between two clusters (Fig. 5), suggesting a strong distinction between *A. rostrata* and *A. cholchol*. The cluster on the right side of the graph corresponds to the shape variation within *A. rostrata* and the cluster on the left corresponds to the shape variation within *A. cholchol*. Relative warp 1 (RW1) accounts for 26.07% and relative warp 2 (RW2) accounts for 17.59%. Together the cumulative variance of RW1 and RW2 is 46.66%. The multivariate test comparing the two species (*A. cholchol* vs. *A. rostrata*) was highly significant ($F= 11.71$, $P<0.0001$).

The distinction between *A. cholchol* and *A. rostrata* was especially evident on the first relative warp. Deformation grids corresponding to extremes of variation along this axis

help visualize shape difference between the two taxa (Fig. 7). *Aegla rostrata* has a longer rostrum (landmark 1) and ornamentation is more exaggerated (landmarks 4 & 5). They also have a more slender anterior and posterior cephalothroax region (to a lesser degree) and the cervical groove area is reduced (landmark 10). *Aegla cholchol* generally has a shorter rostrum and wider anterior cephalothorax region. The posterior cephalothorax is also wider, but to a lesser degree. The cervical groove area is slightly larger as well.

A multivariate test was run on centroid size between the two species with highly significant results ($F=4.60$, $P<0.0001$). This size difference between the two species is easy to see with the naked eye; *A. rostrata* is larger than *A. cholchol*. Thus, the two species show variation in both shape and size.

Intraspecific Variation

Analysis of shape variation between sexes (male vs. female) did not show clear separated clusters (relative warps 3 & 4, Fig. 6). However, the multivariable test shows highly significant difference in sex shape ($F=2.08$, $P<0.0042$). Dimorphism is moving in the same direction for both species. Females of *A. rostrata* have a slightly wider and lower cephalothorax area compared to the males. The same trend is seen in females of *A. cholchol*.

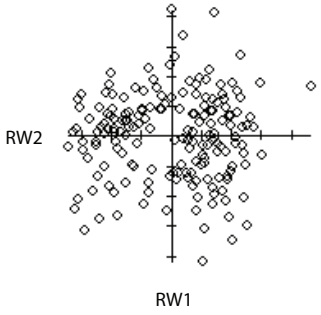

Figure 5. Interspecies comparison (*Aegla cholchol* vs. *Aegla rostrata*) of shape variation on relative warps 1 and 2.

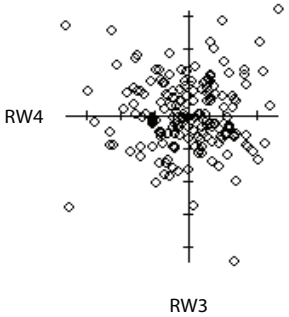

Figure 6. Intraspecies comparison (male vs. female) of shape variation on relative warps 3 and 4.

4 DISCUSSION

4.1 Molecular Phylogenetic Analysis

Our phylogenetic analysis suggests a high level of genetic diversity and structure within the river species, *A. cholchol* (Fig. 3). This level of divergence was unexpected, especially since a majority of the samples were collected from one sampling locality (Quepe River) in the Araucanía Region of Southern Chile. We acknowledge that our sampling was limited, and with the exploration of more populations and potentially cryptic lineages the amount of diversity may increase. Past studies have found increased levels of population differentiation within freshwater aeglid crabs and accredit this phenomenon to a small population size, habitat fragmentation, and poor dispersal ability (Xu et al. 2009).

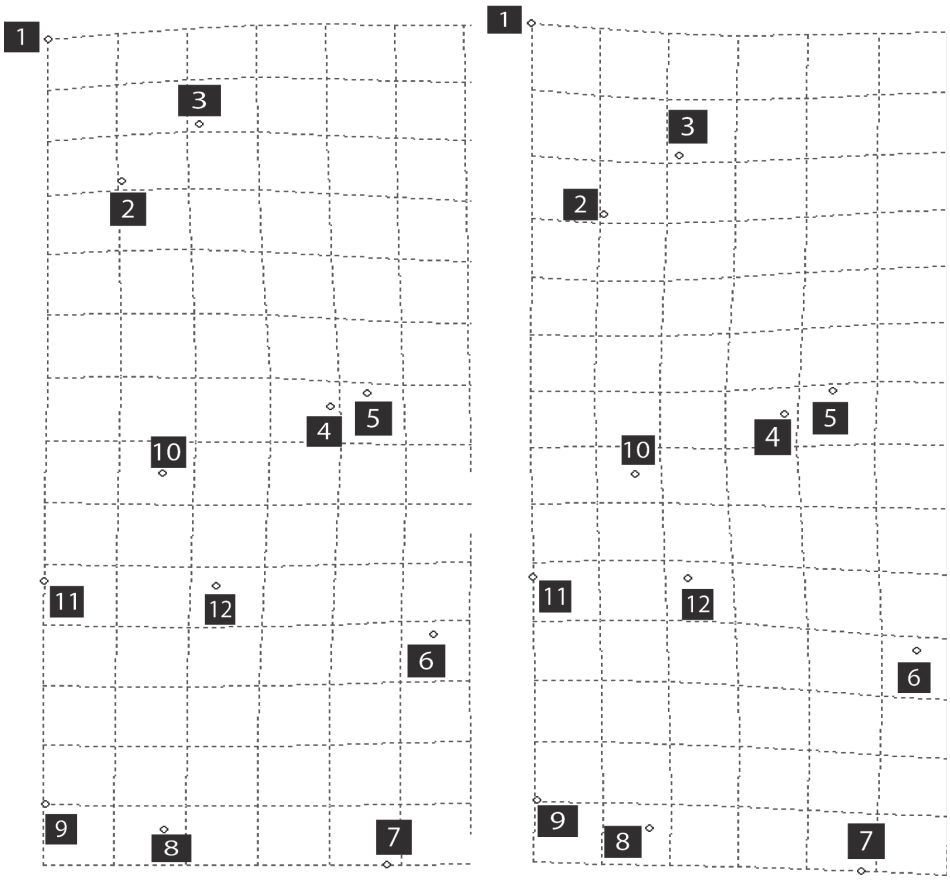

Figure 7. Deformation grids from relative warp 1. The right side is the extreme right, representing *Aegla rostrata*. The left side is the extreme left, representing *Aegla cholchol*.

Additionally, if it is considered that Quaternary glaciation events occurred at least four times in southern Chile (Illies, 1970), range restriction of *Aegla* populations toward the Coastal Cordillera should have occurred the same number of times. Each time the range reduction and the subsequent postglacial range expansion to the east could have caused population fragmentation and incipient differentiation, so that the high level of riverine population differentiation could be the result of those long-term recurrent processes superimposed over time on the same territory. The level of molecular divergence within *A. cholchol* exemplifies the need for species-level studies of aeglid crabs in Southern Chile in an effort to identify and preserve the genetic diversity that exists within these environments. Being *A. cholchol* is a river species, listed as vulnerable and subject to the habitat deterioration and degradation, this study reinforces the fact that genetic diversity, especially in river or stream environments has yet to be discovered and protected.

Our findings suggest the river species, *A. cholchol,* to be non-monophyletic, in congruence with recent molecular studies (Pérez-Losada et al. 2002a, Pérez-Losada et al. 2004, Bond-Buckup et al. 2008). More robust sampling throughout the species distribution may corroborate this conclusion. We are seeing this trend in all three individual gene trees

(not shown), the mitochondrial gene tree (16S/COI combined, Fig. 4), and the combined tree (16S/COI/EF1, Fig. 3). One explanation for the non-monophyly within *A. cholchol* could be attributed to incomplete lineage sorting, especially in recently diverged species. At shallow time depths, this phenomenon may occur when genetic drift has not have enough time to bring loci to fixation (Pamilo & Nei 1988). This may lead to misleading phylogenies that do not accurately portray the true species tree. More specifically, this explanation may apply to the three individuals of *A. cholchol* (KACa1993, KACa1996, KACa1995) that are grouping with *A. rostrata* in the mitochondrial phylogeny (Fig. 4). With the addition of an unlinked nuclear loci (EF1 intron, Fig. 3), these individuals formed a sister group to the *A. rostrata* clade. Although incomplete lineage sorting may explain this particular case, more robust phylogenies exploring deep relationships among aeglid crabs and utilizing a suite of nuclear and mitochondrial genes continue to recover non-monophyletic relationships within *A. cholchol* (Pérez-Losada et al. 2004, Bond-Buckup et al. 2008). A second explanation may be that *A. cholchol* should be split into separate species based on genetic, morphological, and ecological observations. Two morphotypes have been observed for *A. cholchol* (Jara, personal observation) and past research has reported this species to occupy two types of benthic habitats in the Chol-Chol river (pebble/stony vs. sandy) (Jara 1996). In accordance with recent postulation, we agree that habitat partitioning in combination with genetic and morphological differentiation could promote sympatric speciation within *A. cholchol* (Pérez-Losada et al. 2002a).

4.2 Divergence Time Estimates and South American Glacial Cycles

The Last Glacial Maximum (LGM) in southern South America occurred around ~25,000-23,000 years ago (ya) with deglaciation occurring around 17,500-17,150 ya due to a response in climate warming (Hulton et al. 2002, Sugden et al. 2005, Ruzzante et al. 2008). Recent modeling studies have predicted that the ice sheet extended between 38 and 55 degrees S, with a western extension reaching the edge of the continental shelf south of 43 degrees S (Hulton et al. 2002). Upon initiation, deglaciation occurred rapidly in the north and glaciers began to retreat within 10 km of their source within ~2,000 yrs (Hulton et al. 2002). Based on this information it becomes evident that our sampling localities (Quepe River, Chol-Chol River, Lake Panguipulli, Riñihue Lake) fall just within the northern latitudinal limits of the LGM and would have been the first to experience deglaciation in the area. Our chronogram suggests the LGM had little impact on the *A. cholchol* populations, which may have survived in alternative refugia during the glacial cycle or were positioned far enough north to escape the northern extent of the ice sheet. In either case, our results suggest the *A. cholchol* lineage originated around 130,000 ya with subsequent radiations around 55,900-28,700 ya. The origin of *A. rostrata* was estimated around 24,200 ya (35,000-14,600), which corresponds more closely with the LGM and the onset of deglaciation in the area. Previous research examining Lake Panguipulli's bathymetric profile and morphometric parameters have concluded it to be of glacial origins (Campos et al. 1981). It is plausible that *A. rostrata* speciated in this new habitat once the lake basin became free of ice, possibly from invading *A. cholchol*-like forebearers coming from western refuges. Other studies have suggested the current distribution of *A. rostrata* is the result of melting ice sheets following the Pleistocene glaciation (Jara 1977).

4.3 Morphometric Analyses

In this study, we performed a morphometric analysis to examine differences in morphological adaptations associated with living in a lacustrine vs. riverine ecotype. *Aegla cholchol* was chosen to represent the river species whereas *A. rostrata* was chosen to represent the lake species. Despite the overlapping morphological characters of *A. cholchol* and *A. rostrata*, the geometric morphometric analysis revealed a clear distinction in shape. Previous research suggests there is a tendency towards a reduction in rostrum length, width of the forehead, and ornamentation as an adaptation to rivers or stream environments (Ringuelet 1949). These adaptations allow an organism to be more streamline in a system where currents and flow are continuously changing. In the river species *A. cholchol*, the rostrum length is shortened and there is visibly less expressed ornamentation (not statistically shown), however the width in shape of the anterior cephalothorax region is larger. The lake species, *A. rostrata*, have a longer rostrum, thinner anterior cephalothorax, and more pronounced ornamentation. Although the size of the cephalothorax is larger in *A. cholchol* when compared to *A. rostrata*, other morphological features represent adaptations to a dynamic environment such as a river or stream.

In the past, there have been contrasting descriptions of size differences between lake and river species. Giri and Loy (2008) found river crabs to be larger, when comparing *A. riolimayana* and *A. neuquensis*, while Jara (1989) found lake crabs to be larger when describing *A. denticulata lacustris* and *A. denticulata denticulata*. Our findings are similar to Jara's results; size is a significant factor and *A. rostrata* are significantly larger than *A. cholchol*. The smaller overall body size may be another adaption to the river environment, allowing crabs to be more streamline and resistant to drag.

This study also found sexual dimorphism in both species. Significant differences were revealed in shape and size of the cephalothorax between males and females. Females of both *A. cholchol* and *A. rostrata* were found to have a wider posterior cephalothorax when compared to males. This trait should be correlated with the fact that females carry the eggs (Jara 1994). Our study highlights the importance of adaptive morphological evolution in distinguishing populations even when the neutral genetic markers have not had time to completely sort to species (Crandall et al. 2000).

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Table 1. Taxonomy, voucher catalog numbers, & GenBank (GB) accession numbers for gene sequences used in study. An "N/A" (not available) indicates missing sequence data. New sequences are indicated in bold.

Taxon	Locality	Voucher	GB Nos. 16S	GB Nos. COI	GB Nos. EF1 intron
Outgroup taxa					
Anomura MacLeay, 1838					
Aeglidae Dana, 1852					
Aegla abtao Schmitt, 1942		KAC-Aa5	AY050067	AY050113	N/A
Aegla alacalufi Jara & Lopez 1981		KACa1142	FJ472205	FJ471839	FJ472271
Ingroup taxa					
Anomura MacLeay, 1838					
Aeglidae Dana, 1852					
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1992	**HQ236179**	**HQ236258**	**HQ236337**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1993	**HQ236180**	**HQ236259**	**HQ236338**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1994	**HQ236181**	**HQ236260**	**HQ236339**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1995	**HQ236182**	**HQ236261**	**HQ236340**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1996	**HQ236183**	**HQ236262**	**HQ236341**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1997	**HQ236184**	**HQ236263**	**HQ236342**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1998	**HQ236185**	**HQ236264**	**HQ236343**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa1999	**HQ236186**	**HQ236265**	**HQ236344**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2000	**HQ236187**	**HQ236266**	**HQ236345**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2001	**HQ236188**	**HQ236267**	**HQ236346**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2002	N/A	**HQ236268**	**HQ236347**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2003	**HQ236189**	**HQ236269**	**HQ236348**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2004	**HQ236190**	**HQ236270**	**HQ236349**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2005	**HQ236191**	**HQ236271**	**HQ236350**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2006	**HQ236192**	**HQ236272**	**HQ236351**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2007	**HQ236193**	**HQ236273**	**HQ236352**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2008	**HQ236194**	**HQ236274**	**HQ236353**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2009	**HQ236195**	**HQ236275**	**HQ236354**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2010	**HQ236196**	**HQ236276**	**HQ236355**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2011	**HQ236197**	**HQ236277**	**HQ236356**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2012	**HQ236198**	**HQ236278**	**HQ236357**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2013	**HQ236199**	**HQ236279**	**HQ236358**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2014	**HQ236200**	**HQ236280**	**HQ236359**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2015	**HQ236201**	**HQ236281**	**HQ236360**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2016	**HQ236202**	**HQ236282**	**HQ236361**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2017	**HQ236203**	**HQ236283**	**HQ236362**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2018	**HQ236204**	**HQ236284**	**HQ236363**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2019	**HQ236205**	**HQ236285**	**HQ236364**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2020	**HQ236206**	N/A	**HQ236365**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2021	**HQ236207**	**HQ236286**	**HQ236366**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2022	**HQ236208**	**HQ236287**	**HQ236367**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2023	**HQ236209**	**HQ236288**	**HQ236368**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2024	**HQ236210**	**HQ236289**	**HQ236369**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2025	**HQ236211**	**HQ236290**	**HQ236370**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2026	**HQ236212**	**HQ236291**	**HQ236371**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2027	**HQ236213**	**HQ236292**	N/A
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2028	**HQ236214**	**HQ236293**	**HQ236372**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2029	**HQ236215**	**HQ236294**	**HQ236373**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2030	**HQ236216**	**HQ236295**	**HQ236374**
Aegla cholchol Jara & Palacios, 1999	Quepe	KACa2031	**HQ236217**	**HQ236296**	**HQ236375**
Aegla cholchol Jara & Palacios, 1999	CholChol	KAC-A70	AY050049	AY050095	N/A
Aegla cholchol Jara & Palacios, 1999	CholChol	KAC-A69	AY050048	AY050094	N/A
Aegla cholchol Jara & Palacios, 1999	CholChol	KAC-A71	AY050050	AY050096	N/A
Aegla cholchol Jara & Palacios, 1999	CholChol	KAC-A68	AY050047	AY050093	N/A
Aegla rostrata Jara, 1977	Panguipulli	KACa1952	**HQ236141**	**HQ236218**	**HQ236297**
Aegla rostrata Jara, 1977	Panguipulli	KACa1953	**HQ236142**	**HQ236219**	**HQ236298**
Aegla rostrata Jara, 1977	Panguipulli	KACa1954	**HQ236143**	**HQ236220**	**HQ236299**
Aegla rostrata Jara, 1977	Panguipulli	KACa1955	**HQ236144**	**HQ236221**	**HQ236300**
Aegla rostrata Jara, 1977	Panguipulli	KACa1956	**HQ236145**	**HQ236222**	**HQ236301**

Table 1. continued.

Taxon	Locality	Voucher	GB Nos. 16S	GB Nos. COI	GB Nos. EF1 intron
Aegla rostrata Jara, 1977	Panguipulli	KACa1957	**HQ236146**	**HQ236223**	**HQ236302**
Aegla rostrata Jara, 1977	Panguipulli	KACa1958	**HQ236147**	**HQ236224**	**HQ236303**
Aegla rostrata Jara, 1977	Panguipulli	KACa1959	**HQ236148**	**HQ236225**	**HQ236304**
Aegla rostrata Jara, 1977	Panguipulli	KACa1960	**HQ236149**	**HQ236226**	**HQ236305**
Aegla rostrata Jara, 1977	Panguipulli	KACa1961	**HQ236150**	**HQ236227**	**HQ236306**
Aegla rostrata Jara, 1977	Panguipulli	KACa1962	**HQ236151**	**HQ236228**	**HQ236307**
Aegla rostrata Jara, 1977	Panguipulli	KACa1963	N/A	**HQ236229**	**HQ236308**
Aegla rostrata Jara, 1977	Panguipulli	KACa1964	**HQ236152**	**HQ236230**	**HQ236309**
Aegla rostrata Jara, 1977	Panguipulli	KACa1965	**HQ236153**	**HQ236231**	**HQ236310**
Aegla rostrata Jara, 1977	Panguipulli	KACa1966	**HQ236154**	**HQ236232**	**HQ236311**
Aegla rostrata Jara, 1977	Panguipulli	KACa1967	**HQ236155**	**HQ236233**	**HQ236312**
Aegla rostrata Jara, 1977	Panguipulli	KACa1968	**HQ236156**	**HQ236234**	**HQ236313**
Aegla rostrata Jara, 1977	Panguipulli	KACa1969	**HQ236157**	**HQ236235**	**HQ236314**
Aegla rostrata Jara, 1977	Panguipulli	KACa1970	**HQ236158**	**HQ236236**	**HQ236315**
Aegla rostrata Jara, 1977	Panguipulli	KACa1971	**HQ236159**	**HQ236237**	**HQ236316**
Aegla rostrata Jara, 1977	Panguipulli	KACa1972	**HQ236160**	**HQ236238**	**HQ236317**
Aegla rostrata Jara, 1977	Panguipulli	KACa1973	**HQ236161**	**HQ236239**	**HQ236318**
Aegla rostrata Jara, 1977	Panguipulli	KACa1974	**HQ236162**	**HQ236240**	**HQ236319**
Aegla rostrata Jara, 1977	Panguipulli	KACa1975	**HQ236163**	**HQ236241**	**HQ236320**
Aegla rostrata Jara, 1977	Panguipulli	KACa1976	**HQ236164**	**HQ236242**	**HQ236321**
Aegla rostrata Jara, 1977	Panguipulli	KACa1977	**HQ236165**	**HQ236243**	**HQ236322**
Aegla rostrata Jara, 1977	Panguipulli	KACa1978	**HQ236166**	**HQ236244**	**HQ236323**
Aegla rostrata Jara, 1977	Panguipulli	KACa1979	**HQ236167**	**HQ236245**	**HQ236324**
Aegla rostrata Jara, 1977	Panguipulli	KACa1980	**HQ236168**	**HQ236246**	**HQ236325**
Aegla rostrata Jara, 1977	Panguipulli	KACa1981	N/A	**HQ236247**	**HQ236326**
Aegla rostrata Jara, 1977	Panguipulli	KACa1982	**HQ236169**	**HQ236248**	**HQ236327**
Aegla rostrata Jara, 1977	Panguipulli	KACa1983	**HQ236170**	**HQ236249**	**HQ236328**
Aegla rostrata Jara, 1977	Panguipulli	KACa1984	**HQ236171**	**HQ236250**	**HQ236329**
Aegla rostrata Jara, 1977	Panguipulli	KACa1985	**HQ236172**	**HQ236251**	**HQ236330**
Aegla rostrata Jara, 1977	Panguipulli	KACa1986	**HQ236173**	**HQ236252**	**HQ236331**
Aegla rostrata Jara, 1977	Panguipulli	KACa1987	**HQ236174**	**HQ236253**	**HQ236332**
Aegla rostrata Jara, 1977	Panguipulli	KACa1988	**HQ236175**	**HQ236254**	**HQ236333**
Aegla rostrata Jara, 1977	Panguipulli	KACa1989	**HQ236176**	**HQ236255**	**HQ236334**
Aegla rostrata Jara, 1977	Panguipulli	KACa1990	**HQ236177**	**HQ236256**	**HQ236335**
Aegla rostrata Jara, 1977	Panguipulli	KACa1991	**HQ236178**	**HQ236257**	**HQ236336**
Aegla rostrata Jara, 1977	Riñihue	KAC-Ar4	AY050074	AY050120	N/A
Aegla rostrata Jara, 1977	Riñihue	KAC-Ar1	AY050073	AY050119	N/A