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Molecular Systematics and Biogeography of the Southern South American Freshwater "Crabs" *Aegla* (Decapoda: Anomura: Aeglidae) Using Multiple Heuristic Tree Search Approaches

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Abstract.—Recently new heuristic genetic algorithms such as Treefinder and MetaGA have been developed to search for optimal trees in a maximum likelihood (ML) framework. In this study we combined these methods with other standard heuristic approaches such as ML and maximum parsimony hill-climbing searches and Bayesian inference coupled with Markov chain Monte Carlo techniques under homogeneous and mixed models of evolution to conduct an extensive phylogenetic analysis of the most abundant and widely distributed southern South American freshwater "crab," the *Aegla* (Anomura: Aeglidae). A total of 167 samples representing 64 *Aegla* species and subspecies were sequenced for one nuclear (28S rDNA) and four mitochondrial (12S and 16S rDNA, COI, and COII) genes (5352 bp total). Additionally, six other anomuran species from the genera *Munida*, *Pachycheles*, and *Uroptychus* (Galatheoidea), *Lithodes* (Paguroidea), and *Lomis* (Lomisoidea) and the nuclear 18S rDNA gene (1964 bp) were included in preliminary analyses for rooting the *Aegla* tree. Nonsignificantly different phylogenetic hypotheses resulted from all the different heuristic methods used here, although the best scored topologies found under the ML hill-climbing, Bayesian, and MetaGA approaches showed considerably better likelihood scores ($\Delta > 54$) than those found under the MP and Treefinder approaches. Our trees provided strong support for most of the recognized *Aegla* species except for *A. cholchol*, *A. jarai*, *A. parana*, *A. marginata*, *A. platensis*, and *A. franciscana*, which may actually represent multiple species. Geographically, the *Aegla* group was divided into a basal western clade (21 species and subspecies) composed of two subclades with overlapping distributions, and a more recent central-eastern clade (43 species) composed of three subclades with fairly well-recognized distributions. This result supports the Pacific-Origin Hypothesis postulated for the group; alternative hypotheses of Atlantic or multiple origins were significantly rejected by our analyses. Finally, we combined our phylogenetic results with previous hypotheses of South American paleodrainages since the Jurassic to propose a biogeographical framework of the *Aegla* radiation. [*Aegla*; Anomura; biogeography; genetic algorithms; heuristic search; large phylogeny; mitochondrial and nuclear DNA; mixed models.]

"There are no freshwater Crustacea at all like *Aegla* anywhere else in the world" (Schmitt, 1942).

The Aeglidae Dana, 1852, are the most abundant and widely distributed freshwater Decapoda "crabs" in southern South America including Brazil, Argentina, Chile, Uruguay, Paraguay, and Bolivia (Martin and Abele, 1986). They occur in lakes, streams, salt marshes, and caves, ranging from 320 m of depth in Chilean lakes to ~3500 m of altitude in northeastern Argentinean cordilleras. Several features make Aeglidae an interesting group for evolutionary study: first, they are the only anomuran family entirely restricted to the Neotropical region of South America; second, taxonomically the aeglids are included within the superfamily Galatheoidea Samoulle, 1819, but there is some morphological (Martin and Abele, 1986; Tudge and Scheltinga, 2002) and molecular (Pérez-Losada et al., 2002c) evidence that throw into question their taxonomic position; third, several of the known species are threatened (Pérez-Losada et al., 2002a), so the group must be prioritized for conservation efforts; and fourth, from an ecological perspective, aeglids are unique because they are the only anomuran family entirely restricted to freshwater habitats.

The present Aeglidae belong to a single genus, *Aegla* Leach, 1820, consisting of 63 described species and subspecies (see Bond-Buckup and Buckup, 1994); however, new species have been recognized recently and described based on previous molecular phylogenetic analyses (e.g., Jara et al., 2003), and the status of others has been questioned (Pérez-Losada et al., 2002b).

The origin of the group is uncertain. Ortmann (1902) proposed that the aeglids from Chile include the more primitive forms of the genus; however, Schmitt (1942) hypothesized that the *Aegla* from the Atlantic side of South America are more primitive, and species ranging in the Chilean streams are more derived. Feldmann (1986) and Feldmann et al. (1998) considered both conclusions to be speculative, although based on the discovery of the marine fossil *Hamuriaegla glaessneri*† Feldmann, 1984, in New Zealand, they suggested that the primitive aeglids came from the Indo-Pacific region and dispersed through South America from the Chilean coast.

There has been no extensive work in terms of establishing *Aegla* phylogenetic relationships. Partial relationships have been proposed for 7 Chilean and Argentinean species on morphological basis (e.g., Schuldt et al., 1988), and 17 Chilean species using mitochondrial DNA sequences (Pérez-Losada et al., 2002b), but no phylogenetic study has been published on the whole genus. A phylogenetic framework is needed for proper analysis of origin, taxonomic position, biogeography, endangered status, and species recognition within the group. Therefore, as a main goal in this study, we will propose a robust phylogenetic hypothesis of the *Aegla* relationships based on mitochondrial and nuclear genes. To this end we will use standard phylogenetic heuristic approaches such as maximum parsimony (MP),

maximum likelihood hill-climbing methods (MLhc), and Bayesian inference coupled with Markov chain Monte Carlo (BMCMC) techniques, and two new ML genetic algorithms.

MATERIALS AND METHODS

Sampled Taxa

Ingroup taxa.—A total of 167 samples representing 64 *Aegla* from 1 to 7 populations (1 to 3 individuals each) each were collected by hand, dipnet, or trawl from August 1999 to July 2002 (Fig. 1) (see Appendix 1 for detail). This sample collection includes almost all of the described species and subspecies in the genus (58 out of 63) and six new undescribed species, and covers most of their known type-localities (Appendix 1). Those species not sampled include *A. concepcionensis* and *A. expansa* from Chile, presently considered "Extinct in the Wild" (Pérez-Losada et al., 2002a), and *A. franca*, *A. lata*, and *A. microphthalma* from Brazil. *A. franca* and *A. lata* are not found in their restricted areas of occurrence anymore and *A. microphthalma* is a stygobiotic species that only occurs

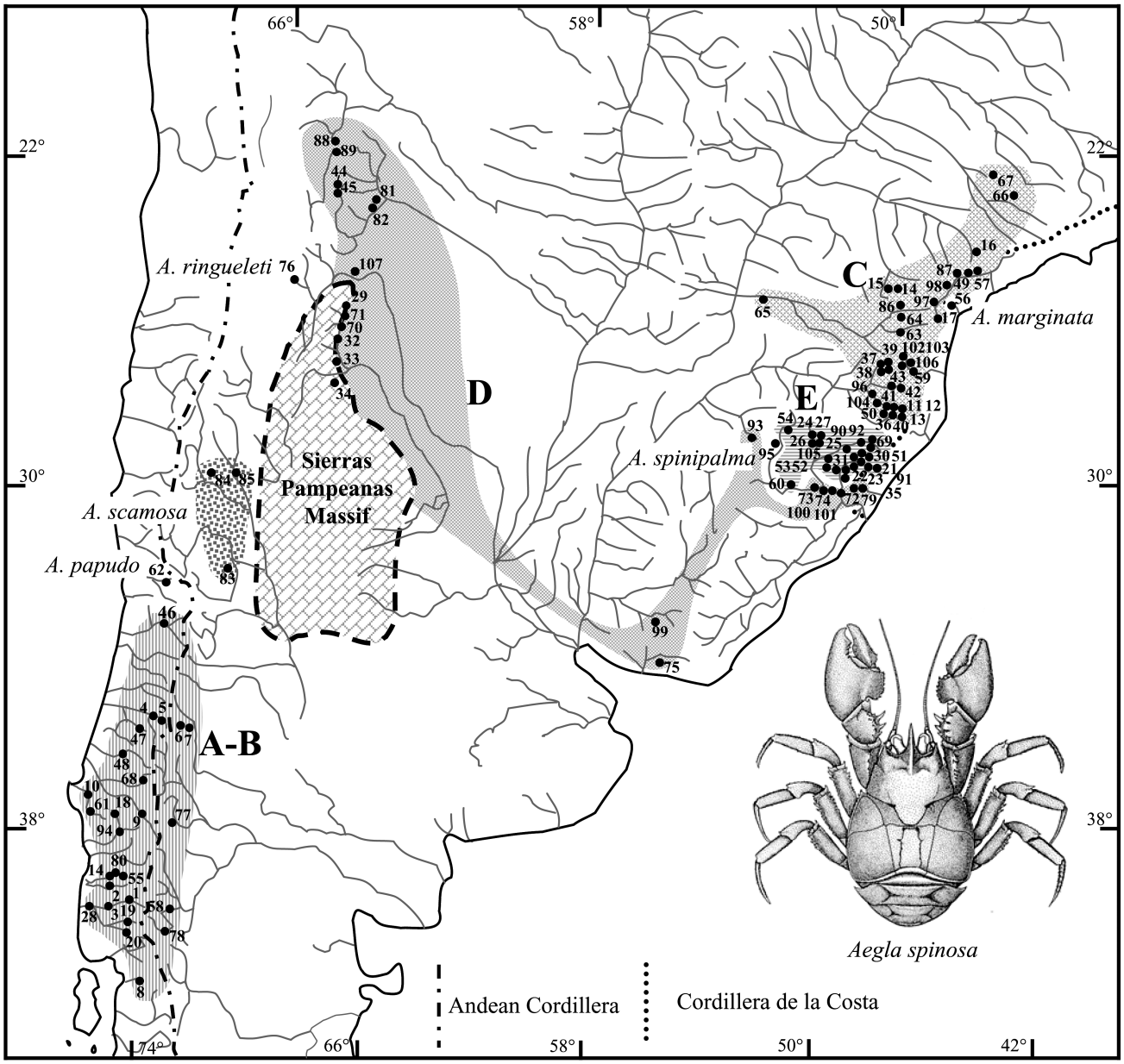

FIGURE 1. Map of southern South America indicating the major river systems and the location of the samples. Key numbers for samples are as in Appendix 1. An approximated geographic delimitation of the phylogenetic clades A to E is indicated (see text for details). Named species are not included in any of the previous clades. A drawing of *A. spinosa* from Bond-Buckup and Buckup (1994) is also shown.

in a single cave that is very difficult to access (GB-B, personal observation).

Outgroup taxa.—Most of the previous systematic studies (e.g., Martin and Abele, 1986), including the last updated classification of the Crustacea (Martin and Davis, 2001), show the superfamily Galatheoidea (Anomura) as constituted by the following families: Aeglidae, Chirostylidae, Galatheidae, and Porcellanidae. However, recent molecular (Morrison et al., 2002; Pérez-Losada et al., 2002c) and morphological (Tudge and Scheltinga, 2002) studies have suggested that the Aeglidae may represent a distinct superfamiliy with a sister relationship to the monospecific Lomisoidea. Therefore, to root the Aeglidae tree, we initially used one or two representatives from all of the previous groups (*Uroptychus*, Chirostylidae; *Munida*, Galatheidae; *Pachycheles*, Porcellanidae; and *Lomis*, Lomisidae) in combination with the paguroid *Lithodes santolla* (Anomoura: Paguroidea), according to the phylogenetic hypotheses proposed by Pérez-Losada et al. (2002c) (Appendix 1). Bayesian, ML (using a genetic algorithm), and MP phylogenetic analyses of four mitochondrial and two nuclear genes placed *Aegla papudo* in the most basal position of the Aeglidae tree, and this relationship was supported by bootstrap proportions (bp) and posterior probabilities (p*P*) of 100%. However, computational limitations did not allow us to execute MLhc searches using ingroup and outgroup sequences combined (see below); hence, to study the evolutionary relationships within the "crabs" *Aegla*, we used *A. papudo* as the functional outgroup (Watrous and Wheeler, 1981).

Molecular Methods

Total genomic DNA was extracted using methods described in Crandall and Fitzpatrick (1996). Polymerase chain reaction (PCR; Saiki et al., 1988) products for the complete nuclear 18S (1964 bp) and 28S (2884 bp), and partial mitochondrial 12S (382 bp; positions 1107 to 1482 in the human mitochondrial genome; Anderson et al. [1981]), 16S (473 bp; positions 2570 to 3034), COI (1045 bp; positions 6197 to 7239), and COII (568 bp; positions 7621 to 8187) genes were amplified using primers from Whiting et al. (1997) for 18S, Whiting (2001) for 28S, and from Pérez-Losada et al. (2002b) for the latter four mitochondrial genes. PCR and sequencing conditions and protocols were described in Pérez-Losada et al. (2002c). The last 633 nucleotide positions of the COI mitochondrial gene failed to amplify within the outgroup, thus they were coded as missing data. In a preliminary analysis of eight *Aegla* species (including *A. papudo*) from Chile, Brazil, and Argentina, the 18S rDNA gene showed very low levels of variation (6 polymorphic sites out of 1827 nucleotide positions). However, we also included this gene for rooting the Aeglidae tree because it has shown adequate levels of variation for solving the basic pattern of relationships between the anomuran families studied here (see Morrison et al., 2002; Pérez-Losada et al., 2002c). To this end, we built a consensus 18S sequence using the previous eight 18S *Aegla* sequences to represent all the aeglids.

Sequence Alignment, Incongruence, and Model Selection

Aeglid and other anomuran nucleotide sequences were aligned using Clustal X (Thompson et al., 1997). Dynamic programming was used under the default settings for the gap opening (10) and gap extension (0.10). Default settings were also used for the multiple alignment parameters gap opening (10), gap extension (0.20), and delay divergent sequence (30%), but a value of 0.3 was used for the DNA transition weight as suggested by the authors for distantly related sequences. Multiple sequence alignments using gap-opening penalties of 7 and 13 and transition weights of 0.5 (default setting) were also examined, but better alignments were not obtained. Alignments were trivial for the protein-coding genes and most of the mitochondrial and nuclear rDNA gene regions. Nevertheless, Clustal rDNA alignments were refined by hand based on the most recent compilation for alignment and secondary structure analyses provided by the European Ribosomal RNA database http://oberon.rug.ac.be:8080/rRNA). A region 506 bp long from the 28S gene, between the primers rd5a and rd6.2b from Whiting (2001), was impossible to align unambiguously because of its variation in base composition and length. In initial analyses, we first coded this hypervariable region and the nonsequenced region of COI as missing data in all the outgroup sequences, in an attempt to use all the phylogenetic information available (7996 nucleotide positions; Long Dataset). However, this strategy became useless for the MLhc analyses because of the extensive computational time required (model parameters as specified below). PAUP* (Swofford, 2002) was not able to find a single optimal ML tree after 2 months of searching on a supercomputer, presumably because of the genetic divergence between outgroup and ingroup sequences. Even when 67 terminal ingroup taxa (see MLhc bootstrap analysis and Fig. 2) were pruned and the 28S and/or the COI regions mentioned above were excluded from the analysis, the search did not converge to an optimal tree. Thus, a new aligned data set of 5601 nucleotides (short data set) was created excluding the six non-Aeglidae sequences (outgroup) and the 18S gene. Under these conditions we were able to compute ML heuristic searches under a less ambiguous and less divergent species-level alignment. However, this strategy is not completely satisfactory because only unrooted *Aegla* trees can be generated. Therefore, to adequately fulfill the aim of this study, we first used the long data set to root the *Aegla* tree; and then, using the short data set and *A. papudo* as the functional outgroup, we reconstructed the *Aegla* phylogeny.

All DNA sequences were deposited in GenBank under the accession numbers AY595421 to AY596101. Part of the 18S sequences, the COII sequence from *Lomis hirta*, and the mitochondrial DNA sequences from 17 Chilean *Aegla* species and two outgroups (*Munida* and *Pachycheles*) were previously collected by Pérez-Losada

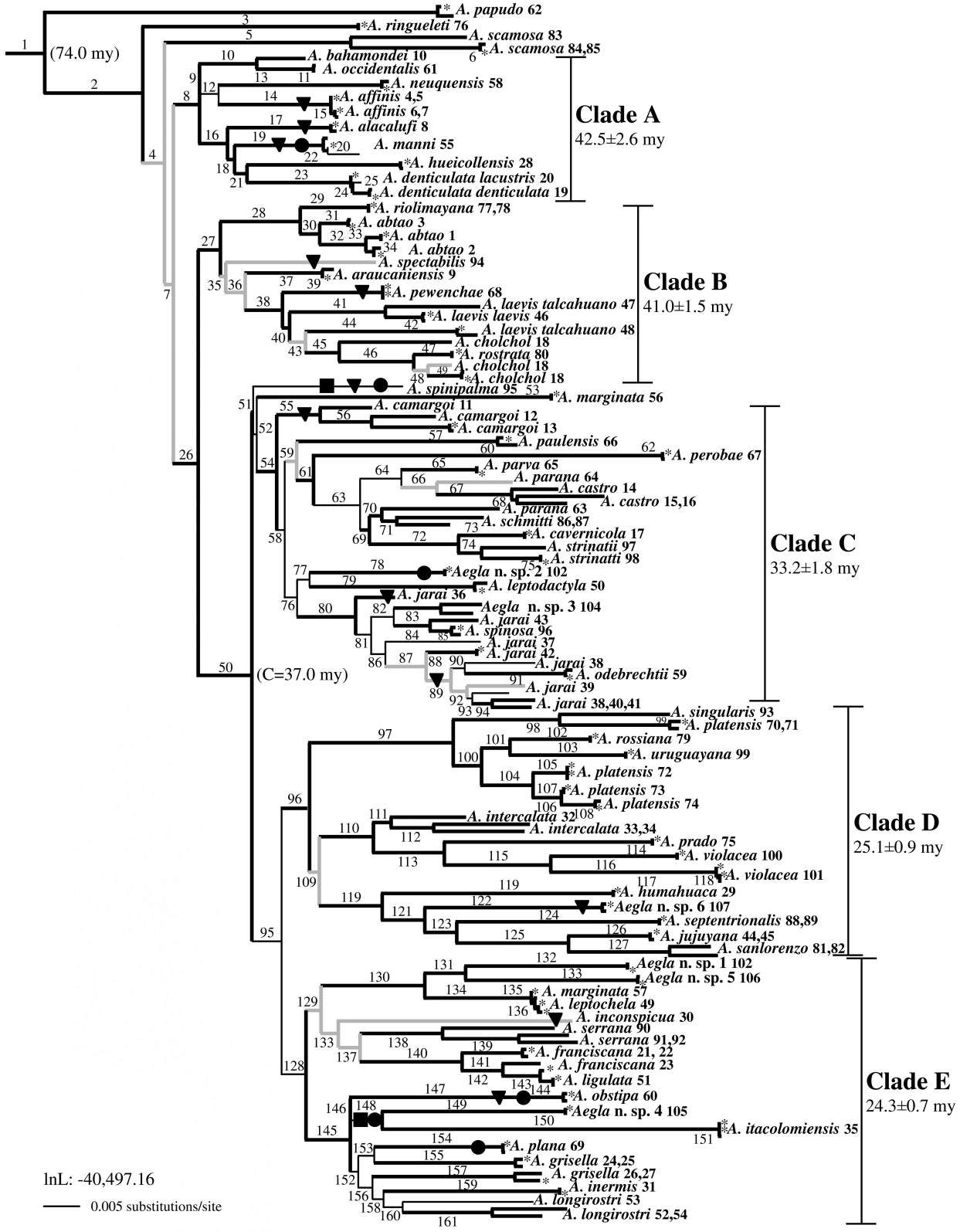

FIGURE 2. MLhc and BMCMC 50% majority-rule consensus trees under the GTR+Γ+I model of evolution. Topological differences resulting from the BMCMC analysis under mixed models (■), MLtgs (▼), and MLmgs (●) are also indicated. Clade support based on the MLhc and BMCMC trees is graphically indicated as follows: ▬ bp ≥ 70% and pP ≥ 0.95; ▬ 50% ≤ bp < 70% and/or 0.75 ≤ pP < 0.95; — bp <50% and/or pP < 0.75. Bp and pP values for each clade are presented in Table 1. Taxa with an * were not included in the MLhc bootstrap analysis. Branch lengths are shown proportional to the amount of change along the branches in the MLhc tree. Divergence dates for the tree root, the major clades A to E, and node 50 (C = calibration) are also indicated.

et al. (2002b, 2002c) and Morrison et al. (2002) and are already deposited in GenBank (accession numbers AF436012, AF436013, AF439382, AF439385 to AF439387, and AF439389 to AF439391; AF437624; and AY049985 to AY050166, respectively).

Incongruence between genes within the short *Aegla* data set was addressed by using the methodology proposed by Wiens (1998). Separate MP and Bayesian phylogenetic analyses were conducted on the four mitochondrial genes combined (because all genes in the mitochondrial genome are linked and should therefore share the same phylogenetic history) and the 28S gene to detect potential areas of strongly supported incongruence (where combined analyses may fail; Wiens, 1998), as indicated by conflicting nodes with bp $\geq$70% or p$P \geq 95\%$, respectively.

We used the model selection procedure outlined by Huelsenbeck and Crandall (1997). The likelihood scores from each model were compared using a likelihood ratio test as implemented in Modeltest v3.6 (Posada and Crandall, 1998). The general time reversible model with invariable sites and rate heterogeneity (GTR+Γ+I) was selected as the best-fit model of nucleotide substitution for both the long and short data sets ($\pi_A = 0.260$, $\pi_C = 0.177$, $\pi_G = 0.238$, and $\pi_T = 0.326$; $r_{CT} = 5.386$, $r_{CG} = 2.039$, $r_{AT} = 0.918$, $r_{AG} = 3.485$, and $r_{AC} = 0.715$; I = 0.62; $\alpha = 0.521$ for the short data set). Therefore, we used this model in the Bayesian analyses to root the *Aegla* tree and the MLhc and BMCMC analyses under homogeneous models of nucleotide substitution to study the *Aegla* phylogeny. Moreover, we performed an alternative BMCMC analysis of the short data set under the mixed models selected by Modeltest for every gene independently (28S: GTR+Γ+I; 12S: TIM+Γ+I; 16S: GTR+Γ+I; COI: TVM+Γ+I; COII: TVM+Γ+I). Variable rate priors among partitions were chosen for all the Bayesian analyses under mixed models. ML genetic searches were performed using the GTR+Γ model under the Treefinder algorithm (Treefinder does not account for invariable sites) and the HKY85+Γ+I model under the MetaGA algorithm—currently MetaPIGA does not implement more complex models. The different models implemented in our phylogenetic analyses make this study inappropriate for a comparative analysis of performance between genetic and standard heuristic approaches. This issue will be properly addressed elsewhere (Pérez-Losada and Crandall, in preparation).

Phylogenetic Analysis, Hypothesis Testing, and Divergence Time Estimation

Maximum parsimony.—We conducted equally weighted MP heuristic searches with 100 random addition (RA) replicates and tree bisection and reconnection (TBR) branch-swapping using PAUP* v4.0b10 (Swofford, 2002). Confidence in the resulting relationships was assessed using the nonparametric bootstrap procedure (Felsenstein, 1985) with 1000 bootstrap replicates, TBR branch-swapping, and 10 RA replicates.

Maximum likelihood.—Hill climbing heuristic searches were performed using PAUP*, with three independent runs using one RA replicate and TBR branch-swapping. One hundred replications, TBR branch-swapping, and starting trees obtained by neighbor-joining (using model-corrected distances) were used for the bootstrap analysis. Because of the long computational time required for MLhc estimation, we did not use all the representatives from every population for the bootstrap analysis; 67 out of 167 terminal branches marked with an * in Figure 2 were excluded. This reduced by about half the time required for analyzing every bootstrap replicate.

Genetic heuristic searches were performed by using the computer programs Treefinder (Jobb, 2002; Treefinder algorithm), hereafter MLtgs, and MetaPIGA (Lemmon and Milinkovitch, 2002; MetaGA algorithm), hereafter MLmgs. Both programs were run under selected population sizes of 16 trees and 10 different replicates were run for every analysis allowing the programs to estimate all the phylogenetic parameters. The strict group consensus option was chosen in MetaPIGA for better accuracy and four rate categories were selected under the discrete gamma model implemented in Treefinder. Default options for all the other parameters were used for both programs. One hundred bootstrap replicates of our short data set were generated using PHYLIP v3.6a (Felsenstein, 2000) and then run through both programs using a population size of 16 trees. The best-scored tree from each replicate was used to perform a majority-rule consensus tree.

Bayesian analyses.—Bayesian phylogeny estimation was performed using MrBayes v3.0 (Ronquist and Huelsenbeck, 2003). Each Markov chain was started from a random tree and run for 4.0×10^6 cycles with every 20,000th cycle sampled from the chain to assure independence of the samples. Model parameters were treated as unknown variables with uniform priors and were estimated as part of the analysis. We ran four chains simultaneously, three heated (temperature = 0.2) and one cold, using Metropolis-coupled Markov chain Monte Carlo to enhance the mixing capabilities of the Markov chains. To check that stationarity had been reached, we monitored the fluctuating value of the likelihood and all the phylogenetic parameters graphically, and repeated each simulation four times starting from different random trees and then comparing means and variances for each model parameter. All sample points prior to reaching stationarity were discarded as "burn in." The posterior probabilities for individual clades obtained from separate analyses were compared for congruence (Huelsenbeck and Imennov, 2002; Huelsenbeck et al., 2002; Nylander et al., 2004), and then combined and summarized on a majority-rule consensus tree (Huelsenbeck et al., 2002).

Hypothesis testing.—Alternative phylogenetic hypotheses were compared using the Shimodaira and Hasegawa (1999; S-H) test. Goldman et al. (2000), Buckley (2002), and Strimmer and Rambaut (2002) have pointed out that the S-H test may be subject to a certain type of bias such that the number of trees included in the confidence set tends to be very large as the number

of trees to be compared increases, which makes the test conservative. However, as the previous authors recognized and Shimodaira (2002) concluded, the S-H test is still safe to use and is a good option when the number of candidate trees is not very large and more data are accumulated. Ten thousand replicates were performed for every topology test resampling the partial likelihoods for each site (RELL model) using PAUP*. Multiple BMCMC, ML genetic, and most-parsimonious alternative hypotheses were first maximized under the GTR+Γ+I model in PAUP*and the resulting best-scored hypothesis on each case was then compared to the single MLhc tree (see below).

Divergence time estimation.—Divergence times within the MLhc *Aegla* tree were estimated using the multiple gene loci local-clock ML method of Yang and Yoder (2003) as implemented in PAML3.14b3 (Yang, 1997). Based on geological information (see below) we calibrated node 50 in Figure 2 as having a fixed age of 37 my (midvalue between 30 and 43 my). Initial analyses were performed under the GTR+Γ model and 0 to 5 local rates but the ML algorithm did not converge, so a less parameterized model (F84+Γ) was used instead. In spite of the differences between the GTR and F84 models, previous studies (see Yang and Yoder, 2003, and references therein) have shown that it is actually the rate variation among sites parameter that has the greatest effect on divergence time estimation. All the parameters within the model as well as the branch lengths were estimated separately for every gene. Previous work has shown that highly parameterized local clocks either do not affect date estimation too much (Yang and Yoder, 2003) or actually generate more accurate divergence estimates (Pérez-Losada et al., 2004); therefore five independent rates (one for each major clade A to E; see Fig. 2) were implemented in our final analysis. Nevertheless, analyses under the clock and less parameterized local-clock models were also performed for comparison.

RESULTS

Separate Gene Analyses

The aligned *Aegla* Short Dataset consists of four mitochondrial genes containing 2502 nucleotide positions total and one nuclear 28S rDNA gene containing 3099 positions. Four independent Bayesian analyses (2×10^6 generations) for each mitochondrial and nuclear data set under the GTR+Γ+I model converged on similar likelihood scores. All samples preceding generation number 120,000 for the mitochondrial data set and number 240,000 for 28S were discarded as "burn-in," and the remaining samples were combined. No areas of strongly supported conflict were identified between either Bayesian or MP mitochondrial and nuclear gene trees. As expected, the 28S trees were more weakly supported than the mitochondrial trees. When mitochondrial and nuclear data sets were combined, an increase of 18% and 42% in resolution, measured respectively as number of nodes supported by bp $\geq$ 70% or p$P \geq$ 95%, was observed compared to the 28S data set alone, and of 20% and 41%, respectively, compared to the mitochondrial data set.

Phylogenetic Relationships

Anomuran relationships.—Two hundred and twenty-three most-parsimonious trees 9005 steps long, 10 MLmgs searches, and four independent Bayesian analyses of the long data set showed *A. papudo* as the most basal *Aegla* species. This result was supported by bp of 100% for the MP and pP of 1.0 for the Bayesian trees. Moreover, this confirms previous genetic results by Pérez-Losada et al. (2002b) who pinpointed *A. papudo* as the most primitive aeglid. *Lomis hirta* (Lomisidae) was shown as the closest relative to *Aegla*, in agreement with the results of Morrison et al. (2002) and Tudge and Scheltinga (2002), whereas the other outgroup families presented the same pattern of relationships described by Pérez-Losada et al. (2002c) depicted as (*Aegla*, (*Lomis*, (*Lithodes*, (*Munida*, (*Pachycheles*, *Uroptychus*))))).

Aegla *relationships.*—Three ML independent hill-climbing heuristic searches of the short data set resulted in the same topology (Fig. 2). Eight independent Bayesian analyses under homogeneous and mixed models of evolution (four each) converged on similar likelihood scores (as optimized in PAUP* under the GTR+Γ+I model) and reached stationarity after <340,000 generations. The corresponding initial samples from every analysis were discarded, leaving a total of 761 combined samples. The pP supporting congruent nodes among these analyses were highly correlated ($r > 0.95$; $P < 0.001$), further indicating that the analyses converged. Overall, the *Aegla* phylogeny resulting from the MLhc analysis was identical to the Bayesian consensus tree under the GTR+Γ+I model and very similar (two topological differences) to the Bayesian consensus tree under mixed models of molecular evolution (Fig. 2). The best-scored Treefinder and MetaPIGA topologies were also similar to the MLhc tree for the most part, showing 12 and 6 topological differences, respectively (Fig. 2). Equally weighted parsimony analysis of 1348 parsimony informative characters out of 5601 nucleotide positions resulted in 97 most-parsimonious trees 5771 steps long (Fig. 3). Thirteen topological differences were found between the MP consensus and the MLhc trees. Clade support is shown graphically on Figures 2 and 3 and numerically in Table 1 and Figure 3.

Most of the observed topological differences among the previous phylogenetic hypotheses involved the poorest resolved parts of the trees and none of them represented conflicting nodes according to Wiens' (1998) criteria. Moreover, none of the alternative hypotheses were significantly different from the MLhc tree (the best-scored hypothesis) according to the S-H test, but the values of significance for the MLtgs and MP phylogenies were close to the 0.05 threshold ($P < 0.08$) and their likelihood difference was greater than 54 units compared to any of the other methods.

FIGURE 3. 50% Majority-rule consensus trees of 97 most parsimonious trees. Consensus (if <100%) and bootstrap proportions (if ≥50%) are shown under and above the branches, respectively. Branch lengths are shown proportional to the amount of change along the branches in one of the most parsimonious trees. Clade support is also indicated graphically as in Figure 2.

TABLE 1. Posterior probabilities under homogeneous and mixed evolutionary models ($BMCMC_{h/m}$) and bootstrap proportions (MLhc, MLtgs, and MLmgs). Node numbers correspond to those in Figure 2.

Node	$BMCMC_{h/m}$	MLhc	MLtgs	MLmgs	Node	$BMCMC_{h/m}$	MLhc	MLtgs	MLmgs	Node	$BMCMC_{h/m}$	MLhc	MLtgs	MLmgs
1	1.0/1.0	—	—	100	55	1.0/1.0	99	99	99	109	0.78/0.87	64	65	67
2	1.0/1.0	100	100	100	56	1.0/1.0	100	100	100	110	1.0/1.0	100	100	97
3	1.0/1.0	—	100	100	57	1.0/1.0	—	100	100	111	0.98/0.99	100	100	95
4	0.95/0.93	54	<50	<50	58	0.98/0.95	<50	<50	<50	112	1.0/1.0	100	100	97
5	1.0/1.0	100	100	100	59	0.96/0.99	60	<50	<50	113	1.0/1.0	100	100	100
6	1.0/1.0	—	100	100	60	1.0/1.0	—	100	100	114	1.0/1.0	—	100	100
7	0.95/0.97	55	<50	<50	61	0.98/0.99	89	76	<50	115	1.0/1.0	100	100	100
8	1.0/1.0	75	74	58	62	1.0/1.0	—	100	100	116	1.0/1.0	—	100	100
9	<0.5/<0.5	<50	<50	<50	63	0.72/0.79	<50	60	87	117	1.0/1.0	—	100	100
10	1.0/1.0	100	100	100	64	0.72/0.79	<50	75	82	118	0.95/0.96	—	<50	<50
11	1.0/1.0	—	100	100	65	1.0/1.0	—	100	100	119	1.0/1.0	99	93	95
12	0.95/0.97	<50	<50	<50	66	0.75/0.79	56	67	69	120	1.0/1.0	—	100	100
13	1.0/1.0	—	100	100	67	1.0/1.0	100	100	100	121	1.0/1.0	94	87	96
14	1.0/1.0	—	100	100	68	1.0/1.0	100	100	100	122	1.0/1.0	—	100	100
15	1.0/1.0	—	100	100	69	0.99/0.99	74	81	68	123	1.0/1.0	74	<50	62
16	1.0/1.0	77	61	60	70	0.99/0.99	79	76	65	124	1.0/1.0	—	100	100
17	1.0/1.0	—	100	100	71	0.99/0.99	72	83	65	125	1.0/1.0	100	100	100
18	1.0/1.0	<50	<50	<50	72	1.0/1.0	100	100	100	126	1.0/1.0	—	100	100
19	1.0/1.0	100	81	100	73	1.0/1.0	—	100	100	127	1.0/1.0	100	100	100
20	0.67/0.80	—	<50	<50	74	1.0/1.0	100	89	97	128	0.96/0.57	<50	<50	<50
21	1.0/1.0	<50	<50	<50	75	1.0/1.0	—	100	100	129	0.96/0.98	61	58	<50
22	1.0/1.0	—	100	100	76	0.77/0.76	<50	<50	<50	130	1.0/1.0	100	100	100
23	1.0/1.0	100	100	100	77	0.79/0.70	<50	<50	<50	131	1.0/1.0	83	57	81
24	0.68/0.66	—	<50	<50	78	1.0/1.0	—	100	100	132	1.0/1.0	—	100	100
25	1.0/1.0	—	100	100	79	1.0/1.0	—	100	100	133	1.0/1.0	—	100	100
26	1.0/1.0	87	55	70	80	1.0/1.0	86	90	91	134	1.0/1.0	—	100	100
27	1.0/1.0	84	79	59	81	1.0/1.0	<50	<50	<50	135	1.0/1.0	—	<50	<50
28	1.0/1.0	100	100	100	82	1.0/1.0	<50	50	51	136	0.97/0.97	68	68	<50
29	1.0/1.0	—	100	100	83	1.0/1.0	100	100	100	137	0.91/0.91	62	51	<50
30	1.0/1.0	75	72	82	84	1.0/1.0	98	90	100	138	1.0/1.0	100	100	100
31	1.0/1.0	—	100	100	85	1.0/1.0	—	100	100	139	1.0/1.0	100	100	100
32	1.0/1.0	100	100	100	86	1.0/1.0	<50	<50	<50	140	1.0/1.0	100	100	100
33	1.0/1.0	—	100	100	87	1.0/1.0	55	<50	52	141	1.0/1.0	—	100	100
34	0.97/0.99	—	100	100	88	1.0/1.0	—	100	100	142	1.0/1.0	93	98	100
35	0.87/0.75	55	<50	<50	89	1.0/1.0	53	51	53	143	1.0/1.0	—	100	100
36	0.99/0.99	52	61	55	90	1.0/1.0	<50	<50	<50	144	1.0/1.0	—	100	100
37	1.0/1.0	—	100	100	91	1.0/1.0	—	100	100	145	1.0/1.0	72	<50	53
38	1.0/1.0	97	100	95	92	1.0/1.0	69	79	81	146	<0.5/<0.5	<50	<50	<50
39	1.0/1.0	—	100	100	93	<0.5/<0.5	<50	<50	<50	147	1.0/1.0	—	100	100
40	0.95/0.92	72	<50	60	94	1.0/1.0	98	100	97	148	0.89/0.80	<50	<50	<50
41	1.0/1.0	100	100	100	95	0.84/0.52	<50	65	<50	149	1.0/1.0	—	100	100
42	1.0/1.0	—	100	100	96	0.99/0.99	72	57	71	150	1.0/1.0	—	100	100
43	0.96/0.94	67	57	76	97	1.0/1.0	100	100	100	151	<0.5/<0.5	—	<50	<50
44	1.0/1.0	—	100	100	98	1.0/1.0	100	100	100	152	<0.5/<0.5	<50	<50	<50
45	1.0/1.0	96	91	97	99	1.0/1.0	—	100	100	153	0.55/<0.5	<50	<50	<50
46	1.0/1.0	100	100	100	100	1.0/1.0	93	84	86	154	1.0/1.0	—	100	100
47	1.0/1.0	—	100	100	101	1.0/1.0	80	75	78	155	1.0/1.0	—	100	100
48	0.91/0.88	58	53	<50	102	1.0/1.0	—	100	100	156	0.55/<0.5	<50	<50	<50
49	1.0/1.0	—	100	100	103	1.0/1.0	—	100	100	157	1.0/1.0	—	100	100
50	1.0/1.0	96	76	91	104	1.0/1.0	100	100	100	158	0.75/0.76	<50	<50	<50
51	<0.5/<0.5	<50	<50	<50	105	1.0/1.0	—	100	100	159	1.0/1.0	—	100	100
52	<0.5/<0.5	<50	<50	<50	106	1.0/1.0	96	98	100	160	0.92/0.96	<50	<50	<50
53	1.0/1.0	—	100	100	107	1.0/1.0	—	100	100	161	1.0/1.0	100	100	100
54	1.0/1.0	77	77	73	108	1.0/1.0	—	100	100					

All the phylogenetic analyses using *A. papudo* as the outgroup showed *A. ringueleti* as the most basal ingroup taxon and *A. scamosa* as the next most basal taxon (except in the MP tree). The remaining 61 species, except *A. marginata* and *A. spinipalma*, were clustered into five major groups informally referred to as clades A, B, C, D, and E (Figs. 2 and 3). The only differences in species composition among these clades included *A. scamosa* as part of clade A in the MP tree and *A. spinipalma* as part of clade E in the ML genetic trees. These same major assemblages also resulted from previous analyses using the long data set, but with lower support and worse resolution—presumably due to the use of a more distant outgroup (data not shown).

Our molecular trees provided strong support for most of the already described and undescribed *Aegla* species (designated here as *Aegla* n. sp.); although samples of the putative species *Aegla laevis*, *A. cholchol*, *A. jarai*, *A. parana*, *A. marginata*, *A. platensis*, *A. franciscana*, and *A. grisella* were shown as paraphyletic by all the phylogenetic

TABLE 2. Shimodaira and Hasegawa (1999) test results and posterior probability estimates for comparisons of alternative monophyletic hypotheses. Δ = difference − ln L; n = no of monophyletic trees.

	S-H test		BMCMC	
Taxon	Δ	P	n	6
A. laevis	<3.4	>0.140	564	0.017
A. cholchol	>54.3	<0.001	0	<0.001
A. jarai	>232.1	<0.001	0	<0.001
A. parana	>85.2	<0.001	0	<0.001
A. marginata	>155.2	<0.001	0	<0.001
A. platensis	>96.4	<0.001	0	<0.001
A. franciscana	>22.7	<0.012	0	<0.001
A. grisella	<1.0	>0.260	862	0.025

methods (Figs. 2 and 3). Monophyly of these taxa was tested using the S-H test and their posterior probabilities were estimated (Table 2) and rejected ($P < 0.05$ and $pP < 0.001$) for all but two species (*Aegla laevis* and *A. grisella*).

The major lineages recovered for all the phylogenetic methods within the *Aegla* suggest a strong pattern (bp > 70% and $pP > 0.95$) of phylogeographic structure and effective partitioning of the genus into two large geographical sections of its range as follows: *A. ringueleti*, *A. scamosa* and clades A and B include the Chilean and southern Argentinean species; *A. marginata* and *A. spinipalma* and clades C, D, and E include the northern Argentinean, Uruguayan, and Brazilian species (Fig. 1). Moreover, our trees suggest a radiation West → East for the group. Alternative hypotheses of radiation East → West from clades C, D, and/or E or of two independent radiations from East and West sides were rejected by the S-H test ($P < 0.001$) and had $pP < 0.001$. Individual clades within these two sections are not completely delimited geographically: clades A and B overlap in Chile and over the Andes Cordillera and a subclade of four species from clade E (species 49, 57, 102 and 106; see Appendix 1) falls within the area delimited by clade C. Nevertheless, for the most part, phylogenetic clades A-B, C, D, and E can be geographically recognized as depicted in Figure 1. Relationships among eastern clades C, D, and E were not strongly supported in our analysis. In fact, alternative rearrangements between these clades to those shown in our trees were not significantly rejected by the S-H test ($P > 0.08$).

Phylogenetic assemblages among the five major clades and the species within do not match the present-day bifurcating pattern of river systems where they occur ($P < 0.001$ for all the comparisons in the S-H test). For example, *A. intercalata* 32, 33, 34 from the Dulce River in northwestern Argentina is more closely related to *A. prado* 75 from the La Plata River in Uruguay and *A. violacea* 100, 101 from the Guaíba River in southeastern Brazil than to *A. humahuaca* 29 or *A. platensis* 70, 71 populations from the Dulce River (see Figs. 1 and 2); or the Argentinean *A. neuquensis* 58 and *A. riolimayana* 77, 78 from the Negro River, which are more closely related to the Chilean species than to each other. This suggests an older pattern of radiation for the group that predates current river drainage patterns.

DISCUSSION

Systematic Implications

Our phylogenetic analyses indicated that at least *A. cholchol*, *A. jarai*, *A. parana*, *A. marginata*, *A. platensis*, and *A. franciscana* samples form significant nonmonophyletic groups, and confirms similar observations by Pérez-Losada et al. (2002b) for *A. cholchol*. These conflicting results between gene trees and alpha taxonomy suggest these samples might represent unrecognized species. Under certain species concepts (e.g., Cracraft, 1983), populations that do not form monophyletic (exclusive) groups qualify as different species. Thus, populations within these six taxa could represent distinct species. Alternative explanations for this nonexclusive association include incorrect estimation of the gene tree or incomplete lineage-sorting of ancestral polymorphisms. Incorrect estimation of the gene tree can be ruled out considering the strong statistical support observed for these discordant clades and the results of the S-H test (Table 2). However, to distinguish the effects of incomplete lineage-sorting from gene flow may be difficult, especially if the populations have split very recently (see Neigel and Avise, 1986). Although considering the smaller *Ne* of the mitochondrial genome and the number of genes analyzed in this study, we may assume that the nonmonophyletic associations observed here are caused by lack of gene flow. Nevertheless, Wiens and Penkrot (2002) have proposed an explicit tree-based species delimitation protocol that emphasizes species recognition based on the older lineages within a putative species (they are less likely to retain ancestral polymorphisms) and does not require species to be exclusive. We applied this protocol to the previous populations and besides *A. chochol*, which does not qualify because it has been collected from a single location, all of the other taxa may be considered multiple species (fig. 1c from Wiens and Penkrot, 2002).

It has been always accepted that the present Aeglidae belong to a single genus; however, there has been no extensive work in terms of establishing the phylogenetic relationships among the species. All of our phylogenetic trees partition the present *Aegla* geographically in two well supported groups: the western clade, constituted by at least 20 species and subspecies from southern Argentina and Chile, and *A. papudo* (when using the long data set); and the central-eastern clade, which includes at least 43 species from northern Argentina, Uruguay, and Brazil. This result may suggest a new subdivision within the present Aeglidae; however, the proposition and diagnosis of a new taxonomic category are beyond the scope of this study.

Biogeographic Implications

Phylogenetic analyses (e.g., Martin and Abele, 1986; Pérez-Losada et al., 2002c) and fossil evidence (Feldmann, 1986; Feldmann et al., 1998) have corroborated the observation made previously (e.g., Schmitt, 1942) that the Aeglidae arose in a marine setting and subsequently adapted and dispersed toward the freshwater

habitats in southern South America. But little is known about how this dispersion occurred and, until now, no extensive phylogenetic approach has been attempted to address this question. Distribution patterns (Ortmann, 1902) and the scarce fossil record suggest the species from the Pacific are the most primitive; however, some descriptive and biometric studies (Schmitt, 1942; Ringuelet, 1949) and panbiogeographic analyses (Morrone and Loppretto, 1994) have argued for an Atlantic origin of the group. Our phylogenies support the Pacific-Origin Hypothesis. Alternative hypotheses depicting an Atlantic origin or two independent origins from both coasts were significantly rejected by the S-H test. Interestingly, our phylogenetic trees agree fairly well with the single generalized track proposed by Morrone and Loppretto (1994) based on the compatibility of individual tracks for three different freshwater Decapoda groups (Anomura, Astacidea, and Brachyura), although our results suggest a different orientation. To establish the direction of individual tracks, Morrone and Loppretto (1994) built a cladogram based on the data presented by Schuldt et al. (1988). Their panbiogeographic analysis pointed out *A. uruguayana* from the Atlantic region as the most primitive species relative to the other Argentinean and Chilean aeglids, resulting in an orientation Northeast → Southwest for the generalized track. Our more complete analysis of the *Aegla* relationships suggests the opposite, *A. uruguayana* has evolved from Pacific relatives.

All of our phylogenetic topologies do not clearly match the present-day drainage systems, which were mostly established ~8 Mya (Potter, 1997; Lundberg et al., 1998). This suggests that the Pleistocene refuge-allopatric divergence model (e.g., Prance, 1982) may only apply to the shallower nodes of the *Aegla* phylogeny. Hence, an older framework must be considered to understand how the radiation of the group took place. The fossil record indicates that all the Anomura superfamilies, except the Hippoidea, started in the Jurassic, ~200 Mya (Schram, 1982). The oldest recognized Aeglidae fossil belongs to *Protaegla miniscula*† from the Early Cretaceous (~110 Mya) in southern Mexico (Feldmann et al., 1998), although a younger Aeglidae fossil (*Haumuriaegla glaessneri*†) from the Late Cretaceous (~75 Mya) was previously found in New Zealand (Feldmann, 1984). However, considering the similarity between these two fossils and the present Aeglidae (most of the differences between them relate to the degree of development of features of the carapace), an earlier origin could even be postulated for the family. Nevertheless, both of these fossils were found in marine strata, which confirms the marine origin of the group, but does not allow us to infer a minimum age for the invasion of the freshwater environments.

Another valuable source of information to study *Aegla* radiation comes from the integration of the history of the southern South American drainages and our own phylogenetic hypotheses. An extensive review of the South American paleodrainage and paleography since the Late Cretaceous including maps and numerous references is presented in Lundberg et al. (1998). As described therein, during the Jurassic and Early Cretaceous periods, almost all of the South American platform and much of adjacent Africa was probably arid or semiarid, hence large master streams may not have existed (Potter, 1997). Drainages from western continental shields were likely predominately westward into the Pacific Ocean (Coney and Evenchick, 1994; Potter, 1997). The separation of America from Africa, which began ~140 Mya, accompanied by the development of broad uplifts along the southeastern Brazilian coast, provoked in the eastern side of the South American Plate an inward-directed and parallel-to-the-coast flow of Gondwanic drainages into the present-day Río de la Plata Estuary (Potter, 1997). In the Late Cretaceous (~90 Mya), western drainage directions changed from westward to eastward as a consequence of the uplift of the early Andes (proto-cordillera), and in the Magallanes and Neuquen Basins (south of ~35°S) rivers flowed into the Atlantic seaway (Coney and Evenchick, 1994; Potter, 1997). During this period to the Early Paleocene (60 Mya), ongoing eastward growth of the proto-cordillera permitted two elongated marine transgressions from the North through the underfilled foreland and parallel to the thrust front as far as northwestern Argentina (~22°S) where they met with a large northward flowing river between the western edge of the Sierras Pampeanas Massif (Fig. 1) and the emerging Andes (Gayet et al., 1993; Sempere et al., 1997). Another two limited marine transgressions from the South Atlantic entered the lower paleo-Paraná basin and overlapped the eastern edge of the Sierras Pampeanas Massif. There is no evidence to indicate that the transgressions from the North and South were ever connected (Gayet et al., 1993). No other large marine transgression has been recorded in southern South America until the Paranan transgression from the south Atlantic in the Late Miocene (~11 Mya). As we mentioned above, our phylogenetic analyses strongly support a Pacific-Origin hypothesis of the present *Aegla*, which, examined under the previous paleodrianage scenarios, suggests that the radiation of the present aeglids from their marine ancestors began at least 60 Mya (2nd marine transgression). From the Late Cretaceous to the Middle Eocene, the northward river flowing along the foreland basin was separated from the paleo-Paraná system by the Sierras Pampeanas Massif. This would explain the basic phylogenetic clustering of *A. ringueleti* and *A. scamosa* to the Chilean species and suggests that the marine Aeglidae came into the continent along with one of the two marine transgressions. The fossil evidence found in southern Mexico also supports this hypothesis. However, a pre-Andean uplift invasive hypothesis cannot be ruled out. The two transgression-regression phases that occurred during this period, the formation of the Chilean Cordillera de la Costa drainage, which flows perpendicular to the present Andes, and the change in drainage directions of the Colorado and Negro Rivers (south of ~35°S) surely created several rearrangements in the western paleodrainage. Multiple occurrences of vicariance and migration can be postulated under this scenario of drainage coalescence and extension that would produce the mixed pattern of

species locations observed between clades A and B in Figure 1.

After the Middle Eocene (~43 Mya), the Sierras Pampeanas Massif lost their influence as a barrier, which is related to an eastward propagation of the Andean thrust front and the breakup of the parallel-to-the-western-edge drainage (Lundberg et al., 1998). As thrusting propagated significantly eastward, waters from the once western drainage (between ~20° and ~35°S) were captured by an already enlarged paleo-Paraná River. This created the possibility for the Chilean aeglids to radiate over the vast eastern territories. A major orogenic phase along much of the Andean chain was initiated during the Late Oligocene (~30 Mya) that reached elevations of 3000 m in some places (Marshall and Sempere, 1993; Sempere et al., 1994, 1997). This significant uplift probably isolated the Chilean species from their eastern relatives.

During the middle Tertiary periods, the present Paraná-Uruguay drainage pattern was largely established, although the details remain to be clarified (Potter, 1997). Based on our phylogenetic trees and the known distribution of the present *Aegla* species (see Bond-Buckup and Buckup, 1994), the eastern radiation of the group seems to have grossly followed the present river pattern: clade C along the Paraná Basin; clade D along the western tributaries and the Uruguay Basin (nowadays this river is mostly represented by *A. prado*, *A. platensis* and *A. uruguayana*); and clade E along the Uruguay Basin. However, some discrepancies still exist between the phylogenetic assemblages in our trees and the current drainage system, which might be a consequence of the paleodrainage modifications that occurred in southern South America over the last Tertiary periods (see Lundberg et al., 1998 and references therein). Among them, the formation of the Paranan Sea and the uplift of the Serra do Mar had the largest effects. During the Late Miocene an extensive marine transgression (the Paranan Sea) flooded the central part of southern South America up to ~17°S. Marine waters, which reached tens of meters in depth in some places, covered most of the western tributaries of the paleo-Paraná and the paleo-Paraguay and the lower parts of the paleo-Uruguay and paleo-Paraná for ~2 my until they regressed to the Atlantic. Under these conditions episodes of isolation into refugia at the drainages flowing off the eastern edge of the Andes followed by recolonization of the reborn freshwater areas can be postulated. This would explain some intriguing phylogenetic associations within clade D. The final uplift of the Serra do Mar (Fig. 1) in southeastern Brazil modified the upper course of the eastern tributaries of the paleo-Paraná, paleo-Uruguay, and the short intermountain rivers flowing eastward. Capture and breakup of headwaters between drainages may have caused the observed overlap between some species locations of clades E and C in this area.

Based on the described geological history of the Sierras Pampeanas Massif and the emerging Andes, it may be inferred that the most recent ancestor of the central-eastern *Aegla* species diverged between 43 and 30 Mya. Hence we used this information for calibrating the node 50 in the MLhc tree (Fig. 2) to a midvalue of 37 my. Then we applied the multilocus local-clock method of Yang and Yoder (2003) to estimated divergence times for all the nodes in the tree and test our biogeographical hypothesis (see Fig. 2). Although here we will only present the results under five local rates ($r = 5$), dates were also estimated under $r = 0$ (clock model) and $r = 2$ (two different rates for the western and central-eastern groups). Similar age estimations were obtained under the local-clock models for the major clades, but unreliable younger dates were generated under the clock model. This is not surprising considering the poor fit of the latter hypothesis to our data compared to the best-fit model (LRT = 724.4; $P < 0.001$). Under the local-clock$_{r=5}$ model, the age of the most basal node in the MLhc tree (node 1) was estimated as 74.0 my. This suggests that the colonization of southern South America and hence the origin of the freshwater Aeglidae is post-Andean and occurred during the Late Cretaceous marine transgression. Moreover, this date agrees well with the scarce Aeglidae fossil information available, which indicates that the marine relatives of the present *Aegla* are at least 75 my old. Estimated ages for the western clades A and B were 42.5 ± 2.6 (95% confidence interval of 39.9 to 45.1 my) and 41.0 ± 1.5 my, respectively, and for the central-eastern clades C, D, and E were 33.2 ± 1.8, 25.1 ± 0.9, and 24.3 ± 0.7 my, respectively, which confirms the older origin of the former *Aegla* clades. Moreover, this confirms that the basic radiation of the central-eastern aeglids along the Paraná-Uruguay drainage occurred before the formation of the Paranan Sea and the final uplift of the Serra do Mar (~12 Mya). However, some groups such as the subclade defined by node 97 (clade D) showed a more recent age (10.7 ± 1.1 my) than their sister relatives ($\geq 19.5 \pm 0.4$ my), which suggests that the former taxa speciated after the regression of the Paranan Sea. This would explain sister associations such as those observed between *A. platensis* 70, 71 (central region) and *A. singularis* 93 (eastern region).

Although the estimated divergence times seem to support our biogeographical hypothesis of the *Aegla* radiation, we are aware that some parts still may look speculative. This is primarily a consequence of the limited knowledge there is about the paleodrainage in South America (see Potter, 1997, for a review of the major questions) and the gaps in our phylogenetic study. Although our analyses included almost all of the described species, there are still key regions such as the middle and lower Paraná and Uruguay Rivers that need to be screened. Our sampling efforts are currently focusing on those areas. Nevertheless, we hope this study provides a framework for future work on the evolution of this unique group and contributes to a better understanding of the history of the southern South American drainages.

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APPENDIX 1. *Aegla* and outgroup samples listed in alphabetical order.

Species	Minor drainage/River/Country	Coordinates	N
A. abtao Schmitt,1942	Rupanco Lake, Bueno River, Chile	40°46′S, 72°36′W	2
	Riñihue Lake, Valdivia River, Chile	39°46′S, 72°27′W	2
	Chifín River, Bueno River, Chile	40°46′S, 73°09′W	2
A. affinis Schmitt, 1942	Maule River, Chile	35°58′S, 70°33′W	1
	Maule Lagoon, Maule River, Chile	36°00′S, 70°33′W	1
	Chico River, Colorado River, Argentina	35°48′S, 70°08′W	1
	Chico River, Colorado River, Argentina	35°51′S, 69°48′W	1
A. alacalufi Jara and López, 1981	Reloncaví River, Continental Chiloé, Chile	41°23′S, 72°17′W	2
A. araucaniensis Jara, 1980	Chaimávida Creek, Bío Bío River, Chile	36°51′S, 72°52′W	2
A. bahamondei Jara, 1982	Huillinco Creek, Tucapel River, Chile	37°44′S, 73°23′W	1
A. camargoi Buckup and Rossi, 1977	Lava Tudo River, Pelotas River, Brazil	28°08′S, 49°41′W	1
	Divisa creek, Pelotas River, Brazil	28°38′S, 49°57′W	1
	Sepultura River, Pelotas River, Brazil	28°34′S, 49°47′W	2
A. castro Schmitt, 1942	Ribeirão Grande, Tibagi-Paranapanema River, Brazil	25°02′S, 49°50′W	1
	Guarauna Creek, Tibagi-Paranapanema River, Brazil	25°17′S, 50°16′W	1
	Taquaral River, Paranapanema River, Brazil	24°03′S, 47°59′W	1
A. cavernicola Türkay, 1972	Areia II Cave River, Ribeira Iguape River, Brazil	26°01′S, 48°47′W	2
A. cholchol Jara, 1999	Chol-Chol River, Imperial River, Chile	38°36′S, 72°52′W	4
A. denticulata denticulata Nicolet, 1849	Chifín River, Bueno River, Chile	40°46′S, 73°09′W	2
A. denticulata lacustris Jara, 1989	Rupanco Lake, Bueno River, Chile	40°46′S, 72°37′W	2
A. franciscana Buckup and Rossi, 1977	Pinto Creek, Caí-Guaíba River, Brazil	29°25′S, 50°30′W	1
	Baio Branco River, Tainhas-Taquari-Guaíba River, Brazil	29°13′S, 50°15′W	1
	Rolantinho Creek, Sinos-Guaíba River, Brazil	29°23′S, 50°24′W	1
A. grisella Bond-Buckup and Buckup, 1994	Capingui River, Taquari-Guaíba River, Brazil	28°21′S, 52°14′W	1
	Capingui River, Taquari-Guaíba River, Brazil	28°21′S, 52°12′W	1
	Sangão River, Taquari-Guaíba River, Brazil	28°32′S, 52°03′W	1
	Sesteado Creek, Taquari-Guaíba River, Brazil	28°25′S, 52°11′W	1
A. hueicollensis Jara, 1999	Hueicolla River, Bueno River, Chile	40°09′S, 73°39′W	2
A humahuaca Schmitt, 1942	Sali River, Dulce River, Argentina	26°14′S, 65°29′W	2
A. inconspicua Bond-Buckup and Buckup, 1994	Pinto Creek, Caí-Guaíba River, Brazil	29°25′S, 50°30′W	1
A. inermis Bond-Buckup and Buckup, 1994	Caará Creek, Sinos-Guaíba River, Brazil	29°52′S, 50°20′W	2
A. intercalata Bond-Buckup and Buckup, 1994	Las Carreras Creek, Dulce River, Argentina	26°56′S, 65°46′W	1
	Chirimayo Creek, Dulce River, Argentina	27°21′S, 65°49′W	1
	Trancas River, Dulce River, Argentina	28°04′S, 65°54′W	1
A. itacolomiensis Bond-Buckup and Buckup, 1994	Demetrio Creek, Gravataí-Guaíba River, Brazil	29°46′S, 50°51′W	3
A. jarai Bond-Buckup and Buckup, 1994	Cascata Avencal, Canoas-Pelotas River, Brazil	28°02′S, 49°36′W	1
	Marombas River, Canoas-Pelotas River, Brazil	27°09′S, 50°27′W	1
	Amola Faca Creek, Canoas-Pelotas River, Brazil	27°44′S, 50°20′W	2
	Pessegueiro Creek, Canoas-Pelotas River, Brazil	27°44′S, 50°20′W	1
	Lava Tudo River, Canoas-Pelotas River, Brazil	28°08′S, 49°41′W	1
	Canoas River, Pelotas River, Brazil	27°47′S, 49°38′W	1
	Espingarda Creek, Itajaí-Açú River, Brazil	27°01′S, 49°09′W	2
	das Pedras River, Canoas-Pelotas basin, Brazil	27°07′S, 50°27′W	1
A. jujuyana Schmitt, 1942	Grande River, Bermejo River, Argentina	23°11′S, 65°20′W	1
	Grande River, Bermejo River, Argentina	23°33′S, 65°23′W	1

(Continued on next page)

APPENDIX 1. *Aegla* and outgroup samples listed in alphabetical order. *(Continued)*

Species	Minor drainage/River/Country	Coordinates	N
A. laevis laevis (Latreille, 1818)	Trebulco Creek, Maipo River, Chile	33°19′S, 70°24′W	2
A. laevis talcahuano Schmitt, 1942	Lircay River, Maule River, Chile	35°32′S, 71°20′W	1
	Torreón River, Ñuble River, Chile	36°30′S, 72°11′W	2
A. leptochela Bond-Buckup and Buckup, 1994	Dos Paiva Cave, Ribeira do Iguape River, Brazil	24°16′S, 48°26′W	2
A. leptodactyla Buckup and Rossi, 1977	Divisa creek, Pelotas River, Brazil	28°38′S, 49°57′W	2
A. ligulata Bond-Buckup and Buckup, 1994	Tainhas River, Taquari-Guaiba River, Brazil	29°15′S, 50°13′W	3
A. longirostri Bond-Buckup and Buckup, 1994	Antas River, Taquari-Guaiba River, Brazil	29°05′S, 51°40′W	1
	Sta. Barbara River, Taquari-Guaiba River, Brazil	29°05′S, 51°41′W	1
	Carreiro River, Taquari-Guaiba River, Brazil	28°35′S, 51°57′W	1
A. manni Jara, 1980	Buenaventura Creek, Valdivia River, Chile	39°48′S, 73°09′W	3
A. marginata Bond-Buckup and Buckup, 1994	Grota Funda Creek, Litoral River, Brazil	25°20′S, 48°53′W	2
	Dos Paiva Cave, Ribeira do Iguape River, Brazil	24°16′S, 48°26′W	2
A. neuquensis Schmitt, 1942	Collón Curá River, Negro River, Argentina	40°00′S, 70°50′W	2
A. odebrechtii Müller, 1876	Atalanta Creek, Itajaí-Açu River, Brazil	27°28′S, 49°48′W	2
A. obstipa Bond-Buckup and Buckup, 1994	Horto Forestal Ramos Creek, Guaíba River, Brazil	30°26′S, 52°06′W	2
1*A. occidentalis* Jara et al., 2002	Huillinco Creek, Tucapel River, Chile	37°44′S, 73°23′W	2
A. papudo Schmitt, 1942	Rabuco River, Aconcagua River, Chile	32°42′S, 70°33′W	2
A. parana Schmitt, 1942	Passa Três Creek, Iguaçu River, Brazil	26°03′S, 49°43′W	1
	Varzea River, Iguaçú River, Brazil	25°39′S, 49°50′W	1
A. parva Bond-Buckup and Buckup, 1994	Zuk Creek, Iguaçu River, Brazil	25°30′S, 53°34′W	2
A. paulensis Schmitt, 1942	Jundiaí Creek, Tiete River, Brazil	23°17′S, 46°56′W	2
A. perobae Hebling and Rodrigues, 1977	Peroba Cave, Tiete River, Brazil	22°31′S, 47°56′W	2
A. pewenchae Jara, 1994	Icalma Lake, Bío Bío River, Chile	38°48′S, 71°16′W	3
A. plana Buckup and Rossi, 1977	Pinto Creek, Caí-Guaíba River, Brazil	29°25′S, 50°30′W	2
A. platensis Schmitt, 1942	Vipos River, Dulce River, Argentina	26°30′S, 65°24′W	1
	Los Membrillos River, Dulce River, Argentina	26°51′S, 65°25′W	1
	Demetrio Creek, Gravataí-Guaíba River, Brazil	29°46′S, 50°51′W	3
	Mariana Pimentel Creek, Guaíba River, Brazil	30°19′S, 51°35′W	2
	Tolotti Creek, Guaíba River, Brazil	30°17′S, 51°36′W	2
A. prado Schmitt, 1942	Montevideo, La Plata River, Uruguay	34°52′S, 56°10′W	2
A. ringueleti Bond-Buckup and Buckup, 1994	Calchaqui River, Salado River, Argentina	25°07′S, 66°09′W	2
A. riolimayana Schmitt, 1942	Moquehue-Aluminé Lake, Negro River, Argentina	38°52′S, 71°12′W	1
	Limay River, Negro River, Argentina	41°04′S, 71°09′W	1
A. rossiana Bond-Buckup and Buckup, 1994	Escangalhado Creek, Mampituba-Litoral River, Brazil	29°34′S, 50°17′W	2
A. rostrata Jara, 1977	Riñihue Lake, Valdivia River, Chile	39°46′S, 72°27′W	2
A. sanlorenzo Schmitt, 1942	Los Berros Creek, Bermejo River, Argentina	23°44′S, 64°39′W	1
	Sta. María River, Bermejo River, Argentina	23°14′S, 64°16′W	1
A. scamosa Ringuelet, 1948	Mendoza River, Desaguadero River, Argentina	32°41′S, 69°21′W	1
	Agua Negra Creek, Desaguadero River, Argentina	30°19′S, 68°42′W	1
	Jachal River, Desaguadero River, Argentina	30°12′S, 69°02′W	1
A. schmitti Hobbs III, 1979	Varzea River, Iguaçú River, Brazil	25°39′S, 49°50′W	1
	Betari River, Ribeira do Iguape River, Brazil	24°31′S, 48°41′W	1
A. septentrionalis Bond-Buckup and Buckup, 1994	Yavi Creek, Pilcomayo River, Argentina	22°05′S, 65°27′W	1
	Colorado Creek, Miraflores River, Argentina	22°24′S, 65°34′W	1
A. serrana Buckup and Rossi, 1977	Guirra Creek, Caí-Guaíba River, Brazil	29°21′S, 50°39′W	1
	Antas River, Guaíba River, Brazil	28°47′S, 49°58′W	1
	Silveira River, Pelotas River, Brazil	28°36′S, 49°58′W	1
A. singularis Ringuelet, 1948	Caxambu River, Ijuí-Pelotas River, Brazil	28°21′S, 53°32′W	1
A. spectabilis Jara, 1986	Chol-Chol River, Imperial River, Chile	38°36′S, 72°52′W	1
A. spinipalma Bond-Buckup and Buckup, 1994	Ivaí River, Jacuí-Guaíba River, Brazil	28°56′S, 53°38′W	1
A. spinosa Bond-Buckup and Buckup, 1994	Aguas Brancas River, Canoas-Pelotas River, Brazil	27°56′S, 49°34′W	2
A. strinatii Türkay, 1972	Ribeirão Grande, Ribeira do Iguape River, Brazil	25°07′S, 49°56′W	1
	do Diabo Cave, Ribeira do Iguape River, Brazil	24°38′S, 48°24′W	2
A. uruguayana Schmitt, 1942	del Palacio Cave, Uruguay River, Uruguay	33°31′S, 56°53′W	2
A. violacea Bond-Buckup and Buckup, 1994	Barra Ribeiro Creek, Guaíba River, Brazil	30°16′S, 51°40′W	2
	Mariana Pimentel Creek, Guaíba River, Brazil	30°21′S, 51°34′W	3
*Aegla n.*sp.1	Passa Quatro Creek, Itajaí-Açu River, Brazil	26°28′S, 50°11′W	2
Aegla n. sp.2	Itajaí River, Itajaí-Açu River, Brazil	26°46′S, 49°11′W	2
*Aegla n.*sp.3	Matador River, Canoas-Pelotas River, Brazil	27°49′S, 49°33′W	2
Aegla n.sp.4	Nova Petropolis, Caí-Guaíba River, Brazil	29°22′S, 51°07′W	2
Aegla n.sp.5	Espingarda River, Itajaí-Açú River, Brazil	27°01′S, 49°09′W	2
Aegla n.sp.6	Pasaje River, Salado River, Argentina	25°07′S, 65°00′W	2
Lithodes santolla (Molina, 1782)	Corral, Valdivia, Chile	40°04′S, 74°02′W	1
Lomis hirta (Lamarck, 1810)	Point Sturt, Victoria, Australia	38°38′S, 143°53′E	1
Munida subrugosa (White, 1847)	Quellón, Chiloé, Chile	43°06′S, 73°40′W	1
Pachycheles laevidactylus (Rodrigues da Costa, 1960) (formerly *P. haigae* Rodrigues da Costa, 1960)	Tramandaí, Brazil	29°55′S, 50°00′W	1
Uroptychus nitidus (A. Milne-Edwards, 1880)	Florida, USA	28°14–16′N, 86°21–28′W	1
Uroptychus parvulus (Henderson, 1885)	Corral, Valdivia, Chile	40°04′S, 74°02′W	1