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PHYLOGENETIC RELATIONSHIPS AMONG THE SPECIES OF *AEGLA* (ANOMURA: AEGLIDAE) FRESHWATER CRABS FROM CHILE

Marcos Pérez-Losada, Carlos G. Jara, Georgina Bond-Buckup, and Keith A. Crandall

(MP-L, correspondence) Department of Zoology, Brigham Young University, Provo, Utah 84602-5255, U.S.A. (mp323@email.byu.edu); (CGJ) Instituto de Zoología, Casilla 567, Universidad Austral de Chile, Valdivia, Chile (cjara@uach.cl); (GB-B) Departamento de Zoologia, Universidade Federal Do Rio Grande Do Sul, Porto Alegre, Brazil (ginabb@vortex.ufrgs.br); (KAC) Department of Zoology and Monte L. Bean Museum, Brigham Young University, Provo, Utah 84602-5255, U.S.A. (keith_crandall@byu.edu)

A B S T R A C T

Nineteen species and subspecies of freshwater crabs from the anomuran family Aeglidae are represented in Chile, 16 of which are endemic to this country. We sequenced ~2,600 nucleotides of the 12S, 16S, COI, and COII genes from mitochondrial DNA to estimate phylogenetic relationships among the Chilean aeglids. We sampled 16 putative Chilean species and subspecies and one morphologically unrecognized taxon according to the most recent Aeglidae classification. In addition to the Chilean aeglids, one sample of *Aegla riolimayana* and two samples of *Aegla affinis* were collected from Argentina to check previous hypotheses about the origin of the group. Two other anomurans, one galatheid (*Munida subrugosa*) and one porcellanid (*Pachycheles haigae*), were sequenced to serve as outgroups in our phylogenetic analysis. Our results show the clear separation of *Aegla papudo* from the other *Aegla* species, as has been suggested previously based on morphology. Its basal position in the Aeglidae trees also supports a Pacific origin for the Aeglidae. Our phylogenies provide strong monophyletic support for the currently recognized species, with the exception of *Aegla laevis* and *Aegla cholchol* samples, which form nonmonophyletic groups.

The Aeglidae Dana, 1852, are freshwater anomuran crabs with several features that make them a fascinating group for evolutionary study. First, they are restricted to the Neotropical region of South America, the only anomuran family thus restricted. Second, morphologically, the aeglids are included with the galatheid, porcellanid, and chirostylid crabs in the superfamily Galatheoidea Samoulle, 1819. However, there are some important morphological differences between aeglids and their relatives (e.g., gill structure and caparace sutures; see Martin and Abele, 1988) that throw into question the taxonomic position of Aeglidae (Martin and Abele, 1986). Finally, from an ecological perspective, aeglids are a unique group because they are the only Galatheoidea family entirely restricted to freshwater habitats.

The present Aeglidae belong to a single genus, *Aegla* Leach, consisting of approximately 70 recognized species and subspecies (see Bond-Buckup and Buckup, 1994) distributed among Chile, Brazil, Argentina, Uruguay and Bolivia. The Chilean aeglids constitute a group of 19 putative species and subspecies with a latitudinal distributional pattern starting at the Choapa River in the north, and ranging to Madre de Dios Island in the Insular Territory (see Fig. 1). From a biogeographical perspective, the Chilean aeglids represent an interesting group within the Aeglidae because of their geographical isolation associated with the uplift of the Andean Cordillera. Sixteen of them are endemic to Chile, some occurring only in a single drainage (e.g., *Aegla bahamondei* Jara, 1982, at Tucapel River and *A. spectabilis* Jara, 1986, at Imperial River), and three are found on both sides of the Andean Cordillera (*A. abtao* Schmitt, 1942, *A. neuquensis* Schmitt, 1942, and *A. affinis* Schmitt, 1942).

The origin of Chilean aeglids as well as their trans-Andean relatives is uncertain. Ortmann (1902) proposed that *Aegla* species from Chile included the more primitive forms of the genus. However, Schmitt (1942) hypothesized that the *Aegla* from the Atlantic side of South America were more primitive, and species ranging in the Chilean streams were more derived. Feldmann (1986) considered both conclusions to be speculative, although based on the discovery of the marine fossil *Hamuriaegla glaessneri* Feldmann, 1984, in New Zealand, he suggested that the primitive aeglids came from the Indo-Pacific

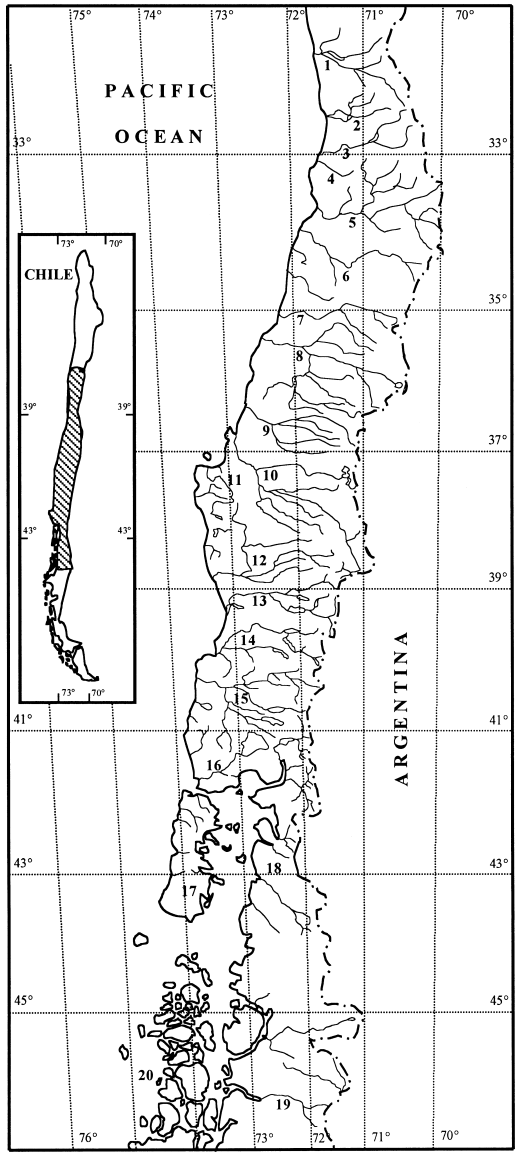

Fig. 1. Chile, showing the main hydrographic basins where *Aegla* occur: 1, Choapa; 2, La Ligua; 3, Aconcagua; 4, Valparaiso; 5, Maipo; 6, Rapel; 7, Mataquito; 8, Maule; 9, Ñuble; 10, Bío Bío; 11, Tucapel; 12, Imperial; 13, Toltén; 14, Valdivia; 15, Bueno; 16, Maullín; 17, Chiloé Island; 18, Chiloé Continental; 19, Aysén; 20, Insular Territory.

region and dispersed through South America from the Chilean coast.

There has been no extensive work in terms of establishing Aeglidae phylogenetic relationships. Partial relationships have been proposed for some Argentinean species (e.g., Schuldt *et al.,* 1988), but no phylogenetic study has been published on the Chilean aeglids. One of the biggest difficulties in constructing morphological-based phylogenies of this group is a lack of enough shared derived characters relative to the large number of extant species. Thus, the analysis demands considerable effort, and a well-established phylogeny is not guaranteed. This makes aeglids ideal candidates for molecular systematics; the use of DNA sequences allows for the collection of more data to investigate the relationships among these taxa. In this paper, we will study the phylogenetic relationships among the Aeglidae from Chile using four mitochondrial genes: 12S, 16S, COI, and COII. The resulting phylogeny is then used to address alternative hypotheses concerning the origin of the group and the monophyly of currently recognized species.

Materials and Methods

Sampling.—Crabs were collected by hand, dipnet, or trawl fishing from August 1999 to February 2000. Abdomen and gill tissues from some specimens were dissected and preserved in 100% EtOH for DNA extraction. The remaining specimens were preserved in 70% EtOH and are housed in the crustacean collection at the Monte L. Bean Life Science Museum, Brigham Young University. We sampled between one and six individuals per species or subspecies (49 individuals sampled total) from one to four localities and for a total of 16 of the 19 putative Chilean species or subspecies (Table 1). A morphologically unrecognized taxon according to the most recent taxonomic classification developed by Jara (1996) was also collected at Tucapel River. This putative new species was included in this study under the name *Aegla* sp. Those species not sampled include: *Aegla concepcionensis* Schmitt, 1942, and *Aegla expansa* Jara, 1992, endemic from the Concepción River and the Hualqui Creek, respectively (Bío Bío Basin); and *Aegla neuquensis,* a trans-Andean species that also occurs marginally in the Insular Territory of Chile. The first two species could be considered extinct due to human pollution and forest exploitation (Bahamonde *et al.,* 1998; Jara, personal observation). In addition to the Chilean aeglids, one sample of *Aegla riolimayana* Schmitt, 1942, and two samples of *Aegla affinis* were collected in Argentina to check previous morphological hypotheses about the origin of the Chilean aeglids. Another two anomurans, one galatheid (*Munida subrugosa* (White, 1847)) from Chile and one porcellanid (*Pachycheles haigae* Rodrigues da Costa, 1960) from Brazil, were collected to be used as the initial outgroup (Table 1).

DNA Extraction and Sequencing.—The DNA was extracted from the preserved tissues using the methods described in Crandall and Fitzpatrick (1996). The PCR products for four mitochondrial genes (~ 2,600 bp) were amplified using the primers 12S-f 5′-GAAACCAGGATTAGATACCC-3′ 12S-r 5′-TTTCCCGCGAGCGACGGGCG-3′ (Mokady *et al.,* 1999); and COI-f 5′-GAGCTCCAGATATAGCATTCC-3′ COI-r 5′-AGTATAAGCGTCTGGGTAGTC-3′ (van Syoc, 1995); and the four newly designed primers 16Saeglid-f 5′-ACTTGATA-

Table 1. *Aegla* species and outgroups (*Munida subrugosa* and *Pachycheles haigae*), and number of specimens (*n*) examined in this study listed in alphabetical order. Key numbers for main Chilean basins in Fig. 1 are also included. AR1, AR2 = Argentina.

Species	Main basin, minor drainage	*n*	Coordinates
A. abtao Schmitt, 1942	(15) Bueno River, Rupanco Lake	2	40°46′S, 72°36′W
	(14) Valdivia River, Riñihue Lake	2	39°46′S, 72°27′W
	(15) Bueno River, Chifín River	2	40°46′S, 73°09′W
A. affinis Schmitt, 1942	(8) Maule River	1	35°58′S, 70°33′W
	(8) Maule River, Maule Lagoon	1	36°00′S, 70°33′W
	(AR1) Colorado River, Chico River	1	35°48′S, 70°08′W
	(AR1) Colorado River, Chico River	1	35°51′S, 69°48′W
A. alacalufi Jara and López, 1981	(18) Continental Chiloé, Reloncaví River	2	41°23′S, 72°17′W
A. araucaniensis Jara, 1980	(10) Bío Bío River, Chaimávida Creek	2	36°51′S, 72°52′W
A. bahamondei Jara, 1982	(11) Tucapel River, Huillinco Creek	2	37°44′S, 73°23′W
A. cholchol Jara, 1999	(12) Imperial River, Chol-Chol River	4	38°36′S, 72°52′W
A. denticulata denticulata Nicolet, 1849	(15) Bueno River, Chifín River	2	40°46′S, 73°09′W
A. denticulata lacustris Jara, 1989	(15) Bueno River, Rupanco Lake	2	40°46′S, 72°37′W
A. hueicollensis Jara, 1999	(15) Bueno River, Hueicolla River	2	40°09′S, 73°39′W
A. laevis laevis (Latreille, 1818)	(5) Maipo River, Trebulco Creek	2	33°19′S, 70°24′W
A. laevis talcahuano Schmitt, 1942	(8) Maule River, Lircay River	2	35°32′S, 70°20′W
	(9) Ñuble River, Torreón River	2	36°30′S, 72°11′W
A. manni Jara, 1980	(14) Valdivia River, Buenaventura Creek	3	39°48′S, 73°09′W
A. papudo Schmitt, 1942	(3) Aconcagua River, Rabuco River	2	32°42′S, 70°33′W
A. pewenchae Jara, 1994	(10) Bío Bío River, Icalma Lake	3	38°48′S, 71°16′W
A. riolimayana Schmitt, 1942	(AR2) Negro River, Moquehue-Aluminé Lake	2	38°52′S, 71°12′W
A. rostrata Jara, 1977	(14) Valdivia River, Riñihue Lake	2	39°46′S, 72°27′W
A. spectabilis Jara, 1986	(12) Imperial River, Chol-Chol River	1	38°36′S, 72°52′W
Aegla sp.	(11) Tucapel River, Huillinco Creek	2	37°44′S, 73°23′W
Munida subrugosa (White, 1847)	Quellón, Chiloé, Chile	1	43°06′S, 73°40′W
Pachycheles haigae Rodrigues da Costa, 1960	Tramandaí, Brazil	1	29°55′S, 50°00′W

TATAATTAAAGGG-3′ 16Saeglid-r 5′-CTGGCGCCG-GTCTGAACTCAAATC-3′; and COIIaeglid-f 5′-CT-TAYTTAGGATTTCAAGATAG-3′ COIIaeglid-r 5′-GGT-ATAAATCTATGATTTGC-3′. These last four primers are based on aeglid sequences obtained in a preliminary screening. Standard PCR conditions were used on a Perkin-Elmer 9600 machine and consisted of the following: an initial denaturation at 96°C for 3 min followed by 50 cycles of 95°C for 1 min, 45°C (12S and COI) or 50°C (16S and COII) for 1 min, 72°C for 1 min followed by an extension at 72°C for 5 min. Successful PCR products were purified using a GeneClean® II kit (Bio 101). Automated sequences were generated in both directions on an ABI 377XL automated sequencer using the ABI Big-dye Ready-Reaction kit, following the standard cycle sequencing protocol, but using a quarter of the suggested reaction size.

Phylogenetic Analyses.—Nucleotide sequences were aligned using Clustal X (Thompson *et al.,* 1994) and then adjusted by eye. Phylogenetic relationships were estimated using maximum parsimony and maximum likelihood (Felsenstein, 1981), recognizing a diversity of opinions relative to the appropriate methodology for reconstructing phylogenetic relationships. Both phylogeny reconstruction methods assume a model of evolution. Maximum parsimony implicitly assumes that all character changes are equally likely. Maximum likelihood, on the other hand, makes explicit assumptions about the relative likelihoods of character change using a model of evolution (Huelsenbeck and Crandall, 1997). Therefore, for this method, the choice of model must be justified relative to the data at hand. This can be accomplished easily within the likelihood framework (Felsenstein, 1998; Goldman, 1993; Huelsenbeck and Crandall, 1997). We used the approach outlined by Huelsenbeck and Crandall (1997) to test hypotheses relating to the molecular evolution of the nucleotide sequences examined in this study. This approach estimates a starting tree using neighbor-joining assuming a Jukes and Cantor model of evolution. With this tree, likelihood scores are calculated for a variety of models of evolution that incorporate different assumptions about the types of changes involved (e.g., base frequencies are equal or not). The likelihood scores are then compared statistically using a likelihood ratio test (Posada and Crandall, 1998). The model choice is then dictated by the null hypotheses rejected. The following null hypotheses were tested: 1) nucleotide frequencies are equal, 2) transition rate equals transversion rate, 3) transition rates are equal, 4) transversion rates are equal, 5) transversions occur at only two rates, 6) rate homogeneity occurs across sites, 7) proportion of invariable sites is not significant. The likelihood values associated with these models were estimated in PAUP* version 4.0b8 (Swofford, 2000). The statistical tests were performed using Modeltest version 3.1 (Posada and Crandall, 1998).

Once a model was selected, phylogenetic relationships were estimated via maximum likelihood incorporating this model of evolution. Maximum likelihood and maximum parsimony searches were heuristic. As such, they

Table 2. Likelihood ratio tests of models of molecular evolution (Huelsenbeck and Crandall, 1997; Posada and Crandall, 1998) for 12S + 16S + COI + COII genes.

Null hypothesis	Models compared	$-\ln L_0$	$-\ln L_1$	$-2\ln\lambda$	*d.f.*	*P*
Equal base frequencies	H_0 : JC69 H_1 : F81	9,499	9,276	447	3	<0.000001
Equal ti/tv rates	H_0 : F81 H_1 : HKY85	9,276	8,913	726	1	<0.000001
Equal ti rates	H_0 : HKY85 H_1 : TrN	8,913	8,912	1.97	1	0.160
Equal tv rates	H_0 : TrN H_1 : K81uf	8,912	8,907	11.1	1	0.0009
Only two tv rates	H_0 : K81uf H_1 : TVM	8,907	8,895	24.09	2	0.000006
Equal rates among sites	H_0 : TVM H_1 : TVM + Γ	8,895	8,268	1,254	1	<0.000001
Proportion of invariable sites	H_0 : TVM + Γ H_1 : TVM + Γ + I	8,268	8,191	153	1	<0.000001

are subject to biases associated with the order of taxon addition (Templeton, 1992) and multiple tree islands (Maddison, 1991). To avoid these biases, 10 random addition heuristic searches were performed for the likelihood and 1,000 for the parsimony analyses. Confidence in the resulting relationships was assessed using the bootstrap procedure with 100 replications (Felsenstein, 1985) for maximum likelihood and 1,000 replications for maximum parsimony. Likelihood and parsimony searches as well as the bootstrap analyses were executed in PAUP* version 4.0b8 (Swofford, 2000). Data sets were analyzed independently with models optimized for each data set. We then performed a partition homogeneity test to see if we were justified in combining data sets (Farris *et al.,* 1994). This test was implemented in PAUP* version 4.0b8 using 1,000 replicates. Phylogenetic signal within the combined data set was assessed for different combinations of taxa using the g_1 statistic of the random tree distribution (Hillis and Huelsenbeck, 1992).

RESULTS

Our sequencing efforts resulted in 196 new 12S, 16S, COI, and COII mtDNA sequences from 18 putative species or subspecies of aeglids and two outgroups (Table 1). The alignment for these sequences can be downloaded from our lab webpage (http://zoology.byu.edu/crandall_lab/cranlabpubs.htm). The new sequences have been deposited in GenBank under the accession numbers AY049985—AY050166.

To root the Aeglidae trees we initially used two species from the Galatheoidea families Galatheidae (*Munida subrugosa*) and Porcellanidae (*Pachycheles haigae*) according to the morphological phylogenies proposed by Martin and Abele (1986) for the anomuran decapods. Because of the large genetic divergence among these three families, the branch connecting outgroup and ingroup taxa was much longer than those relating the ingroup taxa. In all the likelihood and parsimony analyses *Aegla papudo* Schmitt, 1942, was placed in the basal position of the Aeglidae tree. Thus, although we initially used *Munida subrugosa* and *Pachycheles haigae* as the outgroup to check Aeglidae monophyly, once this hypothesis was confirmed and the basal position of *A. papudo* in the tree was established, we used this species as the outgroup to study phylogenetic relationships among the other Aeglidae. We think this strategy avoids the alignment ambiguities and inconsistency (i.e., long branch attraction) produced by a distant outgroup, especially in the parsimony analysis (e.g., Harris *et al.,* 2000). Therefore, only Aeglidae sequences were used for analysis unless otherwise stated.

Different trees based on the four distinct gene regions gave similar topologies (results not shown). Moreover, a partition homogeneity test (Farris *et al.,* 1994) indicated that our Aeglidae data from these gene regions were not significantly heterogeneous ($P = 0.33$), thereby justifying their combination. Phylogenetic signal (g_1) within this data set was –0.6 ($P < 0.01$). To correct for strong contribution in signal of the best-supported clades in the tree, clades with bootstrap values higher than 90% were collapsed and g_1 recalculated. The new g_1 values were always less than –0.45 ($P < 0.01$). The maximum likelihood hypothesis testing procedure resulted in the rejection of all seven null hypotheses tested except hypothesis (3): transition rates are equal (Table 2). Nucleotide frequencies were significantly different from being equal with A = 0.34, C = 0.12, G = 0.14, and T = 0.40. Tran-

sition rates were equal and transversion rates were not equal. Thus, different maximum likelihood estimated rates were used for each of the six reversible rates of change except A↔G and C↔T ($R_1 = 2.22$, $R_2 = 15.93$, $R_3 = 1.55$, $R_4 = 1.45$, $R_5 = 15.93$, and $R_6 = 1.00$). There was also significant rate heterogeneity in these data. Rate heterogeneity is taken into account in models of evolution using a gamma distribution with the shape parameter of the distribution (α) estimated from the data via maximum likelihood (Yang, 1996). The estimated shape parameter for the gamma distribution for these data was $\alpha = 0.57$. There was also a significant proportion of invariable sites in these data estimated at 68.6%. Thus, our justified model was the transversion model plus gamma distributed rate heterogeneity plus a significant proportion of invariable sites (TVM + Γ + I).

Incorporating this model of molecular evolution, we estimated phylogenetic relationships among aeglid taxa using maximum likelihood. Ten random sequence addition searches resulted in the same maximum likelihood tree (Fig. 2). This phylogeny clearly puts *Aegla papudo* apart from the other Chilean aeglids (100% bootstrap support), which are also split into two clades with 70% and 98% bootstrap support. The ML tree also shows that the *Aegla laevis* and *Aegla cholchol* Jara, 1999, populations do not form monophyletic clades, and *Aegla* sp. forms a sister group with *Aegla bahamondei.*

Unlike maximum likelihood methods for which all characters are "phylogenetically informative," maximum parsimony limits phylogenetically informative characters to synapomorphic character changes. Our data set consisted of 366 parsimony-informative characters out of 2,589 characters. The initial maximum parsimony analysis resulted in one island of eight most parsimonious trees with a tree length of 903 steps. The majority rule consensus tree of these eight maximum parsimony trees also supports the separation of *A. papudo* from the other Chilean species (Fig. 3), which are also clustered in the same two groups shown by the ML tree. Again the *Aegla laevis* and *A. cholchol* populations do not form monophyletic groups. The maximum parsimony bootstrap analysis gave similar results to the likelihood analysis with strong support for the same phylogenetic assemblages mentioned above (Fig. 4).

Discussion

Our analyses showed clear support for the separation of *Aegla papudo* from the other Chilean aeglids when *Munida subrugosa* and *Pachycheles haigae* are used as the outgroup (Figs. 2–4). This same result has previously been shown by Cerda (1992) and Jara (1992, 1996) based on morphology: *A. papudo* does not have a ventral *linea aeglica*, the median suture of the telson is obliterated, and the "bar" *linea* is convergent. Therefore, both molecular and morphological data suggest that *A. papudo* could be moved to a new genus. However, the proposition and the diagnosis of a new name for this species are beyond the scope of this study. We will address this issue in a subsequent paper. The position of *A. papudo* as the sister group of the other Chilean and trans-Andean freshwater crabs (*Aegla affinis, A. riolimayana,* and *A. abtao*) also conflicts with Schmitt's (1942) hypothesis about a trans-Andean origin for Aeglidae, but supports the Pacific origin hypothesis suggested by Ortmann (1902) and Feldmann (1986).

Both likelihood and parsimony molecular trees separate Chilean aeglids (except *A. papudo*) that occur mainly in the north basins (*A. laevis* and *A. pewenchae* Jara, 1994) from those occurring in the south basins (*A. hueicollensis* Jara, 1999, *A. denticulata*, *A. manni* Jara, 1980, and *A. alacalufi* Jara and López, 1981), but not completely (e.g., *A. affinis* or *A. abtao*). Therefore, from a biogeographical perspective, this division does not seem to clearly follow the latitudinal distribution pattern indicated by Bahamonde and López (1963) and Jara (1996). Presumably the biogeography of these species is closely related to other major geological events. The uplift of the Andean Cordillera (Pliocene), and the repeated incursions and regressions of marine waters during the ice-ages (Pliocene and Quaternary) associated with the advance and recession of the glaciers (Glacial-Interglacial periods) modified the course of Chilean rivers and provided new upland aquatic habitats where these freshwater crabs could evolve (Lundberg *et al.,* 1998).

The phylogenies suggested by our molecular data provide strong support for the monophyly of most of the currently recognized Chilean Aeglidae species; only *Aegla laevis* and *A. cholchol* are placed in nonmono-

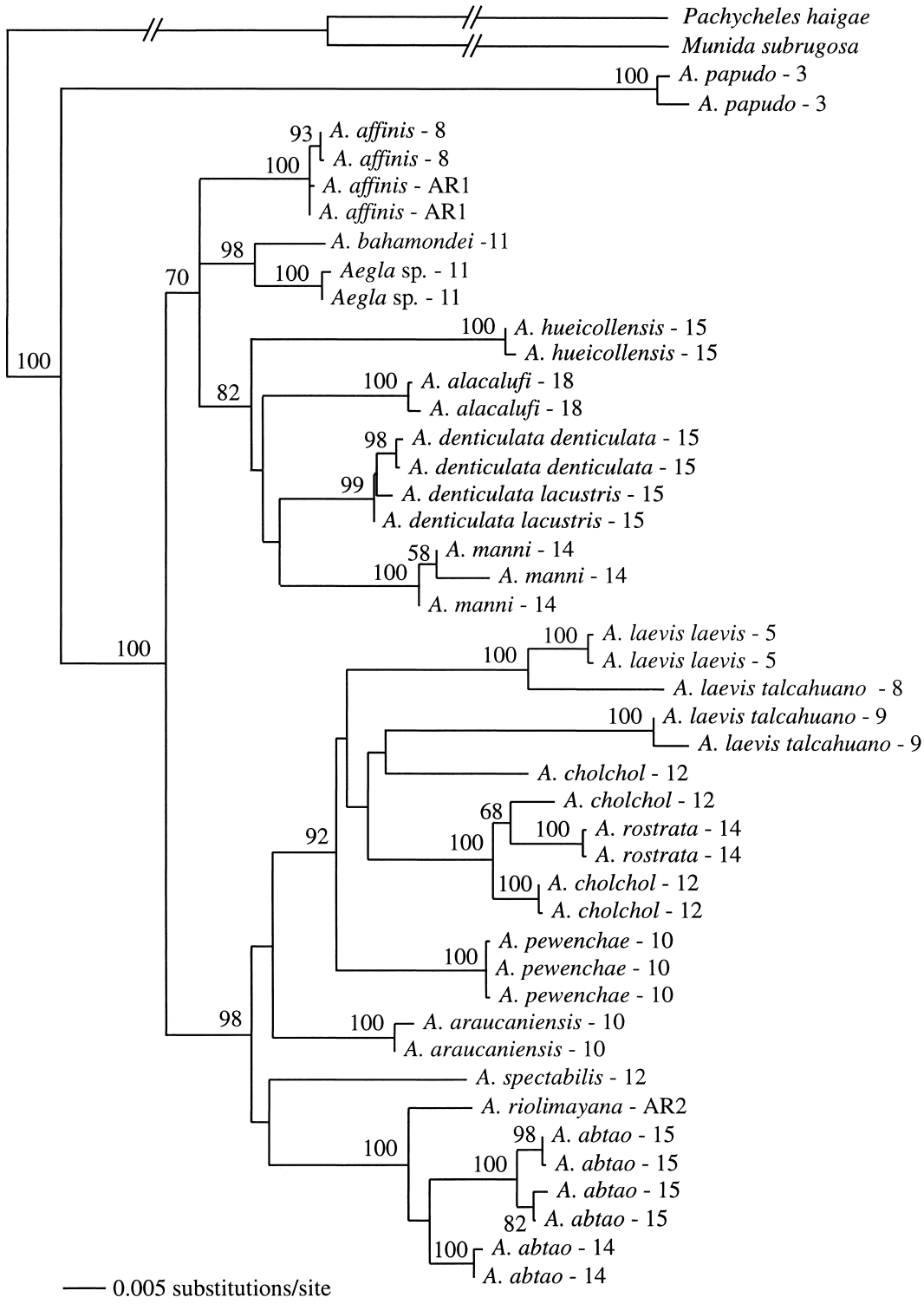

Fig. 2. The maximum likelihood estimate of phylogenetic relationships among the Chilean *Aegla* species assuming the TVM + Γ + I model of evolution (Table 2). Branch lengths are shown proportional to the amount of change along the branches. Bootstrap values are shown as percentages and are based on 100 bootstrap replications. Key numbers for main Chilean basins in Fig. 1 and Table 1 are shown after species names.

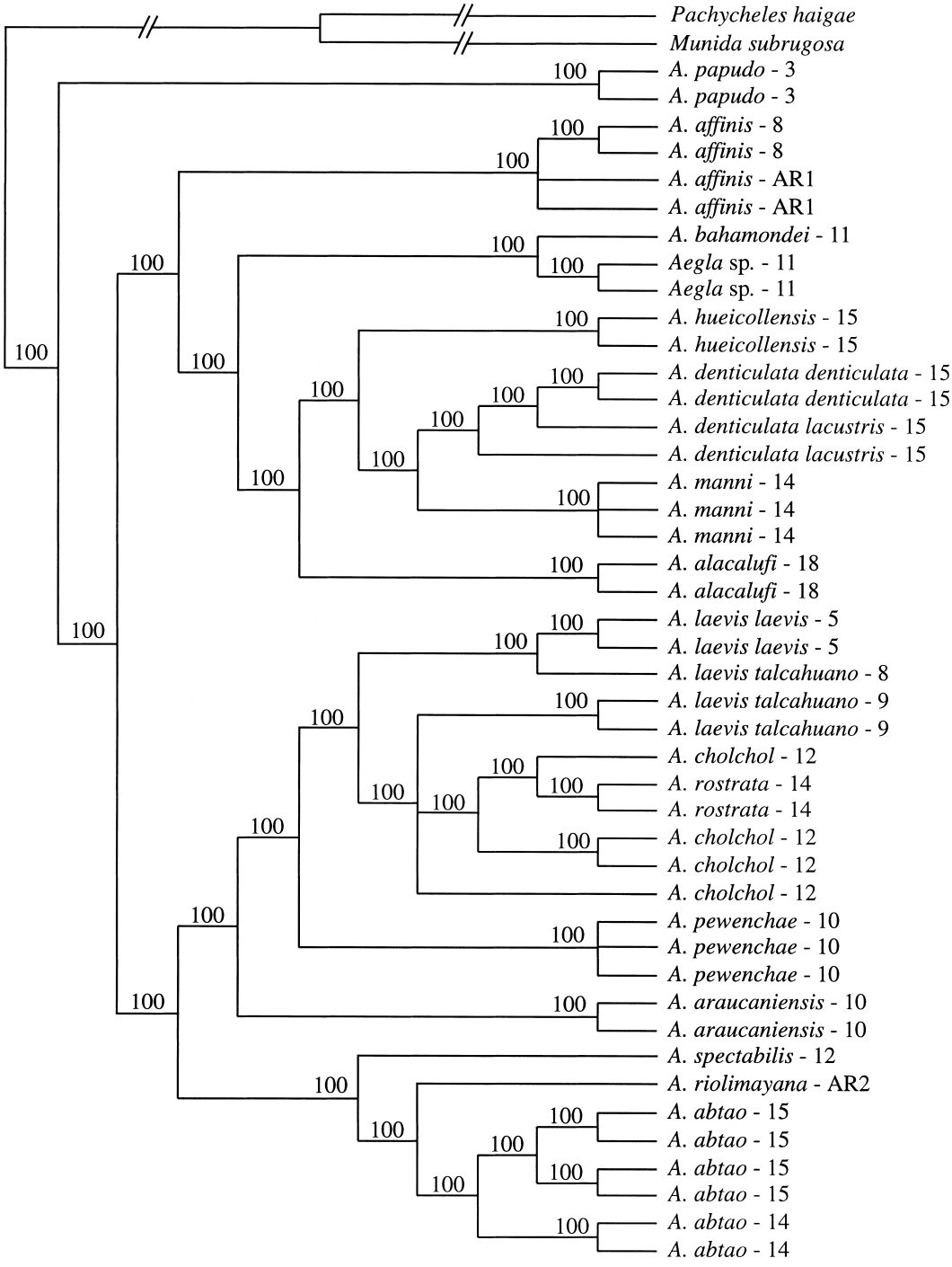

Fig. 3. The 50% majority-rule consensus tree of the eight most parsimonious trees found after 1,000 random addition searches. Key numbers for main Chilean basins in Fig. 1 and Table 1 are shown after species names.

phyletic groups (Figs. 2–4). Under certain species concepts (e.g., Cracraft, 1983), species must form monophyletic groups. Thus, populations within these two species could represent distinct species; whereas, other species concepts (e.g., cohesion species concept, Templeton, 1989) allow for non-monophyletic relationships within a single species. *Aegla* sp. could also be considered an unrecognized species or subspecies based

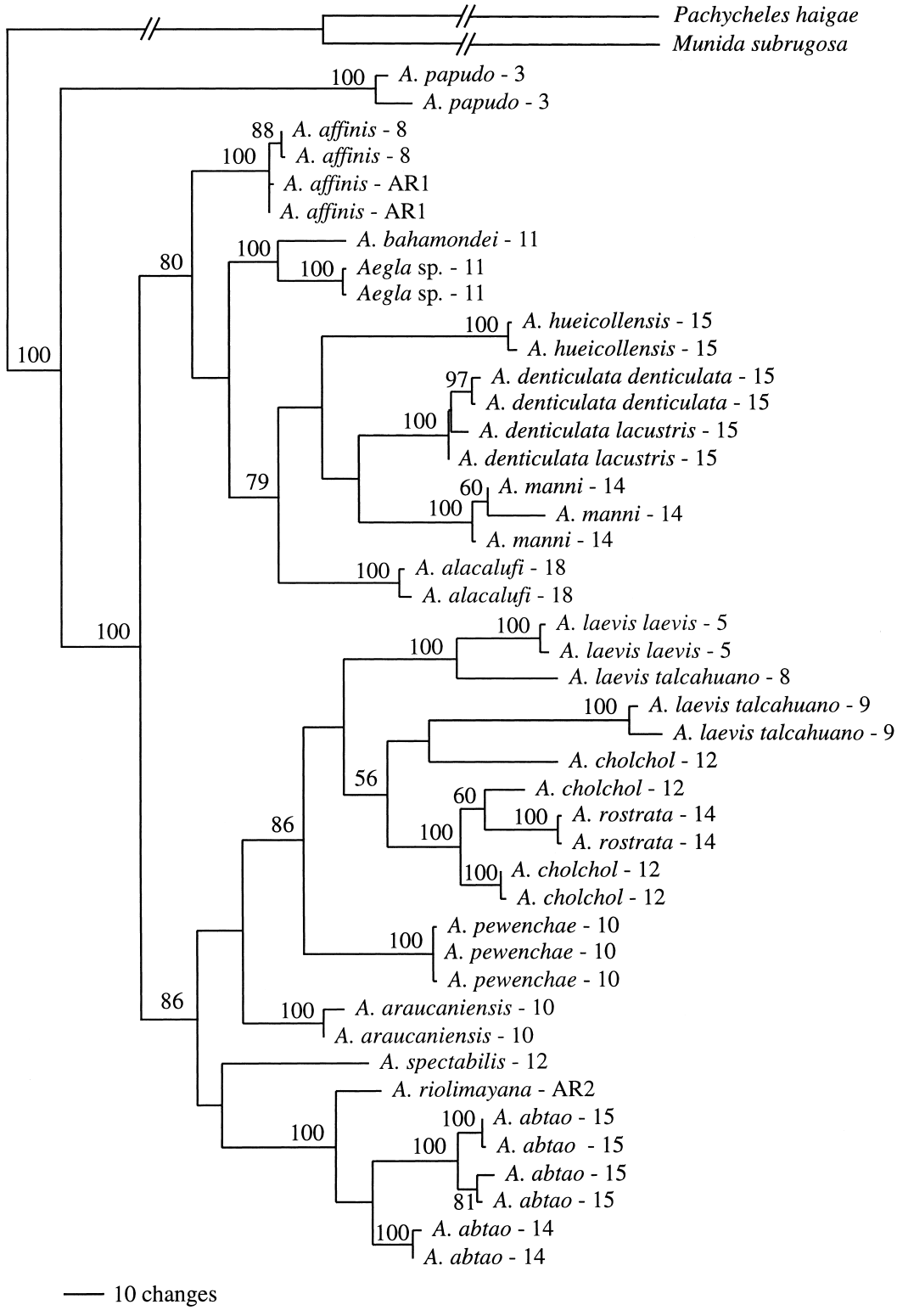

Fig. 4. The maximum parsimony estimate of phylogenetic relationships among the Chilean *Aegla* species. Branch lengths are shown proportional to the amount of change along the branches based on a single representative of the most parsimonious trees. Bootstrap values are shown as percentages and are based on 1,000 bootstrap replications. Key numbers for main Chilean basins in Fig. 1 and Table 1 are shown after species names.

on its position on the trees and the length of the branch connecting it to its closest relative (*A. bahamondei*). The close relationship between *Aegla* sp. and *A. bahamondei* and their occurrence in the same locality (Huillinco Creek) could suggest a common history and a close taxonomic relationship. However, more extensive sampling would be necessary to confirm this hypothesis.

We hope this first analysis of the phylogenetic relationships among these Chilean aeglid species will set the stage for new comparative analyses of the ecology, morphology, and biogeography of these interesting freshwater crabs.

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