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Taxonomic re-examination of the hermit crab species *Pagurus forceps* and *Pagurus comptus* (Decapoda: Paguridae) by molecular analysis

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Abstract

The current taxonomy of two poorly known hermit crab species *Pagurus forceps* H. Milne Edwards, 1836 and *Pagurus comptus* White, 1847 from temperate Pacific and Atlantic coastlines of South America is based only on adult morphology. Past studies have questioned the separation of these two very similar species, which occur sympatrically. We included specimens morphologically assignable to *P. forceps* and *P. comptus* in a phylogenetic analysis, along with other selected anomuran decapods, based on 16S ribosomal gene sequences. Differences between samples putatively assigned to either *P. forceps* and *P. comptus* were moderate, with sequence similarity ranging from 98.2 to 99.4% for the fragments analyzed. Our comparison of mitochondrial DNA sequences (16S rRNA) revealed diagnostic differences between the two putative species, suggesting that *P. forceps* and *P. comptus* are indeed phylogenetically close but different species, with no genetic justification to support their synonymization. The polyphyly of *Pagurus* is not corroborated here among the represented Atlantic species, despite obviously complex relationships among the members of the genus.

Key words: Anomura, molecular systematics, 16S rRNA, South Atlantic

Introduction

The anomurans are one of the most morphologically and ecologically diverse groups of decapod crustaceans, including a large number of extant species such as hermit crabs. More than 800 species in 127 genera are currently reported for the superfamily Paguroidea (McLaughlin 2003), and these inhabit diverse biotopes from intertidal to deep seas. Many more, however, appear to be undescribed. Even though explanations of evolutionary relationships at higher taxonomic levels have been attempted (McLaughlin 1983; Morrison *et al.* 2002), classification and evolutionary history of the group at many taxonomic levels is far from being resolved (McLaughlin 1983; Forest 1987; Ingle 1993; Martin & Davis 2001; McLaughlin 2003; McLaughlin *et al.* 2007).

The family Paguridae is highly diverse, with species widely distributed through all oceans. In recent years, new genera have been added to almost all the families of Paguroidea, but most additions have been to the Paguridae, currently comprised of 74 genera (McLaughlin 2003). Systematic problems remain among these, as for example with the polyphyletic genus *Pagurus* Fabricius, 1775. *Pagurus* exhibits a high degree of

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species proliferation with more than 170 species currently assigned to it worldwide. Some of these species are closely allied to one another, and can be assigned to any one of 11 informal species groups defined on the basis of morphological similarity (see Lemaitre & Cruz Castaño 2004). In some cases, morphology suggests very close relationships between members within these groups, and this raises question as to their status as separate species. This is the case with the identities of two South American hermit crabs of the "*comptus*" group (Group III *sensu* Lemaitre & Cruz Castaño 2004) which have at times been treated as full species and at other times placed in synonymy as varieties of the same species.

Pagurus comptus White, 1847 occurs in the southeastern Pacific Ocean, from Chile (Coquimbo zoogeographic zone, 26–31°S) to the Magellan region, and in the southwestern Atlantic Ocean, from Patagonia (Argentina, 43–44°S) to Uruguay (Montevideo), including the Falkland Islands (Malvinas), from 10–150 m depth (Forest & Saint Laurent 1968; Boschi 1979; Boschi *et al.* 1992; Lancellotti & Vasquez 2000). *Pagurus forceps* H. Milne Edwards, 1836 is found in the southeastern Pacific Ocean, from Chile (Antofagasta, 21–23°S) to Tierra del Fuego, and in the southwestern Atlantic Ocean (Falkland Islands - Malvinas), from the shore to 660 m depth (Haig 1955; Boschi *et al.* 1992; Lancellotti & Vasquez 2000).

Pagurus comptus was originally described by White (1847) from the Falkland Islands (Malvinas). Later, Miers (1881) established the taxon *P. comptus* var. *latimanus,* followed by Henderson's (1988) naming of *P. comptus* var. *jugosa* for specimens from the Magellan region. Lagerberg (1905), in the course of studying crustacean fauna of the south polar region, documented considerable intraspecific variation in the form of the right chelipeds for these taxa, and considered the varieties *latimanus* and *jugosa* to represent normal variation within this species; at the same time he mentioned the possibility of synonymy between *P. comptus* and *P. forceps* from Chile, applying the law of priority. This proposal was first adopted by Stebbing (1914: 277). Haig (1955) agreed, and also noted that *Pagurus gayi* Nicolete (1849) from Chile was undoubtedly synonymous with *P. forceps* since Nicolete´s description and figure of the right cheliped was identical to that of Lagerberg (1905: pl. 1, fig. 1) for *P. forceps*. Forest & Saint Laurent (1968) concluded that *P. forceps* and *P. comptus* were closely related, but considered the latter as a distinct species based on differences in biometric relationships (cephalothoracic shield width/length and right chela propodus length; Forest & Saint Laurent 1968: 140, tab. IV), strong denticulation on the basal margin of the propodus of the right cheliped, a marked crest on the internal face of the carpus, and unique telson shape in *P. forceps* (Forest & Saint Laurent 1968: 141, figs. 107–111). These authors concluded that *P. comptus* was a southwestern Atlantic and subantarctic species, while *P. forceps* was a species of the eastern Pacific coast.

While there has been a long tradition among invertebrate systematics to readily assign separate species rank to geographically separated specimens that could be to some extent be morphologically distinguished, this has been countered by the subsequent tendency of others to synonymize many species names that thusly arose (Spivak & Schubart 2003). To date, most systematic studies on hermit crabs have been based solely on morphology. Molecular tools have been rarely applied to hermits, either to resolve species status (Mantelatto *et al.* 2006) or to determine phylogenetic relationships among major taxa of Anomura (see McLaughlin *et al.* 2007 for review). Considering that 1) members of *P. forceps* and *P. comptus* are similar in general somatic morphology, 2) the current taxonomy of species assignable to both species has been based solely on adult morphology, and 3) there has been no previous attempt to resolve evolutionary relationships among these species with molecular tools, we analyzed phylogenetic relationships among selected species of hermit crabs on the basis of partial sequences of the large ribosomal subunit 16S (mitochondrial), primarily to verify the taxonomic status of *P. comptus* and *P. forceps*.

Material & methods

Sample collection

Hermit crabs were collected between 2004 and 2007 from new localities or were obtained as gifts from colleagues (Tab. 1). Newly collected specimens were preserved directly in 75–90% ethanol. Species

identifications were confirmed on the basis of morphological characters from available references (Stebbing 1914; Haig 1955; Forest & Saint Laurent 1968; Boschi *et al*. 1992). Genetic vouchers from which tissue subsamples were obtained were deposited in the Crustacean Collection of the Biology Department (CCDB) of the Faculty of Philosophy, Sciences and Letters of Ribeirão Preto (FFCLRP), University of São Paulo (USP) (Tab. 1).

Besides *P. comptus* and *P. forceps*, we included varied hermit crabs from the families Diogenidae and Paguridae for comparison, along with representatives of other selected anomuran groups (i.e., Porcellanidae) to more broadly root the analysis. Some comparative sequences were retrieved from GenBank (Tab. 1).

TABLE 1. Hermit crab specimens used for the molecular phylogenetic reconstructions with respective date and site of collection, museum catalogue number, and genetic database accession numbers (GenBank). (CCDB = Crustacean Collection of the Department of Biology, FFCLRP, University of São Paulo; MZUSP = Carcinological Crustacean of Zoology Museum of University of São Paulo; ULLZ = Crustacean Collection of the Department of Biology, University of Louisiana at Lafayette).

Species	Collection site, date	Catalogue No.	GenBank accession No.
Allopetrolisthes spinifrons Miers, 1876	Las Cruces (Chile)	—	AF260617
Calcinus obscurus Stimpson, 1859	Panama City (Panama), 2001	—	AF436058
Calcinus tibicen (Herbst, 1791)	Ubatuba (Brazil), Oct 2001	CCDB 769	DQ369940
Clibanarius antillensis Stimpson, 1859	Florida (U.S.A.), Jul 1998	ULLZ 4683	DQ369941
Coenobita compressus (H. Milne Edwards, 1837)	Amador Causeway (Panama), 2001	—	AF436059
Dardanus insignis (de Saussure, 1858)	Caraguatatuba (Brazil), Apr 2001	CCDB 774	DQ369943
Dardanus venosus (H. Milne Edwards, 1848)	Ubatuba (Brazil), Nov 2001	CCDB 766	DQ369944
Isocheles sawayai Forest and Saint Laurent, 1968	Ubatuba (Brazil), Dec 2000	CCDB 302	DQ369938
Isocheles wurdemanni Stimpson, 1862	Texas (U.S.A.), Oct 1997	ULLZ 3890	DQ369936
Loxopagurus loxochelis (Moreira, 1901)	Ubatuba (Brazil), Nov 2000	CCDB 765	DQ369939
Pachycheles pilosus (H. Milne Edwards, 1837)	Isla Tortuga (Venezuela)	ULLZ 5349	DQ865329
Paguristes calliopsis Forest and Saint Laurent, 1968	Ubatuba (Brazil), Sep 1999	CCDB 768	DQ369932
Paguristes eremita (Linnaeus, 1767)	Costa Alvaria (Portugal), Dec 2003	CCDB 1800	FJ869140
Paguristes erythrops A. Milne-Edwards, 1880	Paraty (Brazil), Oct 1996	CCDB 773	DQ369935
Paguristes robustus Forest and Saint Laurent, 1968	Ubatuba (Brazil), Mar 2001	CCDB 1252	DQ369934
Paguristes wedelli A. Milne-Edwards, 1880	Valdivia (Chile), May 2005	CCDB 1809	FJ869141
Pagurus bernhardus (Linnaeus, 1758)	(France)	—	AF425335
Pagurus brevidactylus (Stimpson, 1859)	Ubatuba (Brazil), Nov 2001	CCDB 771	DQ369945
Pagurus comptus White, 1847	Bahía Gente Grande, Patagonia (Chile), Jul 2004	CCDB 1751	FJ869142
Pagurus comptus White, 1847	Puerto del Hambre, Patagonia (Chile), Jun 2004	CCDB 1805	FJ869143
Pagurus comptus White, 1847	Patagonia (Argentina), Jul 2005	CCDB 1702	FJ869144
Pagurus comptus White, 1847	Patagonia (Argentina), Oct 2007	CCDB 2196	FJ869145
Pagurus criniticornis (Dana, 1852)	Ubatuba (Brazil), Nov 2001	CCDB 779	DQ369947
Pagurus edwardsii Dana, 1852	Antofagasta (Chile), Jan 2004	CCDB 2207	FJ869146
Pagurus exilis (Benedict, 1892)	Ubatuba (Brazil), Nov 2000	CCDB 767	FJ869147

to be continued.

TABLE 1. (continued)

Species	Collection site, date	Catalogue No.	GenBank accession No.
Pagurus forceps H. Milne Edwards, 1836	Punta de Tralca (Chile), Jul 2004	CCDB 1750	FJ869148
Pagurus forceps H. Milne Edwards, 1836	Punta de Tralca (Chile), Jul 2004	CCDB 1806	FJ869149
Pagurus forceps H. Milne Edwards, 1836	Coquimbo (Chile), Oct 2007	CCDB 2078	FJ869150
Pagurus leptonyx Forest and Saint Laurent, 1968	Ubatuba (Brazil), Aug 2001	CCDB 1257	DQ369946
Pagurus mclaughlinae García-Gómez, 1982	Florida (U.S.A.), Jul 2003	ULLZ 5679	FJ869151
Pagurus pollicaris Say, 1817	Louisiana (U.S.A.), Jul 1999	ULLZ 5673	FJ869152
Pagurus prideaux Leach, 1815	Costa Alvaria (Portugal), Dec 2003	CCDB 1798	FJ869153
Pagurus provenzanoi Forest and Saint Laurent, 1968	Santa Catarina (Brazil), no date	MZUSP 13808	FJ869154
Pagurus villosus (Nicolet, 1849)	Valdivia (Chile), Sep 2005	CCDB 1808	FJ869155
Petrolisthes armatus (Gibbes, 1850)	São Sebastião (Brazil), Jan 2006	CCDB 1747	FJ869156
Petrochirus diogenes (Linnaeus, 1758)	Ubatuba (Brazil), Mar 2001	CCDB 776	DQ369942

DNA analysis

We based the analysis exclusively on a partial fragment of the 16S rDNA gene, which has shown its utility in both phylogenetic and population studies for over a decade and is a common choice for use in phylogenetic studies on decapods (see Schubart *et al.* 2000a; Mantelatto *et al.* 2007 for literature review). DNA extraction, amplification, and sequencing protocols followed Schubart *et al.* (2000a) with modifications as in Mantelatto *et al.* (2006, 2007, 2009) and Robles *et al.* (2007). Total genomic DNA was extracted from muscle tissue of the walking legs or chelipeds. Muscle was ground and incubated for 1–12 h in 600 µl of lysis buffer at 65°C; protein was separated by addition of 200 µl of 7.5 M ammonium acetate prior to centrifugation. DNA precipitation was effected by addition of 600 µl of cold isopropanol followed by centrifugation; the resultant pellet was washed with 70% ethanol, dried, and resuspended in 10–20 µl of TE buffer.

An approximately 600-base pair region of the 16S rRNA gene was amplified from diluted DNA by means of polymerase chain reaction (PCR) (thermal cycles: initial denaturing for 10 minutes at 94°C; annealing for 38–42 cycles: 1 minute at 94°C, 1 minute at 45– 48°C, 2 minutes at 72°C; final extension of 10 minutes at 72°C) with the primers designated as follows: 16SH2 (5'–AGATAGAAACCAACCTGG–3'), 16SL2 (5'–TGCCTGTTTATCAAAAACAT–3'), and 16Sar (5'–CGCCTGTTTATCAAAAACAT–3') (for references on the primers see Schubart *et al.* 2000a, b). PCR products were purified using Microcon 100® filters (Millipore Corp.), and sequenced with the ABI Big Dye® Terminator Mix (PE Biosystems) in an ABI Prism 3100 Genetic Analyzer® (Applied Biosystems automated sequencer). All sequences were confirmed by sequencing both strands. A consensus sequence for the two strands was obtained using the computational program BIOEDIT 7.0.5 (Hall 1999, 2005).

Sequences were edited with BIOEDIT 7.0.5 (Hall 2005) and aligned using Clustal X (Thompson *et al.* 1997) with interface to BIOEDIT with default parameters. Ambiguous regions of the alignment were removed. Sequences were submitted to prior analysis in the program MODELTEST (Posada & Crandall 1998) to find the evolutionary model that best fit the data. NJ analysis was performed using the maximum-likelihood distance correction set. Phylogenetic analyses were conducted using PAUP 4.0 beta 10 (Swofford 2003) for Neighbor Joining (NJ) analyses. A Chi-square analysis of base-frequency homogeneity was carried out to estimate whether any saturation was present in the base frequencies. The consistency of topologies was measured by the bootstrap method (1000 bootstraps), and only confidence values > 50% were reported. Nucleotide composition, substitution frequencies, and pairwise distances were calculated with PAUP 4.0 beta 10. To evaluate the range of intrageneric sequence identity found among recognized species, we compared the genetic distances between species pairs using BIOEDIT 7.05. An identity matrix showed the proportion of

identical residues between all of the sequences in the alignment as they were ultimately aligned.

In order to evaluate the robustness of the results of the two-step phylogenetic analysis of the data by a static alignment procedure, we carried out a dynamic analysis in software POY version 4.0 (Varón *et al.* 2007), under direct optimization with parsimony as the optimality criterion (Wheeler 1996). Trees were constructed through random addition sequence followed by a combination of branch-swapping steps (SPR "subtree pruning and regrafting" and TBR "tree bisection and reconnection"). The ratcheting procedure was used to enhance branch swapping by randomly reweighting characters during the SPR and TBR procedure. Sensitivity analysis was carried out using different cost matrices, as suggested by Wheeler (1995). All data sets were analyzed under 10 parameter sets for a range of indels, transition and transversion ratios (Tab. 2).

Morphological data, historical synonymies, and diagnoses for both species were gathered by reviewing the descriptions in the references mentioned in the Introduction. A search was made for diagnostic morphological differences to support our molecular findings (used in Discussion only).

TABLE 2. Matrices used in the sensitivity analysis. The digits matrix corresponds to the ratio of indel/transition/transversion values.

Matrix 111

	A	C	G	T	-
A	0	1	1	1	1
C	1	0	1	1	1
T	1	1	0	1	1
G	1	1	1	0	1
-	1	1	1	1	0

Matrix 112

	A	C	G	T	-
A	0	2	1	2	1
C	2	0	2	1	1
T	1	2	0	2	1
G	2	1	2	0	1
-	1	1	1	1	0

Matrix 113

	A	C	G	T	-
A	0	3	1	3	1
C	3	0	3	1	1
T	1	3	0	3	1
G	3	1	3	0	1
-	1	1	1	1	0

Matrix 211

	A	C	G	T	-
A	0	1	1	1	2
C	1	0	1	1	2
T	1	1	0	1	2
G	1	1	1	0	2
-	2	2	2	2	0

Matrix 212

	A	C	G	T	-
A	0	2	1	2	2
C	2	0	2	1	2
T	1	2	0	2	2
G	2	1	2	0	2
-	2	2	2	2	0

Matrix 221

	A	C	G	T	-
A	0	1	2	1	2
C	1	0	1	2	2
T	2	1	0	1	2
G	1	2	1	0	2
-	2	2	2	2	0

Matrix 411

	A	C	G	T	-
A	0	1	1	1	4
C	1	0	1	1	4
T	1	1	0	1	4
G	1	1	1	0	4
-	4	4	4	4	0

Matrix 412

	A	C	G	T	-
A	0	2	1	2	4
C	2	0	2	1	4
T	1	2	0	2	4
G	2	1	2	0	4
-	4	4	4	4	0

Matrix 812

	A	C	G	T	-
A	0	2	1	2	8
C	2	0	2	1	8
T	1	2	0	2	8
G	2	1	2	0	8
-	8	8	8	8	0

Matrix 411

	A	C	G	T	-
A	0	1	2	1	8
C	1	0	1	2	8
T	2	1	0	1	8
G	1	2	1	0	8
-	8	8	8	8	0

Results

Taxonomic assignments

On the basis of morphology, voucher materials were assigned to species as follow, and DNA was successfully sequenced for those indicated by underlined Shield Lengths (=SL):

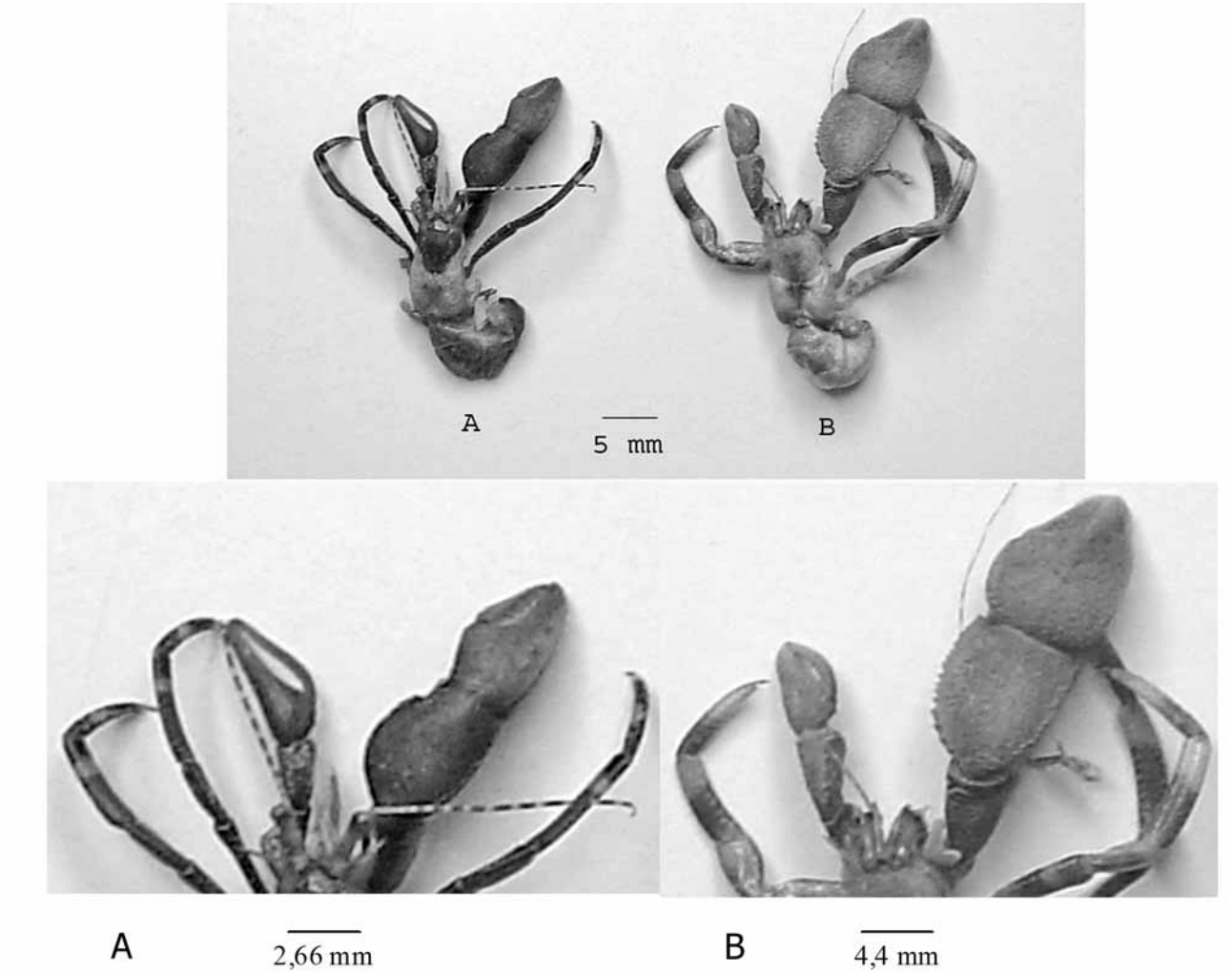

FIGURE 1. Male specimens of *Pagurus forceps* (A) from Punta de Tralca (33°S) (CCDB 1750, SL = 4.76 mm) and *Pagurus comptus* (B) from Bahía Gente Grande (52°S) (CCDB 1751, SL = 8.82 mm).

***Pagurus forceps* H. Milne Edwards, 1836**

Type locality: shores of Chile, probably Valparaíso (see Haig 1955 and Forest & Saint Laurent, 1968 for review).

Material examined: 3 males (3.20, 3.41, 4.36* mm Shield Length = SL), 2 females (3.28, 3.48 mm SL), Coquimbo, Chile, October 2007, CCDB 2078 (DNA voucher); 2 males (4.36, 4.41 mm SL), 1 female (4.23 mm SL), 1 ovigerous female (2.95 mm SL), Punta de Tralca, Central Chile, February 2005, CCDB 2079; 1 male (3.89 mm SL), 1 female (3.99 mm SL), Punta de Tralca, Central Chile, July 2004, CCDB 1806 (DNA voucher); 2 males (3.59, 4.76 mm SL), 1 ovigerous female (3.32 mm SL), Punta de Tralca, Central Chile, July 2004, CCDB 1750 (*underline shield lengths indicates the specimens used for molecular analysis from numbered lot and here considered as DNA voucher).

***Pagurus comptus* White, 1847**

Type locality: Falkland Islands (see Haig 1955 and Forest & Saint Laurent, 1968 for review). *Material examined:* 2 males (6.26, 8.82 mm Shield Length), 2 ovigerous females (3.59, 4.38 mm SL), Bahía Gente Grande, Patagonia, Chile, July 2004, CCDB 1751 (DNA Voucher); 3 males (3.71, 4.24, 4.60 mm SL), Puerto

del Hambre, Punta Arenas, Chile, June 2004, CCDB 1805 (DNA Voucher); 2 males (5.00, 5.28 mm SL), 2 ovigerous females (4.10, 4.25 mm SL), Punta Cavendish, Puerto Deseado, Patagonia, Argentina, July 2005, CCDB 1702 (DNA Voucher); 3 males (3.11, 5.77, 6.26 mm SL), Patagonia, Argentina, October 2007, CCDB 2196 (DNA Voucher); 1 male (6.00 mm SL), Patagonia, Argentina, June 2008, CCDB 2462.

Molecular phylogeny

In the static alignment, 540 positions of the 16S rRNA gene (not including the primer regions) were aligned for 36 anomuran species, including outgroup taxa. The optimal model of nucleotide evolution, selected under the Akaike information criterion (AIC), as recommended by Posada & Buckley (2004), was the general-time reversible model of sequence evolution (Lanave *et al.* 1984; Rodriguez *et al.* 1990) plus gamma distributed rate heterogeneity with a significant proportion of invariant sites (GTR + I + G), with the following parameters: assumed nucleotide frequencies A = 0.4199, C = 0.1087, G = 0.0726, T = 0.3988; proportion of invariant sites I = 0.2340; variable sites followed a gamma distribution with shape parameter = 0.4902 (tree shown in Fig. 2). A chi-square test of homogeneity of base frequencies across taxa showed no significant difference (X^2= 53.11, d.f. = 105, P = 0.99). The higher level of transitional substitutions in comparison with transversions indicated that the 16S rRNA sequences were not saturated.

The sequence identity rates estimated among all species of *Pagurus* included in the analysis ranged from 76.0–94.9% for 16S DNA (data not shown); sequence identity rates for intraspecific individuals ranged from 99.6–100% for 16S DNA. For compared populations of *P. forceps* to *P. comptus*, similarity ranged from 98.2–99.4%. Thus, the measured divergence between populations of the questionably separate species, *P. forceps* and *P. comptus,* was in accord with divergence found among other congeners at the interspecific level. Of the 10 parameter sets under which we analyzed our data using POY, the one that produced the shortest trees was that for an indels/transition/transversion ratio of 1 : 1 : 1 (parameter set 111, Tab. 2) (tree shown in Fig. 3). Parsimony analysis yielded one parsimonious tree of length 1412.

Overall, the two different algorithms (NJ, MP) resulted in similar tree topologies, which were mostly congruent (Figs. 1–2). The resulting molecular phylogeny agrees in several respects with the current morphologically based classification of the two species.

Specimens of *P. forceps* from which mtDNA was obtained shared the same sequence over the 541-nucleotide region of the 16S rRNA gene that was studied, except that the sequence for one of the two sequenced specimens from Punta de Tralca (CCDB 1806) differed by two transitions (positions 176 and 238) from the other. Similarly, specimens of *P. comptus* shared the exact mtDNA sequence except for one from the population at Puerto del Hambre (CCDB 1805), which showed one transition difference (position 286) from the other sequences. The sequences for *P. comptus* differed in only 6 positions (5 transitions and 1 transversion) from all sequences for populations of *P. forceps.*

Polyphyly of *Pagurus,* at least with the set of species used here from Atlantic and Pacific waters, was not corroborated in either analysis. The bootstrap values were significant, supporting both external (members of Paguridae and Diogenidae) and internal nodes. In contrast, the relationships among inner clades of *Pagurus* were weakly corroborated in the sensitive analysis (Fig. 3). The species of *Pagurus*, representing the Paguridae, were grouped into three clades with *P. mclaughlinae* as the sister to remaining congeners (Fig. 1). Clade (A) contained five Atlantic representatives along with the Pacific species *P. villosus*; clade (B) contained three Atlantic species among which was the type species of the genus and the eastern Atlantic and Mediterranean species *P. prideaux*; clade (C) included only the Pacific species *P. edwardsii,* along with *P. comptus,* and *P. forceps* which range across the tip of South American into both oceans.

Within the superfamily Paguroidea we found that all species representing genera of Diogenidae (*Calcinus*, *Clibanarius*, *Dardanus*, *Paguristes*, *Petrochirus*, *Loxopagurus*, and *Isocheles*) were clustered together as expected, in a single clade. This clade also included *Coenobita compressus*, the only member of the Coenobitidae represented in our analysis.

TABLE 3. Sequence identity matrix of the large subunit (16S) between the species *Pagurus forceps* and *P. comptus*.

	1	2	3	4	5	6	7
1. *P. forceps*-Punta de Tralca, Chile	-	0.998	0.996	0,984	0.982	0.984	0.992
2. *P. forceps*-Punta de Tralca, Chile	0.998	-	0.998	0.986	0.984	0.986	0.994
3. *P. forceps*-Coquimbo, Chile	0.996	0.998	-	0.984	0.982	0.984	0.992
4. *P. comptus*-Bahía Gente Grande, Chile	0.984	0.986	0.984	-	0.998	1.000	0.992
5. *P. comptus*-Puerto del Hambre, Chile	0.982	0.984	0.982	0.998	-	0.998	0.990
6. *P. comptus*-Patagonia, Argentina	0.984	0.986	0.984	1.000	0.998	-	0.992
7. *P. comptus*-Patagonia, Argentina II	0.992	0.994	0.992	0.992	0.990	0.992	-

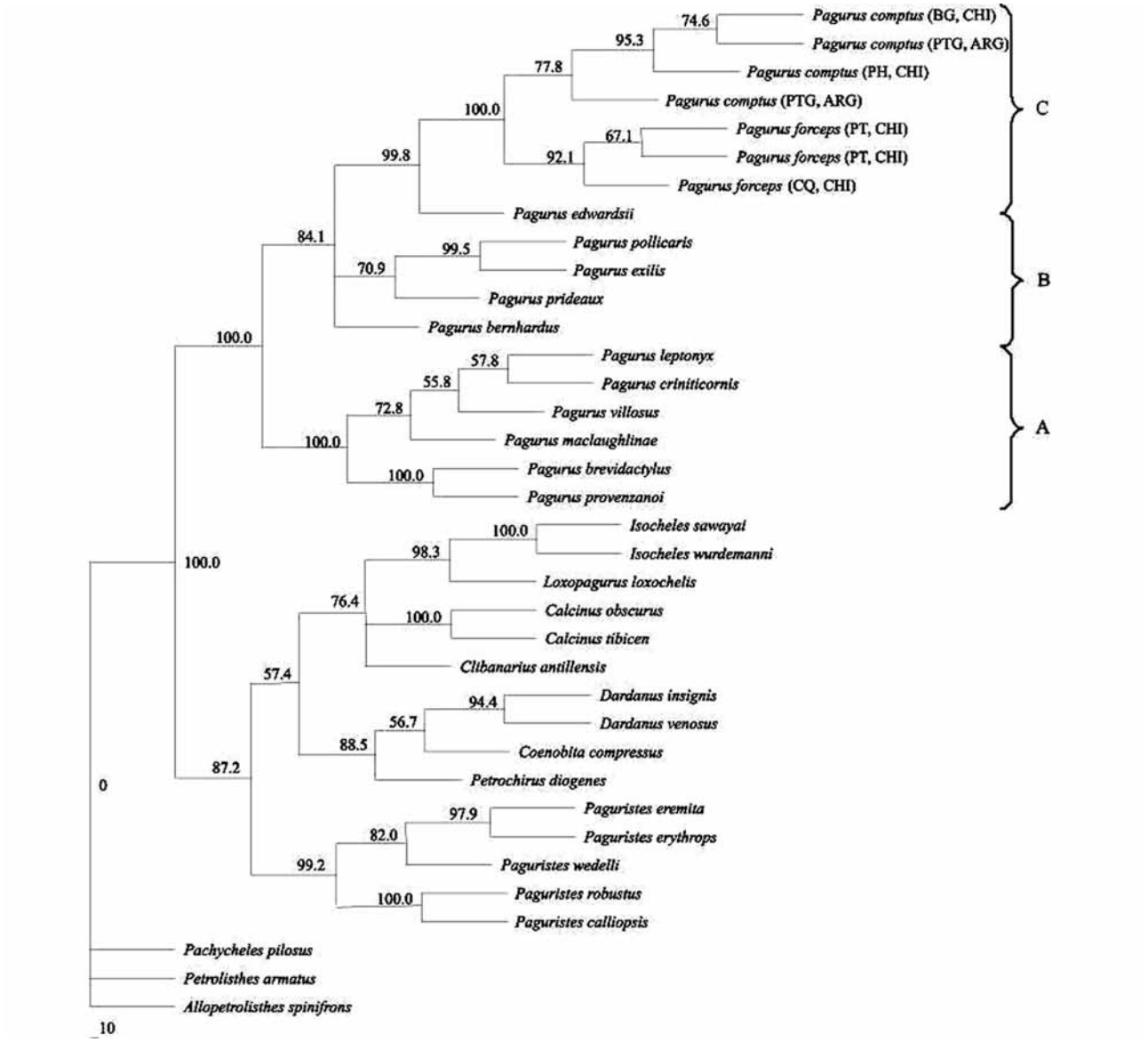

FIGURE 2. Phylogenetic relationships among hermit crab species obtained from analysis of 16S rRNA. ARG: Argentina; BG: Bahía Gente Grande; CHI: Chile; CQ: Coquimbo; PH: Puerto del Hambre; PT: Punta de Tralca; PTG: Patagonia.

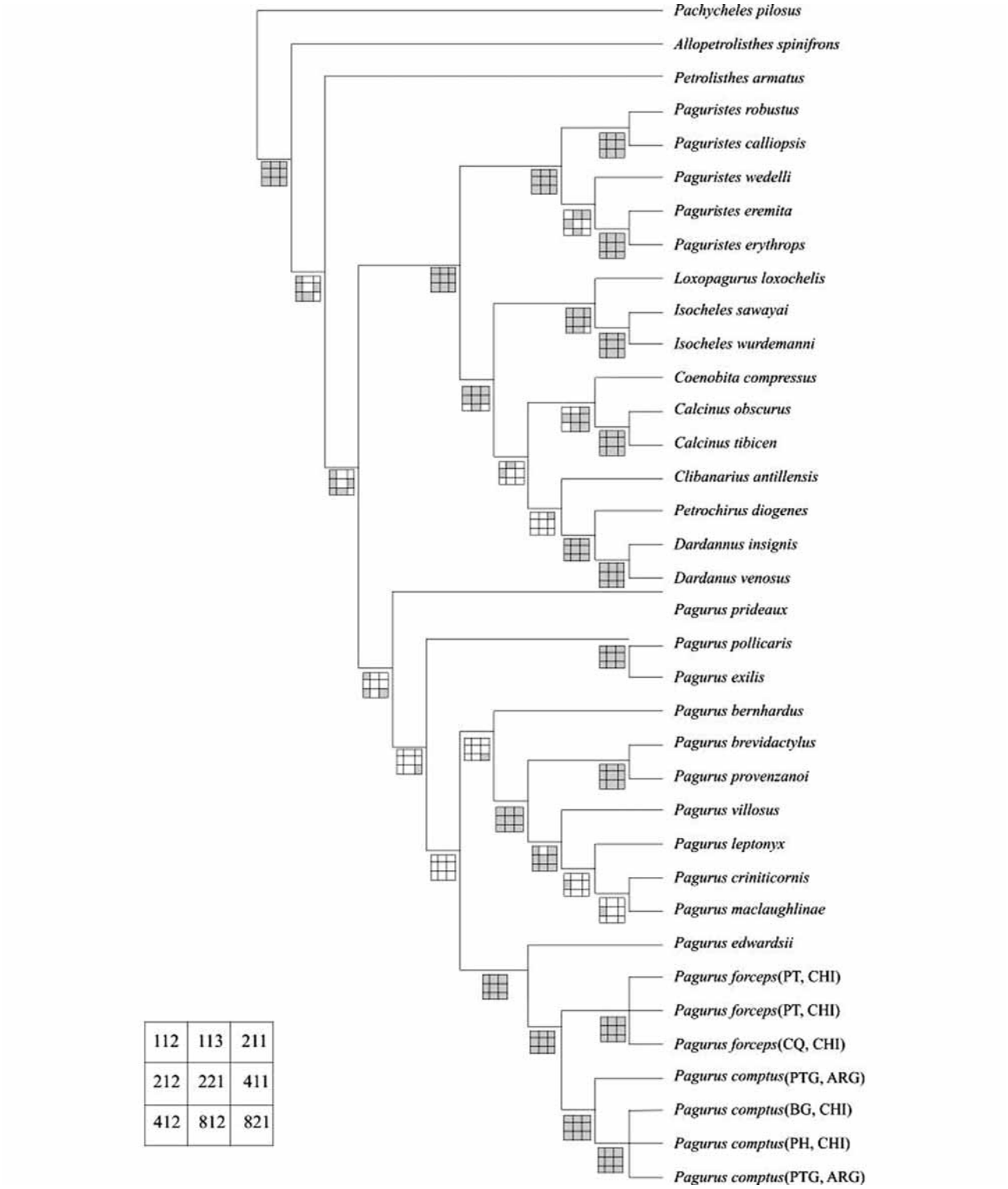

FIGURE 3. Phylogenetic tree based on direct optimization analysis of 16S rRNA data sets under the parameter set that produced the most parsimonious tree. The box on the lower left side indicates the parameter sets used in the analysis. Filled boxes mean that the clade was present. ARG: Argentina; BG: Bahía Gente Grande; CHI: Chile; CQ: Coquimbo; PH: Puerto del Hambre; PT: Punta de Tralca; PTG: Patagonia.

Discussion

The present investigation, based upon analysis of a partial fragment of 16S DNA and a review of morphology, convincingly supported separation of the species *P. comptus* and *P. forceps*. While we acknowledge general

phylogenetic proximity of these species, there is no genetic justification for synonymy of the two. The closest relative to the *P. comptus* and *P. forceps* group in our analysis was found to be *P. edwardsii*, from the eastern Pacific. The Pacific species *P. villosus* was placed in a clade with the Atlantic species, with which it shares several life history traits such as small size and continuous reproduction (Pardo *et al.* 2007).

In recent taxonomy of decapods, some populations that were long regarded as geographical separated morphological variants (morphs) have been assigned to full species rank on the basis of molecular results (Fratini & Vaninni 2002; Spivak & Schubart 2003; Schubart *et al.* 2005; Mantelatto *et al.* 2006). In the present study, morphological differences between were quite evident when two morphs were compared directly, and this supports molecular findings that justify separation at the species level (Fig. 1). In addition to differences in chelipeds, telson, and morphometric proportions of the carpus, propopus, and dactylus (Forest & Saint Laurent 1968: 140, tab. IV), color patterns of pereopods also vary in freshly captured individuals. Marking the carpus of *P. forceps* are a distal blue, middle white, and proximal red band, with the blue and white bands similar in width and the red band three times as wide as the others. The only two bands on the carpus of *P. comptus* are distal white and proximal brownish bands, of equal widths. In addition, morphological criteria adopted by Forest & Saint Laurent (1968) successfully assign specimens from the Magellan region to *P. comptus* and specimens from the central coast of Chile to *P. forceps*.

Forest & Saint Laurent (1968) described some variations in *P. comptus*, and this likely has contributed to questionable separations of this species from *P. forceps*. They described typical "*comptus"* as having the right cheliped with a slight denticulate crest on the inner edge of the distal carpus region, the propodus with the internal contour convex and bearing a strong carina on its dorsal face, an inverse V-shaped swelling in the middle region of the propodus, and strong granulation on the carpus and propodus. However, they also remarked on a high variability in the individuals examined, mentioning that the typical "*comptus"* morph is an extreme form within a morphological gradient. At the other extreme of this perceived gradient, individuals are close to the *"forceps"* morph, with the cheliped exhibiting a strong crest in the carpus region and attenuation of the carina swelling and granulation. Forest & Saint Laurent (1968) also mentioned a high variability in the left cheliped relative to the size and sex of individuals assigned to *P. comptus*, especially in the curvature and length of the dactylus. Other traits, including the form of the shield, ocular peduncle, antennules, antennas, and pereopods were similar in both species (Forest & Saint Laurent 1968). These authors did not give the exact number of individuals examined, but certainly, they inspected more than 15. All of them except one (from Montevideo) were collected in southern Chile and Argentina. Additionally, Lagerberg (1905) postulated that there was considerable intraspecific variation in *P. forceps* in the form of the right chelipeds and that *P. comptus* was a synonym of the former. A restricted collection from one biogeographical region of a species with a wide distribution cannot represent the complete range of morphological variation that is often found in decapods (Cuesta & Schubart 1998; Spivak & Schubart 2003). We observed this intraspecific morphological variability among our set of specimens, but with only minor intraspecific molecular variability. Differences in environmental factors (abiotic and biotic) between biogeographical regions might strongly influence phenotypic plasticity in morphological traits (Brian *et al*. 2006), but also might drive selection for adaptive traits which would promote speciation (Stearns 1989). In the present study, molecular evidence supports the latter statement, as it applies to the Pacific and Atlantic distributions of both *P. forceps* and *P. comptus*. However, as suggested for other cases of similarities among decapods (see Spivak & Schubart 2003 for review), more specimens and more variable molecular markers, as well as other biological aspects (such as reproduction and population features), must be analyzed before morphological variability can simply be attributed to phenotypic plasticity.

On the other hand, size and form in hermit crabs could also be strongly affected by the morphology of the shells that they use (Blackstone 1985). In this context, populations of *P. forceps* and *P. comptus* on the north-central coast of Chile both preferentially use shells of *Tegula* spp. (Soto & George-Nascimento 1991), while on the southern coast they prefer shells of *Adenomelon* and *Polinices* spp. (Soto *et al.* 1999; L.M. Pardo, unpublished), gastropods with morphologically dissimilar shells. Exploring all possible causes of morphological plasticity is not within the scope of the present paper, but our extensive data on shell

occupation lead us to suggest that differences in inhabited shells could significantly account for some variations in shapes of these hermit crabs. This is could be especially true in development of the chelipeds, which are used during shell selection and to protect the shell aperture (Garcia & Mantelatto 2001; Mantelatto & Martinelli 2001).

Recent studies on larval development of these two species do not, as yet, provide additional separating characters to support our findings. Zoeae assigned to both *P. comptus* and *P. forceps* show extended larval development, are planktotrophic, and live in a planktonic habitat (see Bacardit 1986 and Thatje *et al.* 2003 for reviews and additional literature). Unfortunately, details of morphology and diagnostic characters for zoeal stages are thus far unavailable, so we cannot use these to potentially support our proposed separation. For a better understanding of the relationships between *P. comptus* and *P. forceps* and the position of the genus among Diogenidae, the zoeal stages and megalopae of both species must be thoroughly examined.

Previous phylogenetic hypotheses based on limited data have suggested that *Paguristes* has close affinities to *Pagurus* (Morrison *et al.* 2002). However, Mantelatto *et al.* (2006) suggested instead that *Paguristes* is more closely allied to the remaining diogenid species. Our findings corroborated this latter assertion, and position members of *Paguristes* close to other diogenids.

Finally, the results presented here confirmed that some relationships of *Pagurus* are still unclear and that a much more complete subset of species is needed for a comprehensive examination of the evolutionary history in this genus. According to our molecular phylogeny, the genus *Pagurus* as currently defined is monophyletic. Our present phylogeny also suggests that the structure of Clade A should be further explored by inclusion of more taxa. In the present analyses, this clade was dominated by the western-Atlantic species *P. brevidactylus*, *P. provenzanoi*, *P. leptonyx*, *P. criniticornis*, and *P. maclaughlinae*, but also included the eastern-Pacific *P. villosus*. Also, Clade B, so far consisting of the type species *P. bernhardus*, along with *P. prideaux*, *P. pollicaris*, and *P. exilis*, must be analysed with a larger set of populations and species, considering both the intraspecific morphological variations and interspecific morphological similarities that are inherent in these taxa. Some close genetic similarities clearly exist between Atlantic and Pacific and representatives of the genus *Pagurus*, and taxa from throughout these regions should be included if and when morphologically and genetically based taxonomic revisions are undertaken. Future morphological and molecular systematic work will required to reveal whether our concept of the genus *Pagurus* can be retained as is, or whether it must be restricted in order to develop a natural classification based on monophyletic clades.

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