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Phylogeny, phylogeography, and systematics of the American pea crab genus *Calyptraeotheres* Campos, 1990, inferred from molecular markers

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We used mitochondrial cytochrome oxidase I (*COI*) and the large ribosomal subunit (*16S*) genes to establish evolutionary relationships amongst species of *Calyptraeotheres*, evaluate their usefulness as DNA-barcoding genes, and assess molecular diversity at the population level within *Calyptraeotheres garthi*. Bayesian, maximum likelihood, and maximum parsimony phylogenies confirmed the monophyly of *Calyptraeotheres,* showing that the ancestor of *C. garthi*, *Calyptraeotheres hernandezi*, and *Calyptraeotheres granti* radiated after the formation of the Panamanian isthmus. This finding contradicts the austral/tropical hypothesis previously proposed based on morphological data. The COI and 16S distance matrices supported separation of species as well as the genera, and corroborated that DNA barcoding is a useful tool and complements the classical taxonomy in Pinnotheridae. Phylogenetic and genetic distance analyses suggested that *C. hernandezi* is a junior synonym of *C. garthi*. Finally, *C. garthi* did not show a population structure across its distribution range, and showed a pattern consistent with a recent population expansion event that began 230–300 Kya.

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ADDITIONAL KEYWORDS: DNA barcoding – evolution – mitochondrial gene – monophyly – Pinnotheridae.

INTRODUCTION

In recent decades, molecular tools have gained great popularity amongst systematic biologists, in particular those studying decapods (see Martin, Crandall & Felder, 2009 for review). One reason is the power of these tools to build phylogenies and, at the same time, to resolve taxonomic conflicts. Indeed, molecular characters have been dramatically more successful than morphology in the detection of, for instance, cryptic and/or polymorphic species (Xiao *et al*., 2010; Puillandre *et al*., 2011). Moreover, knowledge of intraspecific molecular genealogies allows us to infer how palaeoclimate processes (e.g. the Quaternary glaciations) affected the demography of populations and determined the current genetic structure (Avise *et al*., 1987; Avise, 2009).

DNA barcoding is currently used as a molecular tool for rapid and reliable species identification of specimens. The short sequence obtained from an unknown specimen can be compared to a library of sequences of the same gene in order to identify the species (Meyer & Paulay, 2005). Sequencing of the barcode gene may result in a speedy, objective, and efficient identification tool. The preferred candidate for use as the global 'barcode' gene is mitochondrial cytochrome oxidase I (*COI*; Hebert *et al*., 2003), which

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has shown good results in birds (Hebert *et al*., 2004), fishes (Ward *et al*., 2005), insects (Janzen *et al*., 2005), and crustaceans (Costa *et al*., 2007; Negri, Pileggi & Mantelatto, 2012), amongst others. However, DNA barcoding has not been successful in separating species for some groups (e.g. plants, see Seberg & Petersen, 2009) and it has also failed to properly distinguish recently separated species (Hickerson, Meyer & Moritz, 2006). It also fails in groups of organisms for which hybridization (Nicholls *et al*., 2012) and/or introgressive hybridization (Whitworth *et al*., 2007) occur.

Pinnotheridae De Haan, 1833 is a family of small symbiotic crabs that live in association with a wide variety of benthic invertebrate hosts (Schmitt, McCain & Davidson, 1973; Harrison & Hanley, 2005). With 52 genera and more than 300 currently described species (Ng, Guinot & Davie, 2008; De Grave *et al*., 2009; Palacios-Theil *et al*., 2009), members of Pinnotheridae are collectively known as 'pea crabs' because of their peculiar rounded shape, small size, and smooth carapace. Crabs of this family are known for their remarkable morphological and ecological diversity (Ross, 1983). Pea crabs are endo- or ectosymbionts of a wide range of organisms, including gastropods, bivalves, ascidians, holothurians, and echinoids (Schmitt *et al*., 1973). They also use galleries constructed by echiuran worms, annelid worms, and burrowing crustaceans as refuges (Schmitt *et al*., 1973). Their wide ecological and morphological diversity makes Pinnotheridae an interesting group in which to study evolutionary relationships. Understanding the systematics of this family has been the aim of many studies, which have generally been based on comparative morphology (e.g. Griffith, 1987; Manning & Felder, 1989; Marques & Pohle, 1995; Ahyong & Ng, 2007; Campos, 2009; Campos & Hernández-Ávila, 2010) and more recently have included molecular data (Harrison, 2004; Harrison & Hanley, 2005; Palacios-Theil *et al*., 2009).

Pea crabs lack a large pool of reliable morphological taxonomic characters because of their small size and morphological adaptations to life as symbionts. More importantly, pinnotherid crabs have a complex life history characterized by a postlarval life habit alternating between free-swimming and internal symbiont habits. The dramatically different morphology of these two phases has led to taxonomic errors (Campos, 1989; Mantelatto & Cuesta, 2010). The phylogenetic position of some members is still unclear and under active discussion (Palacios-Theil *et al*., 2009), and the use of DNA characters is an excellent additional tool that complements taxonomy inferred from morphological traits. As far as we know and because of the high biodiversity of the group, molecular data have been implemented in only three studies to resolve taxonomic conflicts and to understand evolutionary relationships within the family Pinnotheridae.

The first two studies used the mitochondrial genes *COI* and *16S* to explain the evolution of the genus *Austinixa* in the Americas (Harrison, 2004; Harrison & Hanley, 2005). These studies present interesting fine-scale results for the speciation patterns within *Austinixa*. The third study was an extensive phylogeny that included more than 18 genera of Pinnotheridae (Palacios-Theil *et al*., 2009), using three mitochondrial genes [*16S*, *tRNA-Leu*, and nitrogen dehydrogenase subunit 1 (*NADH1*)]. This study supported the hypothesis that the subfamilies Xenophthalminae Alcock, 1900, and Asthenognathinae Stimpson, 1856, should be split from Pinnotheroidea. Additionally, this phylogeny revealed the existence of other subfamilies beyond those previously known (Pinnotherinae and Pinnothereliinae).

Calyptraeotheres Campos, 1990, is an American genus belonging to Pinnotheridae, in which all members are obligatory symbionts of slipper limpets of the family Calyptraeidae (Campos, 1999). Within this genus, composed of five species (see Campos & Hernández-Ávila, 2010), *Calyptraeotheres garthi* (Fenucci, 1975) is distributed from the San Matias Gulf in Argentina to the coast of Rio Grande do Sul in southern Brazil (Martins & D'Incao, 1996), where it lives in symbiotic association with almost all naturally occurring limpets of the Calyptraeidae family (Ocampo *et al*., 2012). *Calyptraeotheres politus* (Smith, 1870) is found along the south-eastern Pacific coast, living in limpets of the genera *Crepipatella* and *Calyptraea* (Campos, 1999). *Calyptraeotheres hernandezi* Hernández-Ávila & Campos, 2006, and *Calyptraeotheres granti* (Glassell, 1933) establish symbiosis with limpets of the genus *Crucibulum*, and are present on Cubagua Island, Venezuela, and in the Mexican Pacific, respectively. Finally, *Calyptraeotheres pepeluisi* Campos & Hernández-Ávila, 2010 was described from a single female specimen found in Michoacán, on the Pacific coast of Mexico (Campos & Hernández-Ávila, 2010).

Members of *Calyptraeotheres* have been taxonomically problematic. *Calyptraeotheres politus* was reassigned to the genus *Tumidotheres* on the basis of larval morphology (Marques & Pohle, 1995). Later, based on the morphology of adult crabs, all pinnotherids living as symbionts of limpets of the Calyptraeidae family were reassigned to the genus *Calyptraeotheres* (Campos, 1999). This genus was subdivided into two subgroups, one austral (*C. garthi* and *C. politus*) and one subtropical–tropical (*C. hernandezi* and *C. granti*), based on characters of the third maxilliped; the austral subgroup can be recognized by a three-segmented third maxilliped, whereas this character is two-segmented in the subtropical–tropical subgroup

(Hernández-Ávila & Campos, 2006). Last, two studies, one based on larval morphology (Ocampo *et al*., 2011) and another based on larval and adult morphological characters (Campos & Hernández-Ávila, 2010), supported the hypothesis that *Calyptraeotheres* is a monophyletic clade.

Although our knowledge of the systematics of Pinnotheridae has increased in recent years, fine-scale relationships within most pinnotherid genera and species are still overlooked. At the intraspecific level, nothing is known about DNA phylogeography and/or population genetics in almost all species of this vast family. In view of this taxonomic situation, three main objectives were addressed in the present study. First, mitochondrial *COI* and rDNA *16S* sequences were used to determine phylogenetic relationships amongst species of *Calyptraeotheres*. Second, *COI* and *16S* genes were evaluated for their usefulness for DNA barcoding in some American species of Pinnotheridae. Third, *COI* sequences were used to assess the molecular diversity at the population level and the historical demography of *C. garthi* from Argentinean waters.

MATERIAL AND METHODS

Sample collection

Pinnotherid species and the number of specimens from each species included in this study are listed in Table 1, with the corresponding collection sites and geographical distribution shown in Figure 1. To test for monophyly of the genus *Calyptraeotheres*, we included phylogenetically close representatives of two other genera of the subfamily Pinnotherinae (see Palacios-Theil *et al*., 2009), *Tumidotheres maculatus* (Say, 1818) and *Dissodactylus crinitichelis* Moreira, 1901. Unfortunately, in GenBank there were no available *COI* sequences of other members of the subfamily Pinnotherinae and we only had fresh specimens of *Tumidotheres* and *Dissodactylus*. As outgroups, we included two species of *Austinixa* (subfamily Pinnothereliinae): *Austinixa patagoniensis* (Rathbun, 1918) and *Austinixa aidae* (Righi, 1967). For the phylogenetic analyses only four specimens of *C. garthi* were included. Fresh specimens were fixed in 96% ethanol, and prior to dissection were identified on the basis of their external morphology (Fenucci, 1975; Manning & Felder, 1989; Campos, 1999; Hernández-Ávila & Campos, 2006). When necessary, the mouthparts were dissected to better observe the maxillipeds.

DNA extraction, PCR amplification, and sequencing

DNA extraction, amplification and sequencing protocols followed Mantelatto *et al*. (2007, 2009). Total genomic DNA was extracted from muscle tissue. Muscle was ground and then incubated for 12–24 h in 600 µL lysis buffer [100 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris(hydroxymethyl) aminomethane (TRIS) pH 7.5, 1% sodium dodecyl sulphate] at 55 °C; protein was separated by the addition of 200 µL of 7.5 M ammonium acetate and subsequent centrifugation. DNA was precipitated by the addition of 600 µL cold absolute isopropanol to the supernatant from the previous step, followed by centrifugation; the resulting pellet was washed with 70% ethanol, dried, and resuspended in 20 µL Tris-EDTA buffer (10 mM TRIS, 1 mM EDTA). A fragment (~650 bp) of the barcode region of mitochondrial gene *COI* was amplified using the universal primers COL6b (5′-ACA AAT CAT AAA GAT ATY GG-3′), COH6 (5′-TAD ACT TCD GGR TGD CCA AAR AAY CA-3′) (Schubart & Huber, 2006), HC02198 (5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′), and LCO1490 (5′-GGT CAA CAA ATC ATA AAG ATA TTG G-3′) (Folmer *et al*., 1994). The fragment (~550 bp) of the mitochondrial 16S ribosomal region was amplified using the primers 1472 (5′-AGA TAG AAA CCA ACC TGG-3′) (Crandall & Fitzpatrick, 1996) and 16 SL/16Sa (5′-CGC CTG TTT AAC AAA AAC AT-3′) (Xiong & Kocher, 1991). Reactions were performed in 25 µL volumes (200 µM deoxynucleotide triphosphates, 1× buffer, 0.5 µM of each primer, 1 unit Taq polymerase, 20–30 ng extracted DNA). PCR cycling was performed starting with denaturation for 5 min at 95 °C, followed by 36–40 cycles of 1 min at 94 °C, 1 min at 42–52 °C, and 1.5–2 min at 72 °C, with a final extension of 10 min at 72 °C. Our profiles changed depending upon the gene being amplified, quality of DNA, and success of the PCR reaction. Successful PCR reactions were confirmed with the aid of GelRed in an agarose gel, purified using SureClean, and then sequenced with the ABI Big Dye Terminator Mix (Applied Biosystems, Carlsbad, CA) in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems automated sequencer) following Applied Biosystems' protocols. Only a 16S sequence of *C. granti* was retrieved from GenBank (Table 1). New sequences were deposited in GenBank, and genetic vouchers, from which tissue samples were obtained, were deposited at the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia' (MACN), Argentina and in the Crustacean Collection of the Department of Biology (CCDB), Faculty of Philosophy, Sciences and Letters of Ribeirão Preto (FFCLRP), University of São Paulo (USP), Brazil (Table 1). All sequences were edited and a consensus of complementary sequences was obtained using the BioEdit v.7.08.0 software (Hall, 1999). Consensus sequences were aligned independently for both genes using Clustal W (Thompson, Higgins & Gibson, 1994), as implemented in BioEdit, and verified by eye.

Table 1. List of specimens included in this study for which cytochrome oxidase I (*COI*) mtDNA and large ribosomal subunit (*16S*) mtDNA were sequenced

Species	Sample site	*N*	GB *COI*	GB *16S*	Catalogue number
Calyptraeotheres garthi	Sótano (SOT), San Matias Gulf, Argentina	7	JX839480–JX839486	JX839469	MACN-In 38931 CCDB-3586
	Ría de San Antonio (RIA), Argentina	9	JX839487–JX839495	JX839466	MACN-In 38932 CCDB-3588
	Mar del Plata (MDP), Argentina*	8	JX839496–JX839503	JX839465	MACN-In 38933 CCDB-3587/3589
	San Clemente (SCL), Argentina	6	JX839504–JX839509	JX839467–JX839468	CCDB-3590
Calyptraeotheres granti	San Felipe, Mexico	1	–	EU 934979‡	ULLZ 9599
Calyptraeotheres hernandezi	Isla Cubagua (ICU), Venezuela†	2	JX839510	JX839470–JX839471	MACN-In 38936 CCDB-3109
Calyptraeotheres politus	Arica (ARI), Chile	3	JX839511–JX839513	JX839472	MACN-In 38934
	Puerto Montt (PMO), Chile	7	JX839514–JX839520	JX839473–JX839474	MACN-In 38935 CCDB-3724
Dissodactylus crinitichelis	Ilha Prumirim (IPR), Ubatuba, Brazil	2	JX839521–JX839522	JX839476	MACN-In 38937
	SCL, Argentina	2	JX839523– JX839524	JX839475	CCDB-3591
Tumidotheres maculatus	MDP, Argentina	1	JX839525	JX839477	CCDB-3585
Austinixa patagoniensis	Ilha Comprida (ICO), São Paulo, Brazil	2	JX839526– JX839527	JX839479	CCDB-3667
Austinixa aidae	Caraguatatuba (CAR), São Paulo, Brazil	2	JX839528– JX839529	JX839478	CCDB-3100

*Type locality of *C. garthi* – Fenucci, 1975.
†Type locality of *C. hernandezi* – Hernández-Ávila & Campos, 2006.
‡Palacios-Theil *et al*. (2009).
N, number of specimens; GB, GenBank accession number.
CCDB, Crustacean Collection of the Department of Biology, Faculty of Philosophy, Sciences and Letters of Ribeirão Preto, University of São Paulo; MACN, Museo Argentino de Ciencias Naturales; ULLZ, University of Louisiana, Lafayette Zoological Collection.

Figure 1. Distribution of species of *Calyptraeotheres* in the Americas (dark grey) with collection sites (black stars). Abbreviations: ARI, Arica; CAR, Caraguatatuba; ICO, Ilha Comprida; ICU, Isla Cubagua; IPR, Ilha Prumirim; MDP, Mar del Plata; PMO, Puerto Montt; RIA, Ría de San António; SCL, San Clemente; SOT, El Sótano.

Phylogenetic analyses

We performed independent analysis of the two databases (*COI* and *16S*, figures not included) and obtained the same overall topology. Thus, we concatenated the two databases and performed a single analysis with the combined data. There are two possible approaches to combining these types of data: simply combine or combine only if the different genes have the same evolutionary history. The latter approach can be accomplished, amongst other methods, by using the incongruence length difference (ILD) test (Bull *et al*., 1993). Therefore, we conducted an ILD test (also known as a partition homogeneity test) (Bull *et al*., 1993), as implemented in PAUP, to determine whether the *COI* and *16S* genes could be considered to be samples of the same underlying phylogeny. When dealing with concatenated samples, most authors simply concatenate sequences and apply a single substitution model to the combined alignment (e.g. Rokas *et al*., 2003; Hedges *et al*., 2004; Wolf, Rogozin & Koonin, 2004). Thus, prior to conducting the Bayesian (BAY) and maximum likelihood (ML) analyses, the model of evolution that best fit the data was determined with the software MODELTEST (Posada & Crandall, 1998). Phylogenetic analyses of *COI*/*16S* concatenated sequences were conducted using MrBayes v.3.0b4 (Huelsenbeck & Ronquist, 2001) for BAY analysis, RAxML v.7.0.4 (Stamatakis, 2006) for ML analysis, using the online version on the Cyber Infrastructure for Phylogenetic Research (CIPRES) website (Stamatakis, Hoover & Rougemont, 2008), and PAUP 4.0 beta 10 (Swofford, 2003) for maximum parsimony (MP) analysis. To determine confidence values for the obtained ML trees, we

selected the option to automatically determine the number of bootstraps to be run in RAxML. Thus, 150 bootstrap pseudoreplicates were run, and values > 50% are shown on the resulting trees. BAY analysis was conducted by sampling two sets of four chains, one cold and three heated, for 10 000 000 generations, starting with a random tree. One tree was sampled every 1000 generations, thus obtaining 10 001 trees. Using TRACER v.1.4 software (Rambaut & Drummond, 2007), we determined that stasis was reached well before 10%. Using a conservative approach, we discarded the first 1001 trees corresponding to the first 1 000 000 generations (10%) and obtained a 50% majority rule consensus tree from the remaining 9000 saved trees. On the tree thus obtained, values were reported for posterior probabilities of the respective nodes amongst all the saved trees, expressed as percentages. The MP analysis was performed as a heuristic search with random sequence addition, including tree bisection and reconnection as a branch-swapping option. On the resulting molecular trees, bootstrap confidence values > 50% were reported for MP (1000 bootstraps) analysis.

Genetic distances

To evaluate the usefulness of *COI* and *16S* as DNA-barcoding genes in selected pinnotherid species, two general genetic distance matrices were constructed, one based on *COI* and the other on *16S* sequences. Matrices were calculated under the Kimura two-parameter (K2P) model in MEGA v.5 (Tamura *et al*., 2011). Genetic distances within and between groups were computed only in cases in which more than one specimen per species was sequenced. To visualize barcoding gaps between intraspecific and interspecific genetic distances, we constructed two frequency histograms, one with pairs of *COI* sequences and one with pairs of *16S* sequences. A gap between intraspecific and interspecific sets of genetic distances is a requirement to validate the usefulness as a DNA barcode of a molecular marker (Meyer & Paulay, 2005). The presence of a barcoding gap can be used as a species threshold (Puillandre *et al*., 2011).

Population genetic, phylogeographical, and demographic analysis

Haplotype diversity (h), nucleotide diversity (π), number of haplotypes (N_h), number of polymorphic sites (N_p), and average number of nucleotide differences between pairs of sequences (k) for *COI* sequences of *C. garthi* were computed using DnaSP v. 4.10.3 (Rozas *et al*., 2003). To examine the *COI* population structure of *C. garthi*, an analysis of molecular variance (AMOVA) was performed; its significance was tested by running 10 000 permutations in ARLEQUIN v. 3.1 (Excoffier, Laval & Schneider, 2005). The AMOVA was run with no hierarchical structure (all populations in a single group) and with pairwise comparisons between sampling sites from Argentina. Additionally, pairwise F_{st} values were obtained using the same software with 10 000 permutations. The F_{st} values were computed using haplotypic frequencies. The genealogical relationships amongst *COI* sequences of *C. garthi*, *C. politus*, and *C. hernandezi* were determined by a haplotype network generated with the median-joining method (Bandelt, Forster & Röhl, 1999) in NETWORK v. 4.6 (http://www.fluxus-engineering.com). The question of whether the populations had undergone a sudden population expansion or had remained stable over time was investigated using mismatch distributions (Rogers & Harpending, 1992), and any deviation from the sudden-expansion model was evaluated by calculating the raggedness index (r) (Harpending, 1994) and R_2 (Ramos-Onsins & Rozas, 2002) in DnaSP. The history of demographic changes was also assessed by calculating Tajima's D-test (Tajima, 1989) and Fu's Fs test (Fu, 1997), also using DnaSP. Tajima's and Fu's neutrality tests are used to verify whether a population is in mutation/drift equilibrium or, in contrast, if it is expanding (Ramos-Onsins & Rozas, 2002). Negative and significant values of these parameters are indicative of population expansion and/or negative selection. Selective sweeps and selection against slightly deleterious mutations can result in a pattern of haplotype diversity similar to that produced by a population expansion (Whittam *et al*., 1986; Bertorelle & Slatkin, 1995); these analyses cannot differentiate between the effects of either of the two processes.

Two methods were applied to estimate the time of population expansion of *C. garthi*. First, we incorporated the statistic τ (tau), calculated from mismatch distribution analysis, into the equation $t = \tau/2\,\mu$, where μ is the per-locus per-year mutation rate. Confidence intervals of t, at 95% significance, were obtained using a parametric bootstrap approach in ARLEQUIN. Second, we characterized past changes in the effective population size (N_e) of *C. garthi* by generating COI Bayesian skyline plots (BSPs) with BEAST v. 1.4.8 (Drummond *et al*., 2005; Drummond & Rambaut, 2007). These analyses were run under the Hasegawa-Kishino-Yano with invariable positions and unequal rates of substitutions under a gamma distribution (HKY + I + G) model that was selected by jModelTest (Posada, 2008) using the Akaike information criterion (AIC). The analysis was performed using a relaxed molecular clock, with three runs of 10 000 000 generations each, in which trees and

parameters were sampled every 1000 generations. As the mutational rate for *COI* is not available in pinnotherid crabs, we used a rate of 1.66% million years^{-1} for the *COI* gene of grapsid crabs (Schubart, Diesel & Hedges, 1998) to estimate the absolute and BSP population divergence times.

RESULTS

Segment of 624 and 482 bp in length of *COI* and *16S*, respectively, were obtained from seven species of Pinnotheridae (Table 1). We were unable to amplify three specimens of *C. garthi* from Rio Grande do Sul (south of Brazil) obtained from the Universidade Federal do Rio Grande (FURG 1239/1241). These specimens most likely were fixed in formaldehyde, a strong inhibitor of PCR amplifications. In pea crabs, owing to their small size and the high penetrability of fixatives, unsolvable problems can result from inappropriate fixation and/or storage procedures. Similarly, and despite many attempts, it was impossible to obtain a workable sequence of *COI* (but not *16S*) from one of the two specimens of *C. hernandezi* (Venezuela).

PHYLOGENETIC ANALYSES

The alignments of both *COI* and *16S* gene sequences were unambiguous. The ILD test showed no significant incongruence ($P = 0.383$). Thus, all phylogenetic analyses were performed with a single database (1106 bp) including the two genes. The optimal model, selected using the AIC in MODELTEST (Posada & Crandall, 1998), was the general time reversible model, which accounted for invariable positions and unequal rates of substitutions under a gamma distribution (GTR + I + G) with the following parameters: assumed nucleotide frequencies A = 0.321, C = 0.174, G = 0. 136, T = 0.369; substitution model with rates A–C = 4.124, A–G = 15.219, A–T = 9.606, C–G = 0.029, C–T = 66.053, and G–T = 1.0000; proportion of invariable sites I = 0.649; variable sites followed a gamma distribution with shape parameter = 2.429. Our three phylogenetic analyses derived from concatenated sequences of *COI* and *16S* under BAY, ML, and MP resulted in similar topologies (Fig. 2) in which all members of *Calyptraeotheres* were clustered in a monophyletic clade. Within this clade, the Chilean species *C. politus* was sister to all other species of *Calyptraeotheres*. *Calyptraeotheres hernandezi* and

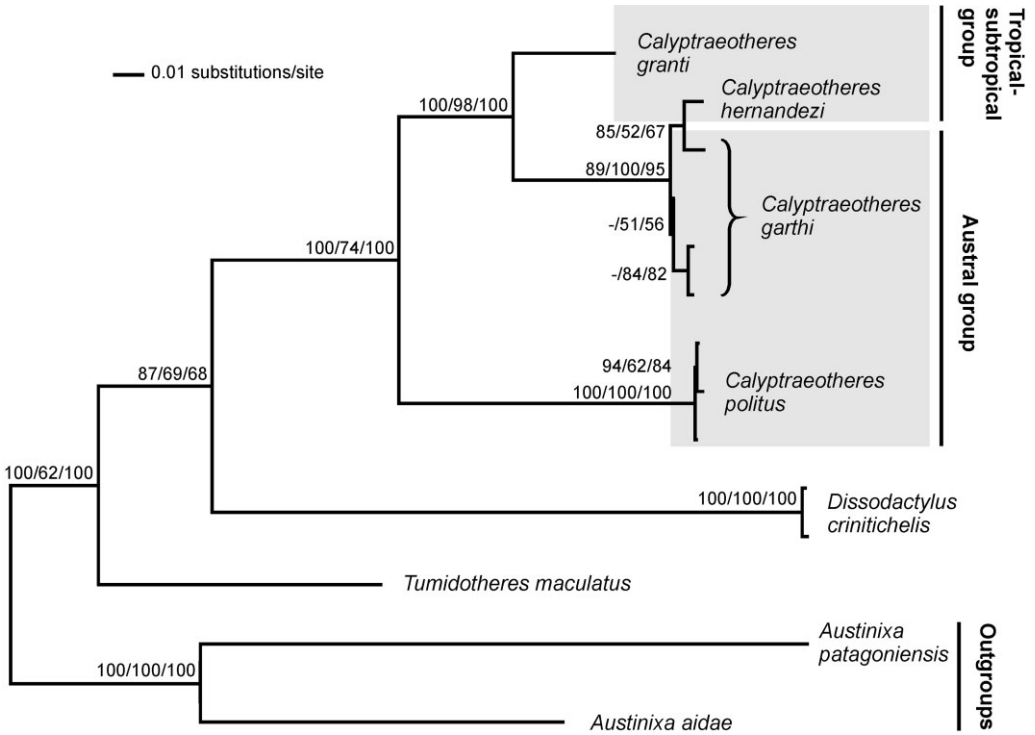

Figure 2. Bayesian (BAY) tree for *Calyptraeotheres* species, *Tumidotheres maculatus*, *Dissodactylus crinitichelis*, and selected outgroups (*Austinixa aidae* and *Austinixa patagoniensis*) based on the cytochrome oxidase I (*COI*) and large ribosomal subunit (16S) concatenated data set. Values represent bootstrap and Bayesian posterior probabilities (maximum likelihood/maximum parsimony/BAY) expressed as percentages. Values ≤ 50% are not shown. The *Calyptraeotheres* subdivision proposed by Hernández-Ávila & Campos (2006) is highlighted in grey.

C. garthi were clustered together in the same clade in what appears to be a paraphyletic *C. garthi* (Fig. 2). This clade is sister to the subtropical Pacific species *C. granti*. With respect to other genera of the subfamily Pinnotherinae, our phylogenetic analyses supported the hypothesis that *Calyptraeotheres* is more closely related to *Dissodactylus crinitichelis* than to *Tumidotheres maculatus*.

Genetic distances and DNA barcoding

The distances between pairwise sequences revealed two well-defined sets of values for *COI* sequences, which were widely separated by a gap (Fig. 3A). The group of smaller values ranged from 0 to 0.022 (mean ± SD = 0.009 ± 0.004) and included all the pairwise distances observed between individuals of the same species. Notably, the genetic distance values observed amongst sequences of specimens of *C. garthi* and *C. hernandezi* were found in this set of values. In the second group, the values ranged from 0.155 to 0.246 (mean ± SD = 0.183 ± 0.021) and included interspecific (e.g. *C. garthi* × *C. politus*) and intergeneric (e.g. *C. garthi* × *T. maculatus*) distances. The group of larger values was subdivided as a bimodal distribution that clearly separated interspecific from intergeneric distances (Fig. 3A). In the case of *16S*, the intraspecific distances ranged from 0 to 0.058 (mean ± SD = 0.0023 ± 0.0017), whereas interspecific and intergeneric distances ranged from 0.035 to 0.163

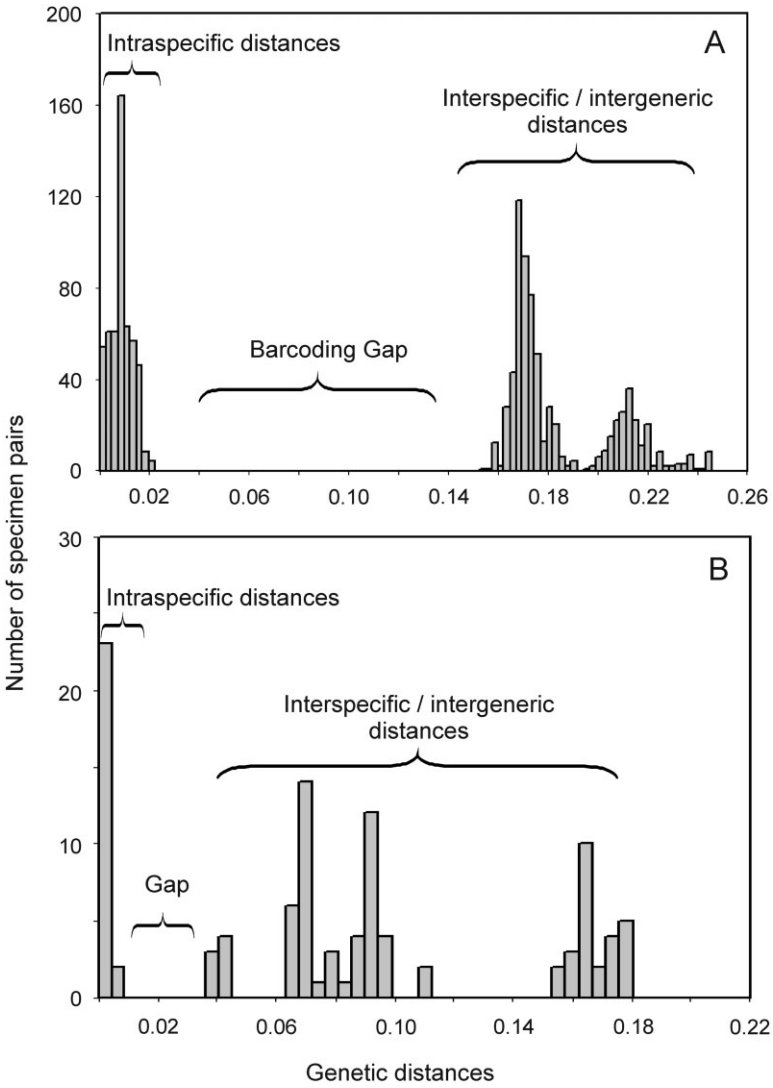

Figure 3. Histogram of Kimura two-parameter genetic distances for (A) the cytochrome oxidase I and (B) the large ribosomal subunit (16S) data sets. Species and number of specimens used for intraspecific and interspecific distance calculations are detailed in Table 1.

Table 2. Number of haplotypes (N_h), number of polymorphic sites (N_p), haplotype diversity (h), nucleotide diversity (π), mean number of nucleotide differences between pairs of sequences (k), and neutrality (Tajima's D and Fu's Fs) and demographic tests (r and R_2) for the 624 bp of the cytochrome oxidase I mtDNA of *Calyptraeotheres garthi* examined in this study

Sampling sites	N_h	N_p	$k \pm$ SD	$h \pm$ SD	$\pi \pm$ SD	D	Fs	r	R_2
SCL	6	15	5.400 ± 3.029	1.000 ± 0.096	0.008 ± 0.006	–	–	–	–
MDP	8	15	5.000 ± 2.717	1.000 ± 0.062	0.008 ± 0.005	–	–	–	–
SOT	7	13	4.285 ± 2.412	1.000 ± 0.076	0.006 ± 0.004	–	–	–	–
RIA	9	26	8.083 ± 4.145	1.000 ± 0.052	0.013 ± 0.007	–	–	–	–
Total	28	38	5.862 ± 2.881	0.993 ± 0.012	0.009 ± 0.005	–1.501*	–25.22**	0.09**	0.161**

*$P < 0.05$; **$P < 0.001$.
MDP, Mar del Plata; RIA, Ría de San António; SCL, San Clemente; SOT, El Sótano.

(mean ± SD = 0.078 ± 0.019). Similarly to the *COI* pairwise distances, a gap separated the intraspecific from the interspecific/intergeneric group of pairwise distances (Fig. 3B); pairwise distances between *C. hernandezi 16S* sequences and any of the *16S* sequences of *C. garthi* fell into the group of intraspecific distances.

Phylogeography, population genetics, and historical demography

Forty-one substitutions (35 transitions, six transversions) and 38 polymorphic sites in 30 *COI* sequences of *C. garthi* obtained from Argentina defined 28 mitochondrial haplotypes. The mean number of differences (±SD) between pairs of COI sequences was 5.86 ± 2.88 (Table 2). Overall nucleotide diversity was 0.009 (ranging from 0.006 to 0.013 across sites) and haplotype diversity was 0.993 (1.00 for each site) (Table 2). Most of the genetic diversity was the result of variability within rather than amongst populations (AMOVA: within populations = 99.97%, amongst populations = 0.03%, $F_{st} = 0.0003$, $P = 0.473$). Furthermore, no significant differences were obtained for any pairwise comparison between sampling sites from Argentina, e.g. El Sótano (SOT)–Mar del Plata ($F_{st} \sim 0$, $P = 0.991$), Ría de San António (RIA)–San Clemente ($F_{st} \sim 0$, $P = 0.991$), SOT–RIA ($F_{st} \sim 0$, $P = 0.991$). Accordingly, the *COI* haplotype network for *C. garthi* shows a complex and unstructured phylogeographical pattern, with the central haplotype absent (Fig. 4A). Amongst the 28 haplotypes, 27 (96%) represented single individuals. Only one haplotype was shared by three geographical sites; the rest were unique haplotypes separated from each other by one to three mutational steps. The haplotype network of *C. garthi* was connected with *C. hernandezi* by only three mutational steps, and with *C. politus* by 74 mutational steps (Fig. 4A). The COI network of the eastern Pacific species *C. politus* showed a central haplotype shared by specimens from the northern and southern populations and three exclusive individual haplotypes.

As no genetic differentiation amongst sites was observed in the AMOVA, we pooled all the samples to calculate neutrality tests, mismatch distribution, and the historical population sizes. Both Tajima's D and Fu's Fs were negative and significant (Table 2). The mismatch distribution was unimodal (Fig. 4B) and the r and R_2 indexes did not detect a significant deviation from expectations under the spatial expansion model (Table 2). The population expansion time of *C. garthi* was estimated to be 303 Kya (216–366 Kya) ($\tau = 6.055$; confidence intervals at $\alpha = 0.05$: 4.330–7.307). The BSP analysis revealed that the haplotypes of *C. garthi* coalesced nearly 224 Kya, when a significant decline in population size was noted (Fig. 4C). Assuming the neutrality of this mitochondrial marker, these results indicate that a strong population expansion took place around that time and that the population then remained stable until the present.

DISCUSSION

Phylogeny of *Calyptraeotheres*

Our phylogenetic results based on the *COI*/*16S* concatenated data set strongly support the monophyly of the genus *Calyptraeotheres*, as previously postulated based on morphological comparisons (Campos & Hernández-Ávila, 2010; Ocampo *et al*., 2011). Moreover, our results show *Calyptraeotheres* positioned closer to *Dissodactylus crinitichelis* than to *Tumidotheres maculatus*. These results partly disagree with a recent molecular study on the phylogeny of the family, in which *Tumidotheres* was found to be the closest relative to *Calyptraeotheres* (Palacios-Theil *et al*., 2009). Whereas these authors

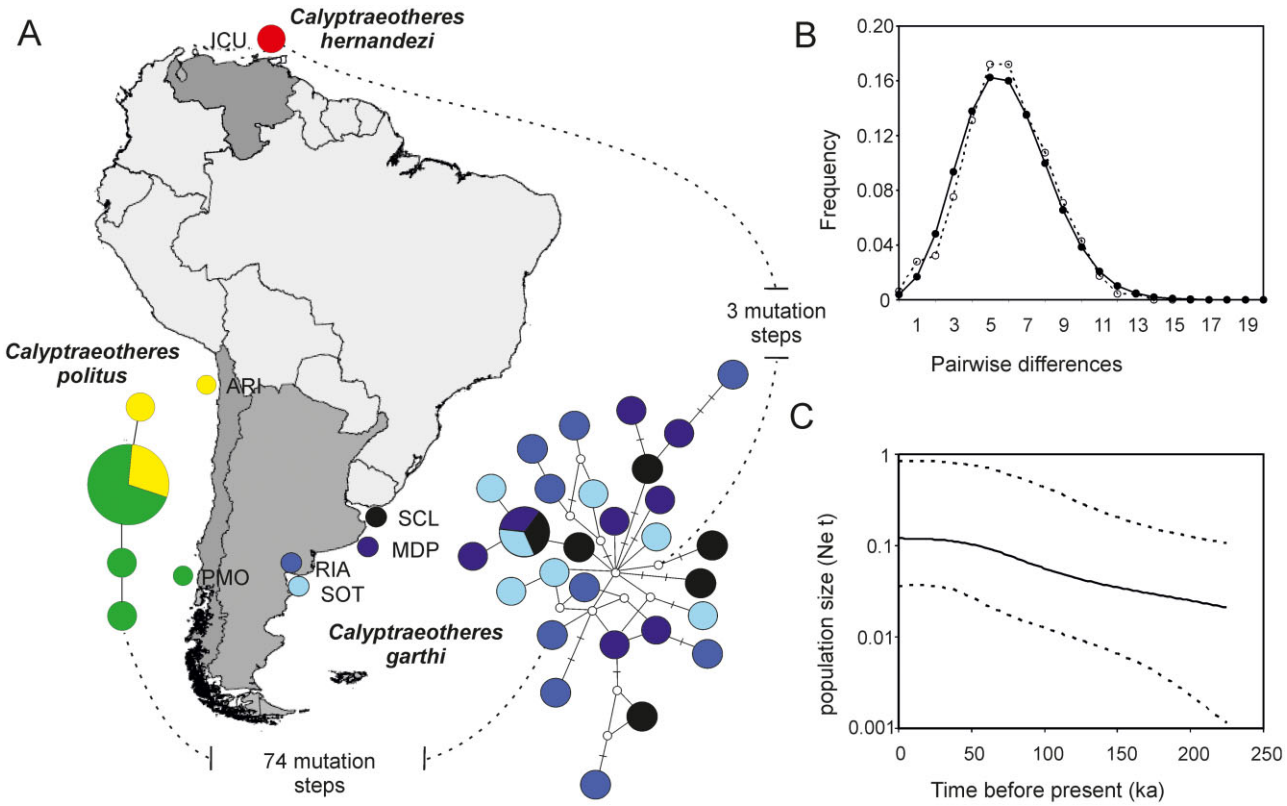

Figure 4. A, median-joining haplotype network for cytochrome oxidase I (*COI*) mtDNA sequences of *Calyptraeotheres garthi*, including some samples of *Calyptraeotheres hernandezi* and *Calyptraeotheres politus*. Area of the circles is proportional to the number of individuals of each haplotype found. White dots represent missing, probably unsampled, haplotypes or extinct lineages. Lines between circles represent additional mutational steps. B, mismatch distributions of *C. garthi*. Solid lines indicate the observed distribution, and dashed lines indicate the expected distribution. C, Bayesian skyline plot based on *COI* sequences of *C. garthi* showing change in population size through time. The *y*-axis is the product of effective population size (N_e) and generation length (t) on a log scale. The heavy solid line is the median estimated under the assumption of a per site mutation rate of 1.66% million years^{-1}, and the dotted lines indicate 95% highest posterior density regions. Abbreviations: ARI, Arica; CAR, Caraguatatuba; ICU, Isla Cubagua; MDP, Mar del Plata; PMO, Puerto Montt; RIA, Ría de San António; SCL, San Clemente; SOT, El Sótano.

used three mitochondrial genes (*16S/NADH1/t-RNA-leu*), we used *COI* and *16S* to construct both phylogenies. Differences in the set of genes as well as the number of species and genera included in the two studies might explain the discrepancies in the topology between our study and the findings of Palacios-Theil *et al*. (2009). Nevertheless, our analysis included all species of the genus, and our results are supported by morphology because *Calyptraeotheres* more closely resembles *Dissodactylus* than *Tumidotheres* in the shape of the carapace and general morphology of the third maxilliped (see Campos & Hernández-Ávila, 2010).

Based on features of the third maxilliped, Hernández-Ávila & Campos (2006) subdivided *Calyptraeotheres* into two subgroups, one austral formed by the south-eastern Pacific species *C. politus* and the south-western Atlantic species *C. garthi* and another composed of the Caribbean species *C. hernandezi* and the north-eastern Pacific species *C. granti* (see Fig. 2). In contrast, our results show a strong relationship between the Atlantic species, with a wide genetic differentiation from the Pacific species. Thus, the patterns of evolutionary history found here cannot be interpreted without accurate knowledge of the historical biogeography of these species. Our results show that the lineage of *C. politus* probably spliced out early in the evolutionary history of *Calyptraeotheres*, prior to the emergence of the Panamanian land bridge (3.5 Mya). The other lineage, which included the ancestor of *C. garthi*, *C. hernandezi*, and *C. granti*, radiated as a result of the formation of the Panamanian isthmus. The closure of the Panamanian land bridge is considered

one of the most important geological events in the last 15 Myr, with dramatic consequences for marine and terrestrial life (Woodring, 1966; Collins, 1996; Fortunato, 2008). The progressive isolation of the Atlantic and Pacific oceans triggered, in turn, genetic isolation of the populations located on either side of the newly emerged land bridge, which has been proposed to explain speciation amongst a wide diversity of marine organisms across the isthmus (Knowlton & Weigt, 1998). The evolution of *Calyptraeotheres* is clearly one more example of how the formation of the Panamanian land bridge affected the diversification of decapod crustaceans, as has been shown previously for *Callinectes* (Robles *et al*., 2007), *Austinixa* (Harrison, 2004), *Alpheus* (Knowlton *et al*., 1993), and *Pachygrapsus* (Schubart, Cuesta & Felder, 2005), amongst others.

Our analysis also showed that the species from the Venezuelan Caribbean, *C. hernandezi* and *C. garthi*, might in fact represent a single species. A more detailed discussion follows.

Barcoding and systematic position of *Calyptraeotheres hernandezi* and *Calyptraeotheres garthi*

For the pinnotherids analysed herein, the K2P distance between pairs of mitochondrial sequences forms two groups of values with a discrete gap between them (see Fig. 3). The lower boundary of the large-distance group of values is almost an order of magnitude greater than the upper boundary of the lower range of values, both in *COI* and *16S*. Indeed, the short-distance group has no overlap with the large-distance group. The average intraspecific and interspecific K2P values calculated in the present study are similar to those observed for other crustaceans. For example, the intraspecific *COI* distance computed in 150 crustacean families has an average value of 0.0046 (Costa *et al*., 2007), whereas in our study the same parameter reaches only a value of 0.009. For the same families mentioned above, the average interspecific distance is 0.17 (Costa *et al*., 2007), whereas in our case it reaches an average value of 0.183. Therefore, the DNA barcode in pinnotherids, as in other crustaceans (Costa *et al*., 2007; Puillandre *et al*., 2011; Negri, Pileggi & Mantelatto, 2012), promises to be a good system to determine the limits amongst species and can be used to complement classical taxonomy.

By contrast, the genetic distances of *COI* and *16S* between specimens of *C. hernandezi* and *C. garthi* fall into the short-distance group. The *COI* sequences between these species differ by only three mutations, which caused, on average, a divergence of 0.010. Likewise, the two *16S* sequences of *C. hernandezi* differ by one mutational step from the *16S* of *C. garthi*. Genetic divergence between *16S* sequences of *C. hernandezi* and *C. garthi* was on average 0.0036. Therefore, our molecular results do not support a species status for *C. hernandezi*.

According to Hernández-Ávila & Campos (2006), the most important character distinguishing *C. hernandezi* from *C. garthi* is the morphology of the adult third maxilliped, which is two-segmented (dactyl absent) in the former species and three-segmented (dactyl present) in the latter. Three characters can also be used to differentiate *C. hernandezi* from the north-eastern Pacific *C. granti*: females of *C. hernandezi* have eyes that are visible in dorsal view, the propodi of the walking legs have subparallel margins, and the ventral margin of the pollex bears a small fringe of setae (Hernández-Ávila & Campos, 2006). These three features were present in the specimens of *C. hernandezi* from Cubagua Island, Venezuela, examined in our study. However, it is our opinion that these characters are shared with *C. garthi.* In this context, Fenucci (1975) mentioned that the margins of the propodi of the first and second walking legs are almost straight in the female of *C. garthi*, and the illustrations (Fenucci, 1975: 170) show that these margins are not tapered distally. Additionally, the illustrations of Fenucci (1975) reveal that the female eyes are clearly observed from the dorsal view and that a fringe of setae covers the ventral margin of the pollex. As expected, these three characters were also observed in the specimens of *C. garthi* included in the present study, therefore contradicting the observations of Hernández-Ávila & Campos (2006).

At this point, we believe that an exhaustive morphological study applying high-resolution techniques (e.g. scanning electron microscopy), accompanied by analysis of more specimens and DNA markers of *C. hernandezi*, is perhaps necessary to clarify whether *C. garthi* and *C. hernandezi* are separate clades or are populations of the same species. For now, our results support the idea that *C. hernandezi* represents a junior synonym of *C. garthi*.

Under the hypothesis that *C. hernandezi* and *C. garthi* belong to a single species, the question arises as to whether there is genetic flow between these two putative populations of *C. garthi*. *Calyptraeotheres garthi* occurs in the south-western Atlantic Ocean from San Matias Gulf, Argentina, to Rio Grande do Sul, Brazil (Martins & D'Incao, 1996), whereas *C. hernandezi* is known only from Cubagua Island, Venezuela. Between the northern boundary of the former species and the location of the latter is a geographical gap of ~6400 km. The coast of Brazil harbours a large number of potential (see Simone, 2006) and probable (e.g. the limpet *Bostrycapulus odites* Collin, 2005; Ocampo *et al*., 2012) hosts of *Calyptraeotheres*. Nevertheless, there is no record of

Calyptraeotheres from intermediate sites between southern Brazil and Venezuela. Attempts to obtain *Calyptraeotheres* from intermediate areas through examination of scientific collections (the museums of Rio Grande do Norte; LABOMAR, Fortaleza, Recife; MOUFPE, Recife; UESC, Bahia; MNRJ, Rio de Janeiro; MZUSP, São Paulo; and MACN, Buenos Aires), sampling on the coast of São Paulo and Rio de Janeiro, Brazil (hosts: *Crepidula* sp. and *Bostrycapulus odites* present but with no crabs associated), and also consultation of a malacologist who studies potential hosts of *Calyptraeotheres* (L. Simone, pers. comm.), were unsuccessful. Apparently, *Calyptraeotheres* are absent from this area or, perhaps, are present only at low levels of abundance. Therefore, *C. hernandezi* and *C. garthi* appear to be genetically isolated, and the single-species hypothesis becomes weak and difficult to credit. Alternatively, there may have been a recent speciation event, which could explain the low genetic distance observed above. By contrast, the lack of genetic differentiation between *C. garthi* and *C. hernandezi* could be a consequence of the introduction of specimens, either in the larval stage through ballast water or in the adult stage along with some of the multiple hosts (Ocampo *et al*., 2012), as has been observed in other marine organisms (Reise, Gollasch & Wolff, 1992; Orensanz *et al*., 2002), including decapods (Hidalgo, Barón & Orensanz, 2005; Taylor & Komai, 2011).

Phylogeography and population genetics of *Calyptraeotheres garthi*

Calyptraeotheres garthi has a high degree of genetic homogeneity across its distribution range, as indicated by the AMOVA and the haplotype network. Genetic structure is observed when ongoing or historical processes have limited gene flow (Avise, 2009). However, marine species commonly exhibit low levels of global population differentiation, even when physical barriers limit gene flow (Ward, Woodwark & Skibinski, 1994; Waples, 1998; Fernández Iriarte *et al*., 2011). Although the San Matias Gulf has been considered to be a closed system with larvae retention (Guerrero & Piola, 1997), this does not seem to lead to genetic structure in *C. garthi*. Similarly to almost all other pinnotherids, *C. garthi* has a long larval cycle and requires about 30 days to settle (Ocampo *et al*., 2011), giving the species sufficient time for marine dispersal and to maintain connectivity amongst populations across its geographical range.

Calyptraeotheres garthi displayed high haplotype (28 haplotypes in 30 individuals) and low nucleotide diversity (overall 0.009), which may be attributable to rapid population growth and accumulation of mutations after a period of low effective population size. In support of this idea, the mismatch distribution showed a unimodal distribution that, together with the results of the neutrality tests (negative and significant), can be interpreted as indicators of sudden expansion. Population expansion of *C. garthi* started ~300 Kya according to the estimate from the mismatch distribution, and ~220 Kya using BSP analysis.

Climate changes in the Quaternary shaped the diversity of marine and terrestrial species (Avise, 2000, 2009). One of the climate events that played a crucial role in determining the abundance and distribution of natural populations was the last glacial maximum (LGM) in the late Pleistocene (~20 Kya; Hewitt, 2004; Provan & Bennett, 2008). Although strongly disputed by some authors (Rabassa, 2008; Lessa, D'Elía & Pardiñas, 2010), during the LGM many species are generally thought to have remained in refuges and then expanded when the glaciers retreated. Recent population expansions resulting from post-LGM recolonization have been detected mainly for terrestrial (Hewitt, 2000, 2004) and for some marine species (see examples given by Marko *et al*., 2010). Nevertheless, our results show that *C. garthi* underwent a population expansion earlier than the LGM (~220 Kya based on BSP and ~300 Kya calculated by τ). Thus, if this expansion was a result of climate change, this event would have occurred before the LGM. Consistent with our results, several marine species underwent population growth before the LGM in the south-western Atlantic (Fernández Iriarte *et al*., 2011; Ceballos *et al*., 2012) and elsewhere (Marko, 2004; Wilson, 2006; Wang, Li & Li, 2008; Marko *et al*., 2010).

Glaciations in the mid-Pleistocene appear to have left traces in the evolutionary history of several marine species (Wilson, 2006; Marko *et al*., 2010). Indeed, after the Great Patagonian Glaciation during the Miocene (~1 Mya; Rabassa, 2008) and before the LGM, three glacial periods strongly affected the region. Two of these occurred at or around 145 Kya (Kaplan *et al*., 2005) and 260 Kya (Hein *et al*., 2009). In these periods, the decrease in sea level and water temperature and changes in marine currents may have forced species to take refuge at lower latitudes, as has been suggested for the sub-Antarctic fish *Eleginops maclovinus* (Cuvier, 1830) (Ceballos *et al*., 2012) and the south-western Atlantic fish *Cynoscion guatucupa* (Cuvier, 1830) (Fernández Iriarte *et al*., 2011). *Calyptraeotheres garthi* may have undergone a northward retraction during glacial phases, similar to the process that has been suggested either for marine species such as those mentioned above or for other continental organisms in Patagonia (Ruzzante *et al*., 2008; Lessa *et al*., 2010). *Calyptraeotheres garthi* populations expanded at the end of the glacial period, by ~260 Kya, either recolonizing or colonizing higher latitudes for the first time.

Given the species' obligate symbiont lifestyle, a *C. garthi* population requires the prior existence of its hosts for growth and survival. It is plausible that the evolution of this crab is intimately related to the history of its host. The presence of limpet hosts [e.g. *Crepidula protea* (d'Orbigny, 1841), *Crepidula onyx* (Sowerby, 1824)] on the Argentinean coast dates from the Miocene (~20 Mya; Aguirre, 1993; Aguirre & Farinati, 1999), which would have enabled the crab population to establish. However, whether these host species either expanded or maintained stable population sizes over the last 300 000 years is unknown. It would be interesting to assess, in future studies, the historical demography of the limpet hosts of *C. garthi*.

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