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Taxonomic status of two South American sympatric squat lobsters, *Munida gregaria* and *Munida subrugosa* (Crustacea: Decapoda: Galatheidae), challenged by DNA sequence information

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We investigated the taxonomic status of two sympatric morphospecies of squat lobsters from southern South America (Beagle Channel, Strait of Magellan, and Burdwood Bank), *Munida gregaria* and *Munida subrugosa*, by DNA sequence analysis of three mitochondrial (mt)DNA gene fragments [416 bp of 16S rDNA(165), 566 bp of cytochrome *c* oxidase subunit I(COI) and 418 bp of NADH dehydrogenase subunit 1 (ND1)]; and the nuclear rDNA internal transcribed spacer (ITS) 1 (883–952 bp). We obtained a total of 79 sequences from 32 individuals. The 16S sequences of all *M. gregaria* and *M. subrugosa* were invariant and identical, whereas COI and ND1 showed 12 and 15 variable sites, respectively. These polymorphisms were shared between morphospecies. Interspecific Tamura–Nei distances for COI and ND1 sequences were 0.0024 and 0.0032, respectively, and were not significantly different from intraspecific distances (Kruskal–Wallis tests: $P = 0.58$ and $P = 0.69$, for COI and ND1, respectively). Similar to the results obtained from the mtDNA sequences, no relationship was found between the ITS1 maximum parsimony tree topology and the morphologic classification of specimens in *M. gregaria* and *M. subrugosa*. We conclude that *M. gregaria* and *M. subrugosa* from southern South America may either represent a case of a dimorphic species, or a case of incomplete lineage sorting. The fact that these two morphospecies did not show fixed differences over a total of 1947 bp analysed reinforces the hypothesis of a single dimorphic species. *Biological Journal of the Linnean Society*, 2008, **94**, 421–434.

ADDITIONAL KEYWORDS: 16S – COI – ITS1 – genetic variation – ND1 – speciation.

INTRODUCTION

Molecular genetic techniques have proved to be useful in the resolution of taxonomic problems of closely-related crustacean species (Gusmão, Lazoski & Solé-Cava, 2000; Daniels *et al.*, 2003; Machordom & Macpherson, 2004). Decapods are known to display low levels of enzyme polymorphisms (Nelson & Hedgecock, 1980; Hedgecock, Tracey & Nelson, 1982). Yet, the study of mitochondrial (mt)DNA sequence polymorphisms has been used in many decapods, and has provided important information for taxonomic studies and phylogenetic reconstructions of closely-

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related species (Schubart, Neigel & Felder, 2000; Pérez-Losada *et al.*, 2002; Macpherson & Machordom, 2005). However, as the mitochondrial genome represents a single linkage group, the information present in its components (genes) may be correlated and reflect a partial history of the species (Neigel & Avise, 1986). This problem can be overcome by studying multiple loci (e.g. organelle and nuclear genes; Pamilo & Nei, 1988; Nichols, 2001).

Sequences of the eukaryotic nuclear rDNA such as the two internal transcribed spacers, ITS1 and ITS2, have been used in phylogenetic reconstructions of crustaceans (Harris & Crandall, 2000; Chu, Li & Ho, 2001; Daniels, Hamer & Rogers, 2004) and other arthropods (Schlötterer *et al.*, 1994; Vogler & DeSalle, 1994). The level of divergence observed in crustacean ITS1 is appropriate for molecular systematic studies at the species level. However, because the rDNA array consists of several hundred tandemly repeated copies, the existence of polymorphisms among repeat units may cause extensive intragenomic variation. This phenomenon needs to be taken into consideration whenever concerted evolution is slower than speciation because a single genome will contain divergent paralogs (Vogler & DeSalle, 1994; Harris & Crandall, 2000; Chu *et al.*, 2001).

The systematics and phylogenetic history of the family Galatheidae has been under much revision lately (Macpherson & Machordom, 2001, Macpherson & Machordom, 2005; Machordom & Macpherson, 2004). A total of 122 unknown species of the genus *Munida* and related genera have been described for the West Pacific since 1988 (Machordom & Macpherson, 2004). The genus *Munida* is the most speciose within the galatheids, with approximately 210 described species around the world. The evolution of this group in the West Pacific has been marked by rapid speciation accompanied by stasis in morphological characters, or certain constraints in its morphological evolution, leading to the existence of very similar morphotypes that are genetically clearly divergent (Machordom & Macpherson, 2004).

Munida gregaria (Fabricius, 1793) and *Munida subrugosa* (Dana, 1852) occur in large numbers mainly off the coasts of New Zealand and South America (Tapella *et al.*, 2002). In South America, *M. gregaria* and *M. subrugosa* occur in Atlantic waters on the continental shelf from Uruguay (35°S) to Cape Horn (55°S), including the Malvinas (Falkland) Islands whereas, on the Pacific side, they occur up to the island of Chiloé (41°S, Chile) (Tapella *et al.*, 2002) (Fig. 1).

Galatheids are almost exclusively benthic as adults (Zeldis, 1985); however, pelagic aggregations of young stages of *Munida* spp. have been reported in New Zealand and South America (Matthews, 1932; Jillett & Zeldis, 1985). In New Zealand, pelagic shoals of *Munida* occur frequently, and are composed by specimens of *M. gregaria*, *M. subrugosa*, and intermediate forms between them (Williams, 1973). Off South America, particularly in the Beagle Channel, the occurrence of pelagic shoals of *Munida* spp. are occasionally reported by local fishermen, but no taxonomic identification of these pelagic forms has been ever performed. In the Beagle Channel, both morphospecies are almost exclusively benthic, and both young and adult individuals are captured in benthic samples (Tapella *et al.*, 2002).

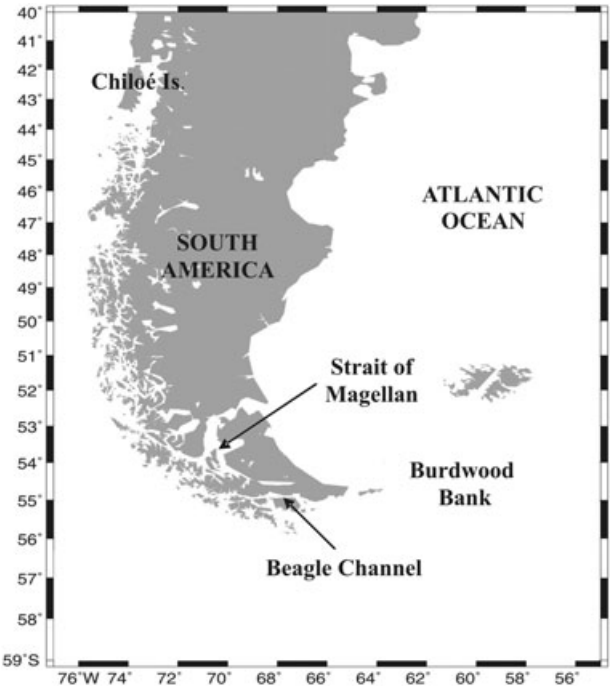

Figure 1. Study area.

Munida gregaria and *M. subrugosa* have been described on the basis of the following morphologic characters: shape of the carapace; shape, size, and direction of the rostral spine; the length and shape of the eyestalk and details of the junction of the cornea and the peduncle, and the form of the terminal segments of the endopodite of the third maxilliped (Lagerberg, 1906). However, the species status of these squat lobsters has long been discussed (Williams, 1973, 1980). Williams (1973) proposed that *M. gregaria* and *M. subrugosa* off Otago–New Zealand (45°S, 170°50′E) are the same species. In captivity and after three or four molts (Williams, 1980), pelagic animals gradually change their morphology accordingly to the acquisition of the benthic habit (Williams, 1973). The '*gregaria*' features were associated with a pelagic habit, and the '*subrugosa*' features with a benthic one (Williams, 1973). Yet, the method used by

Williams to classify specimens in *M. gregaria* and *M. subrugosa* is not clear, and appears to have relied on the type of habitat in which specimens were caught, rather than the use of taxonomically consistent characters, therefore rendering these results questionable.

Because of their large abundances in sub-Antarctic ecosystems, species of *Munida* are of potential commercial interest (Lovrich *et al.*, 1998). Their multiple uses in the food and aquaculture industries foresee their commercial exploitation (Lovrich *et al.*, 1998), hence being of crucial importance the determination of their taxonomic status for the application of proper management programmes, and the conservation of the resource (Tapella, 2002).

In the present study, we used DNA sequence information of three mitochondrial genes: 16S rDNA (16S), cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase subunit 1 (ND1); and a nuclear non-coding region, the internal transcribed spacer 1 (ITS1), to investigate whether the conspicuous morphologic differentiation between *M. gregaria* and *M. subrugosa* from South America is correlated with genetic divergence, which would validate their species status.

MATERIAL AND METHODS

Sample collection

Munida gregaria ($N = 20$) and *M. subrugosa* ($N = 20$) were collected with an epibenthic trawl in the Beagle Channel (55°S, 68°W) during April 2003. Samples of *M. subrugosa* ($N = 20$) from the Strait of Magellan (53°30′S, 70°W) were captured with baited traps. Specimens of *M. subrugosa* ($N = 10$) and *Munida spinosa* Henderson, 1885 ($N = 4$) from the Burdwood Bank (54°30′S, 55°W) were collected with Agassiz and epibenthic trawls during the LAMPOS 2002 cruise on board of the RV Polarstern (Fig. 1). Type localities for each morphospecies are: *M. gregaria* South Atlantic Ocean at latitude 37°30′S, and *M. subrugosa* Rendezvous Cove, Auckland Islands (50°42′S 166°20′E) (Haig, 1955).

Animals were fixed in EtOH 96% and kept at the Crustacean Biology Laboratory Collection for further morphologic analysis and DNA extraction.

Classification of specimens

All *M. gregaria* and *M. subrugosa* were classified using taxonomically important characters (Lagerberg, 1906), by means of two different methodologies. On the one hand, species determination was performed visually on the basis of rostral and eyestalk morphology, and carapace shape. The rostrum has a wider basis and the ocular peduncles are longer in *M. gregaria*. The lateral margins of the carapace converge to meet its anterior edge at an obtuse angle, the cornea has a kidney shape, and the terminal segment of the endopodite of the third maxilliped is more slender in *M. subrugosa* (Fig. 2A, B).

On the other hand, the discriminant function (DF) analysis developed by Tapella & Lovrich (2006) was applied. The following morphometric measures were taken with a digital calliper to the nearest 0.01 mm: carapace length, anterior carapace width (ACW), rostral basis width (RBW), widths of dactylopodite (DaW) and propodite (PW) of the third maxilliped, and eyestalk length (EL) (Fig. 2C).

$$DF(1) = 2.080\ ACW - 8.487\ RBW - 9.494\ DaW - 12.201\ PW + 23.694\ EL - 28.697$$

$$DF(2) = 0.175\ ACW - 5.299\ RBW - 0.282\ DaW - 12.282\ PW + 26.421\ EL - 38.470$$

If $DF(1) - DF(2) > 0$, the specimen belongs to *M. subrugosa*; if $DF(1) - DF(2) < 0$, the specimen belongs to *M. gregaria*.

DNA extraction, amplification, and sequencing

DNA was extracted from gills dissected from the specimens preserved in EtOH 96%. The tissue was digested in 600 μL of 2% cetyltrimethylammonium bromide (CTAB) lysis buffer (Corach, 1991) with 5 μL of proteinase K (10 mg mL^{-1}), and incubated at 60 °C overnight. DNA was extracted using the standard phenol/chloroform–isoamylalcohol protocol (Sambrook, Fritsch & Maniatis, 1989).

Fragments of the three mitochondrial genes 16S, COI and ND1 with different mutation rates (from slower to faster; Saccone *et al.*, 1999) were applied as they differ in their level of resolution. Although the 16S rDNA and COI genes are generally applied to study species phylogenies (Macpherson & Machordom, 2001, Macpherson & Machordom, 2005; Machordom & Macpherson, 2004), the polymorphisms in ND1 (and sometimes COI) are applied for phylogeographical or population inference (Zane *et al.*, 2000; Jarman *et al.*, 2002). We used the primers 16Sar and 16Sbr (Cunningham, Blackstone & Buss, 1992), LCO1494 and HCO2198 (Folmer *et al.*, 1994), ND1af and ND1ar (Zane *et al.*, 2000), and ND1af-P (5′-CGG TTG ATC TTC AAA TTG TAA-3′) and ND1ar-P (5′-AAG CTT ATC ATA TCG TAA ACG A-3′) specifically designed for our study. The nuclear ITS1 was amplified using the primers SP-1-5′138 and SP-1-3′ (Chu *et al.*, 2001).

Polymerase chain reaction (PCR) reactions were performed using a Geneamp PCR System 2700 (Applied Biosystems) in 10-μL reactions consisting of 1 μL of DNA, 0.2 mM of each dNTP, 2 mM (16S, COI and ITS1) or 3 mM (ND1) $MgCl_2$, 0.15 μM of each

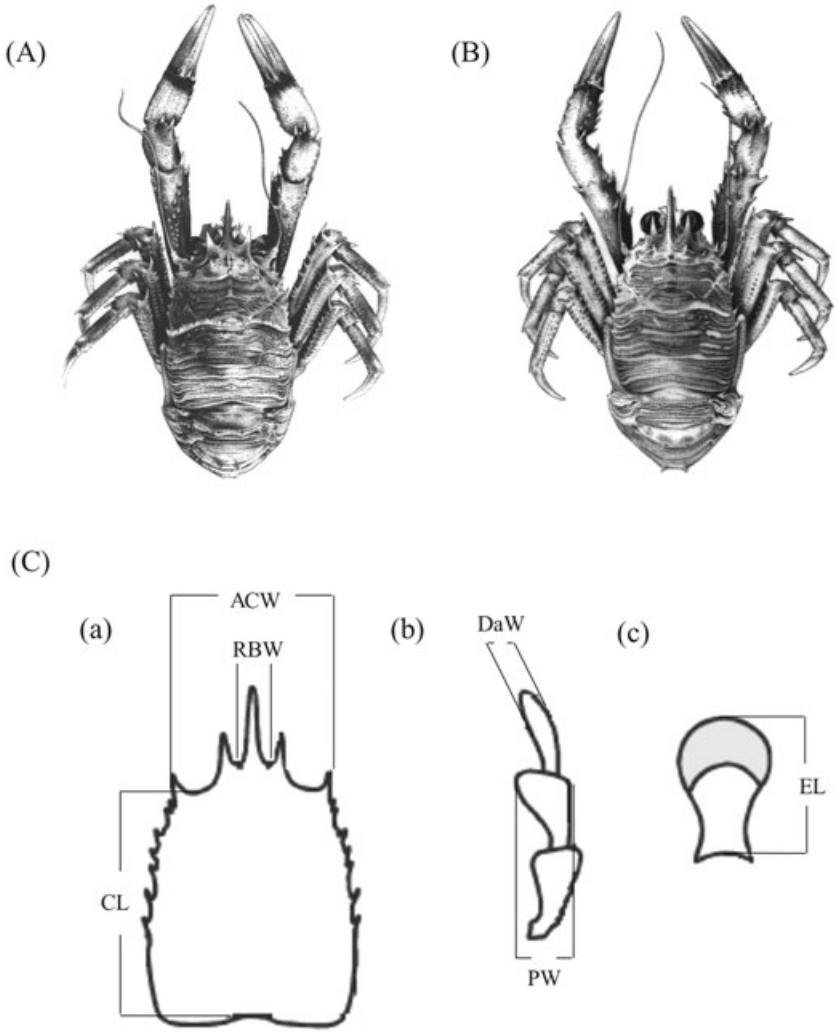

Figure 2. A, *Munida gregaria*. B, *Munida subrugosa*. C, measurements made on the (a) carapace (b) endopodite of the third maxilliped and (c) eyestalk (modified from Tapella & Lovrich, 2006). CL, carapace length; ACW, anterior carapace width; RBW, rostral basis width; DaW, width of the dactylopodite of the third maxilliped; PW, width of the propodite of the third maxilliped; EL, eyestalk length.

primer, 0.25 U of Bioline BioTaq, the corresponding buffer and ddH_2O. Thermal cycling conditions consisted of an initial denaturation step of 94 °C for 3 min followed by 35 cycles at 94 °C for 30 s, 50 °C (16S and COI), 40 °C (ND1af and ND1ar), 55 °C (ND1af-P and ND1ar-P), or 60–65 °C (ITS1) for 50 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min.

To investigate the presence of internal genomic variation, ITS1 PCR products were cloned and sequence information was obtained from seven individuals. Before cloning, ITS1 amplification products were purified from 1% agarose gels by using the QIAquick Gel Extraction kit (Qiagen), according to the manufacturer's recommendations. DNA concentration and purity was assessed from A_{260}/A_{280} and A_{260}/A_{230} ratios (Johnson, 1994). Purified products were cloned using the pGEM-T Easy Vector System II (Promega) according to the manufacturer's recommendations. Ten colonies from each sample containing the vector with the cloned PCR product were selected, re-streaked out on Luria–Bertani plates and used for insert verification. Plasmid extraction from transformed colonies using the alkaline method according to Sambrook *et al.* (1989) rendered the expected plasmid-insert size for all cases. Verified colonies were used for additional PCR amplification using the original ITS1 primers.

All products were visualized under ultraviolet light in 1% agarose gels stained with ethidium bromide, with a comigrating 100-bp ladder molecular-weight marker to confirm the correct amplification. Amplifi-

Table 1. Mean intraspecific Tamura–Nei distances (*d*) and standard error (SE)

	16S		CO *I*		ND1	
	d	SE	*d*	SE	*d*	SE
MGB	0	0	0.0022	<0.0012	0.0021	<0.0012
MSB	0	0	0.0036	<0.0018	0.0024	<0.0016
MSM	0	0	0.0027	<0.0014	0.0037	<0.0022
MSBUR	0	0	0.0018	<0.0012	0.0053	<0.0019
MSALL	0	0	0.0027	<0.0009	0.0043	<0.0013
MSPI	0	0	0.0048	<0.024	(–)	(–)

COI, cytochrome *c* oxidase subunit I; ND1, NADH dehydrogenase subunit 1; MGB, *Munida gregaria* from the Beagle Channel; MSB, *Munida subrugosa* from the Beagle Channel; MSM, *M. subrugosa* from the Strait of Magellan; MSBUR, *M. subrugosa* from the Burdwood Bank; MSALL, *Munida subrugosa* from all three locations (Beagle Channel, Strait of Magellan, and Burdwood Bank) pooled together; MSPI, *Munida spinosa*; –, no amplification product obtained.

cation products were cycle-sequenced using the Big Dye Terminator v3 following the manufacturer's recommendations (Applied Biosystems). ITS1 was sequenced using the original forward and reverse ITS1 primers. Reactions were analysed on ABI Prism 3100/3110 Genetic Analysers (Applied Biosystems).

Data analysis

Nucleotide sequences were aligned using BioEdit, version 5.0.9 (Hall, 1999), and then manually adjusted by eye.

Mitochondrial DNA sequence analysis

Pairwise Tamura & Nei (1993) (TN93) phylogenetic distances were calculated in MEGA, version 3.1 (Kumar, Tamura & Nei, 2004). Phylogenetic relationships among haplotypes were reconstructed as median-joining networks using Network 4.2.0.1 (Bandelt, Forster & Röhl, 1999). Nucleotide (Nei, 1987) and haplotype diversities were calculated using DNAsp, version 4.00.5 (Rozas *et al.*, 2003). As implemented in DNAsp, version 4.00.5 (Rozas *et al.*, 2003), departures from neutral expectations were investigated applying the F_s test of Fu (1997), which detects excess of rare alleles or young mutations. This test is more powerful than other neutrality tests in detecting population growth and background selection (Fu, 1997). Negative values indicate excess of recent mutations. Partition of genetic variances within and among the three sampling sites, and the F_{ST} analogs of Excoffier, Smouse & Quattro (1992) were calculated with analysis of molecular variance (AMOVA) for COI and ND1 haplotype frequencies using Arlequin, version 3.0 (Excoffier, Laval & Schneider, 2005). Significance of these statistics was estimated with 1000 replications. A hierarchical analysis was also performed pooling the continental samples (Magellan Strait and Beagle Channel) together.

Nuclear rDNA sequence information

Percentage sequence divergence within, and between individuals and morphospecies was calculated handling gaps and missing data with the pairwise deletion option in MEGA, version 3.1 (Kumar *et al.*, 2004). To reconstruct hypothesis of phylogenetic relations between sequences, indels were coded according to the 'simple indel coding' procedure (Simmons & Ochoterena, 2000) using SeqState software (Müller, 2005). The phylogenetic reconstruction was performed using maximum parsimony with PAUP, version 4.0 (Swofford, 1998). Trees were generated using the branch and bound option. Confidence in resulting nodes was evaluated by performing 100 bootstrap replicates.

RESULTS

We obtained a total of 79 squat lobster sequences: 19 sequences of 16S (417 bp), 20 of COI (566 bp), 24 of ND1 (498 bp), and 12 complete (883–952 bp) and four partial (669–762 bp) sequences of ITS1 (GenBank accession numbers: AY700158-AY700162 for 16S, AY700163-AY700179 for COI, AY704638-AY704649 for ND1, and EF653432-EF653447 for ITS1).

Mitochondrial DNA sequence information

The 16S sequences of all *M. gregaria* and *M. subrugosa* individuals of this study were identical (Table 1) and differed in one transition from the

M. subrugosa sequence from Chiloé, Chile (Pérez-Losada *et al.*, 2002) (GenBank AF439382). *Munida spinosa* sequences were identical to each other, but differed from those of *M. gregaria* and *M. subrugosa* by a TN93 distance of 0.1053.

The 17 COI sequences of *M. gregaria* and *M. subrugosa* showed a total of 11 different haplotypes, determined by 12 variable (segregating) sites and no parsimony-informative sites (Table 2). The most common haplotype was shared by the two morphospecies, and differed in one or two substitutions from the other ten haplotypes, two of which were *M. gregaria* and eight of *M. subrugosa* (Fig. 3A). Haplotype diversities for *M. gregaria* and *M. subrugosa* were 0.7 ± 0.2 and 0.91 ± 0.08, respectively. Nucleotide diversities for *M. gregaria* and *M. subrugosa* were 0.0021 ± 0.0009 and 0.0027 ± 0.0005, respectively. The segregating sites at positions 343 and 535 (Table 2) determined amino acid changes. The mean interspecific TN93 distance between *M. gregaria* and *M. subrugosa* for COI was 0.0024, and was not significantly different from the mean intraspecific distance (Kruskal–Wallis test: $P = 0.58$) (Table 1). The mean TN93 interspecific distances between *M. spinosa* and *M. gregaria* and *M. spinosa* and *M. subrugosa* were 0.2225 and 0.2215, respectively, two orders of magnitude higher than the distance between *M. gregaria* and *M. subrugosa*.

The total of 24 ND1 sequences of *M. gregaria* and *M. subrugosa* showed ten different haplotypes, determined by 15 variable (segregating) sites, one of which was parsimony informative and was shared by two *M. subrugosa* specimens from the Burdwood Bank (at position 293) (Table 3). The most common haplotype was present in both morphospecies (Fig. 3B). Of the other nine haplotypes, three belonged to *M. gregaria* and six to *M. subrugosa* (Table 3). Haplotype diversities for *M. gregaria* and *M. subrugosa* were of 0.7 ± 0.2 and 0.57 ± 0.14, respectively. Nucleotide diversities for *M. gregaria* and *M. subrugosa* were of 0.0021 ± 0.0007 and 0.004 ± 0.002, respectively. Three of the variable sites (at positions 66, 100, and 285) determined a change of amino acid. The mean interspecific distance between haplotypes was 0.0032, and not significantly different from the mean intraspecific distance (Kruskal–Wallis test: $P = 0.69$) (Table 1). No amplification products were obtained for *Munida spinosa* samples.

Median-joining networks for both COI and ND1 (Fig. 3) showed no correspondence between COI and ND1 sequences and taxonomy. In both cases, the haplotype with the highest frequency, displayed in the centre of the network, was present in the two morphological types (Fig. 3A, B). Some correspondence with geographical location can be seen in Figure 3B (ND1), where two *M. subrugosa* specimens from the Burdwood Bank apparently suggest the existence of a separate lineage in this location. MSBUR53 showed eight nucleotide substitutions compared with the most frequent haplotype, only one of which implied an amino acidic change from Thr to Ser, both hydrophylic.

Given the apparent gene flow between the two morphospecies, the partition of variation among sampling sites was investigated without distinction of phenotype. Thus, AMOVA was applied to the overall COI and ND1 datasets separately. A regional analysis between the continental samples (Magellan Strait and Beagle Channel) and the shelf sample (Burdwood Bank) was also investigated using a hierarchical approach. The results, summarized in Table 4, indicate that there is no geographical structure among the samples or regions, as most of the variance is contained within samples.

Fu's F_s test was highly significant for COI ($F_s = -9.407$, $P < 0.001$) and ND1 ($F_s = -5.418$, $P < 0.001$) sequences. The departures from neutrality expectation may indicate the effect of selection and/or historical demographic processes such as population growth.

Nuclear rDNA sequence information

Three clones of each of two individuals of *Munida gregaria* from the Beagle Channel, three clones of two *M. subrugosa* from the Beagle Channel, two clones of a *M. subrugosa* from the Strait of Magellan and three clones of each of two *M. subrugosa* from the Burdwood Bank, adding a total of 16 ITS1 sequences for seven individuals, were recovered after cloning. The length of the ITS1 amplification products in different clones was between 883 and 952 bp.

Similarly to the difficulties that Chu *et al.* (2001) experienced in sequencing the ITS1 region of *Panulirus japonicus* from the 3′ (5.8S) end, we had difficulties in obtaining the complete sequence on the proximity of the 5.8S region. Due to this difficulty, further analysis was based on approximately 600 bp (547–599 bp) from the 5′ (18S) end of ITS1. These partial sequences were used for the phylogenetic analysis. Aligned sequences revealed the existence of indels among clones of the same individual, as well as indels between ITS1 sequences of different individuals. Of the 624 positions aligned in the data matrix, nine variable characters were parsimony-uninformative and two were parsimony-informative. Nine regions of the alignment presented indels, representing between 25 and 75 bp missing in the total alignment. Percentage sequence divergence (calculated handling indels with the pairwise deletion option) between morphospecies (0.4%) was similar to percentage sequence divergence within individuals

Table 2. Cytochrome *c* oxidase subunit I (COI) haplotypes and variable (segregating) sites

Code	51	93	117	129	285	300	309	342	343	354	477	535	MGB	MSB	MSM	MSBUR
MGB2	A	A	T	T	A	T	A	A	G	A	T	C	3/5	1/4	1/4	2/4
MGB8	–	–	–	–	–	–	–	–	–	–	–	T	1/5	0	0	0
MGB9	–	–	C	–	–	C	–	–	–	–	–	–	1/5	0	0	0
MSB2	G	G	–	–	–	–	–	–	–	–	–	–	0	1/4	0	0
MSB5	–	–	–	–	–	–	–	–	–	–	C	–	0	1/4	0	0
MSB8	–	–	–	–	–	–	T	–	–	–	–	–	0	1/4	0	0
MSM33	–	–	–	–	–	–	–	T	–	–	–	–	0	0	1/4	0
MSM34	–	–	–	C	–	–	–	–	–	–	–	–	0	0	1/4	0
MSM39	–	–	–	–	G	–	–	–	–	–	–	–	0	0	1/4	0
MSBUR52	–	–	–	–	–	–	–	–	–	G	–	–	0	0	0	1/4
MSBUR61	–	–	–	–	–	–	–	–	A	–	–	–	0	0	0	1/4

Numbers indicate nucleotide positions. Dashes indicate identity with the first sequence. Last four columns indicate frequency of each haplotype in each population.

MGB, *Munida gregaria* from the Beagle Channel; MSB, *Munida subrugosa* from the Beagle Channel; MSM, *M. subrugosa* from the Strait of Magellan; MSBUR, *M. subrugosa* from Burdwood Bank.

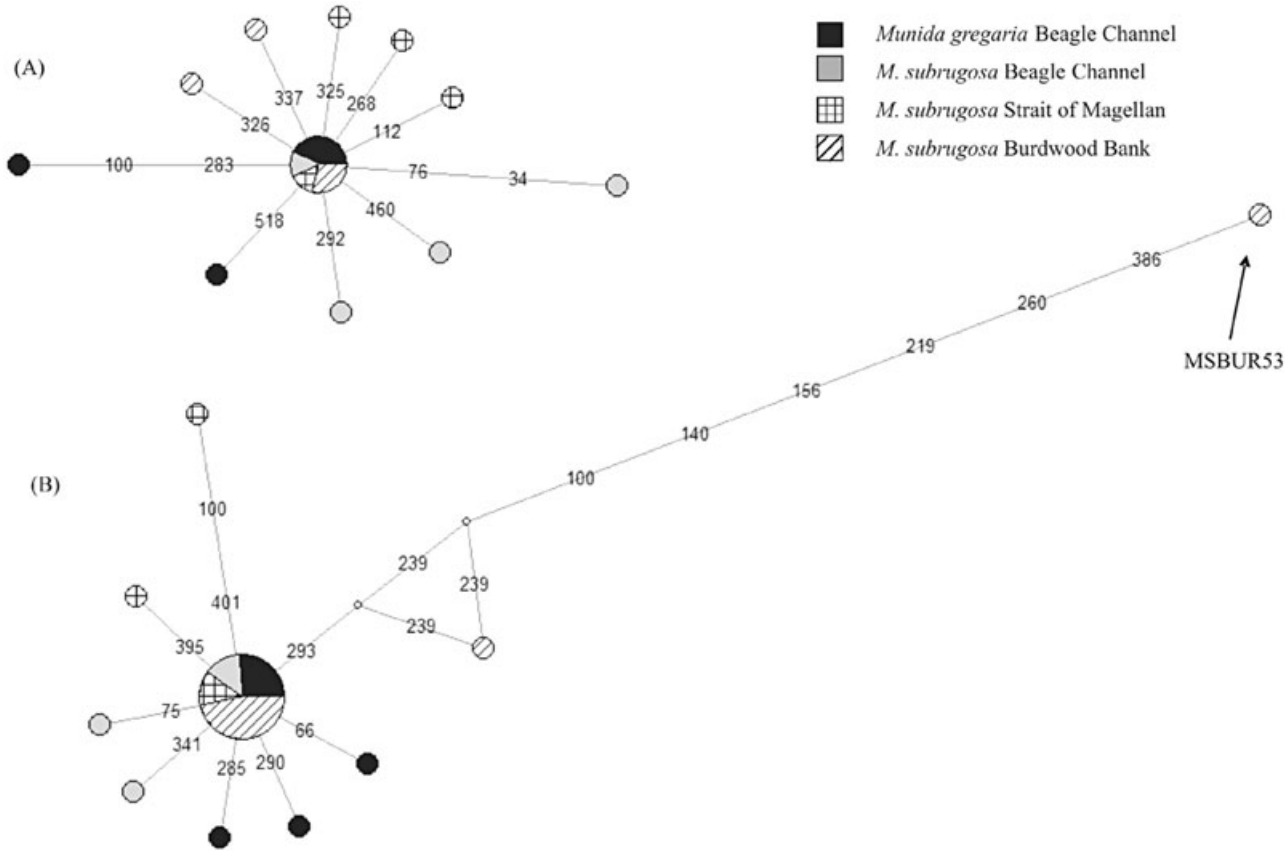

Figure 3. Median-joining networks for: (A) cytochrome *c* oxidase subunit I (COI) and (B) NADH dehydrogenase subunit 1 (ND1) sequences of *Munida gregaria* and *Munida subrugosa*. Circles represent different haplotypes, with their area proportional to their frequency, except for small white circles which represent median vectors (haplotypes not found in our samples). Numbers correspond to mutated positions. MSBUR, *M. subrugosa* from Burdwood Bank.

(between 0.2% and 0.6%) or among individuals within morphospecies (0.2% between individuals of *M. subrugosa* and 0.5% between individuals of *M. gregaria*).

Taking indels into account, no two sequences were identical. Most indels appeared to be a result of single insertion/deletion events because they consist of a variable number of 3 or 4 bp repeats identical to those upstream. After coding indels, the aligned matrix contained 651 characters, 613 constant and 38 variable of which 17 were parsimony-informative. The parsimony analysis recovered a single tree length of 50 steps. An unrooted strict consensus tree of the 224 equally parsimonious trees found is shown in Figure 4. All possible rootings of this tree showed that there are certain clones that belong to different morphospecies that are more related to each other than to clones of their same morphospecies. Similar to the results obtained from the mtDNA sequences, no relationship was found between the ITS1 tree topology and the morphologic classification of specimens in *M. gregaria* and *M. subrugosa*.

DISCUSSION

Molecular markers and taxonomic status

The molecular genetic analyses carried out in the present study revealed no genetic differences to validate the phenotypic differentiation of individuals of *M. gregaria* and *M. subrugosa* and the recognition of two different species. Neither the mitochondrial, nor the nuclear markers employed in the present study revealed differences between the two morphospecies. Yet, two of these markers were useful to separate *M. gregaria* and *M. subrugosa* from the sympatric *M. spinosa*.

In recent years, the use of molecular markers in the identification of species has yielded two different scenarios. These are: (1) a correspondence between morphologic and molecular data (Gusmão *et al.*, 2000; Macpherson & Machordom, 2001; Pérez-Losada *et al.*, 2002; Spivak & Schubart, 2003), probably due to subtle morphological differences being found once the genetically differentiated forms are recognized

Table 3. NADH dehydrogenase subunit 1 (ND1) haplotypes and variable (segregating) sites

Code	66	75	100	140	156	219	239	260	285	290	293	341	386	395	401	MGB	MSB	MSM	MSBUR
MGB2	C	G	C	T	T	T	A	T	G	T	T	T	T	A	T	1/7	0	0	0
MGB3	T	–	–	–	–	–	–	–	A	–	–	–	–	–	–	1/7	0	0	0
MGB4	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4/7	2/4	2/4	7/9
MGB6	T	–	–	–	–	–	–	–	–	C	–	–	–	–	–	1/7	0	0	0
MSB6	T	–	–	–	–	–	–	–	–		–	C	–	–	–	0	1/4	0	0
MSB8	T	A	–	–	–	–	–	–		–	–	–	–	–	–	0	1/4	0	0
MSM33	T	–	T	–	–	–	–	–	–	–	–	–	–	–	C	0	0	1/4	0
MSM39	T	–	–	–	–	–	–	–	–	–	–	–	–	G	–	0	0	1/4	0
MSBUR53	T	–	G	C	C	C	G	C	–	–	C	–	C	–	–	0	0	0	1/9
MSBUR60	T	–	–	–	–	–	T	–	–	–	C	–	–	–	–	0	0	0	1/9

Numbers indicate nucleotide positions. Dashes indicate identity with the first sequence. Last four columns indicate frequency of each haplotype in each population.
MGB, *Munida gregaria* from the Beagle Channel; MSB, *Munida subrugosa* from the Beagle Channel; MSM, *M. subrugosa* from the Strait of Magellan; MSBUR, *M. subrugosa* from Burdwood Bank.

Table 4. Analysis of molecular variance (AMOVA) table for the evaluation of genetic differences between sampling sites using haplotypes defined by cytochrome *c* oxidase subunit I(COI) and NADH dehydrogenase subunit 1 (ND1) sequence information

Mitochondrial DNA fragment	AMOVA comparison	Variance component	% of total variance	ϕ-statistics	*P*
COI	Three populations	Among populations	2.9	0.0289	0.26
		Within populations	97.1		
	Two groups	Among groups	–14.84	$\phi_{CT} = -0.14$	0.26
		Among populations within groups	12.55	$\phi_{SC} = 0.1$	1
		Within populations	102.29	$\phi_{ST} = -0.0022$	0.35
ND1	Three populations	Among populations	101.13	–0.011	0.45
		Within populations	–1.13		
	Two groups	Among groups	3.24	$\phi_{CT} = 0.032$	0.33
		Among populations within groups	– 3.57	$\phi_{SC} = -0.037$	1
		Within populations	100.33	$\phi_{ST} = -0.0032$	0.52

A hierarchical analysis was performed grouping Magellan Strait and Beagle Channel populations to compare this group with the Burdwood Bank population. Significance of the Φ-statistics was calculated with 1000 permutations.

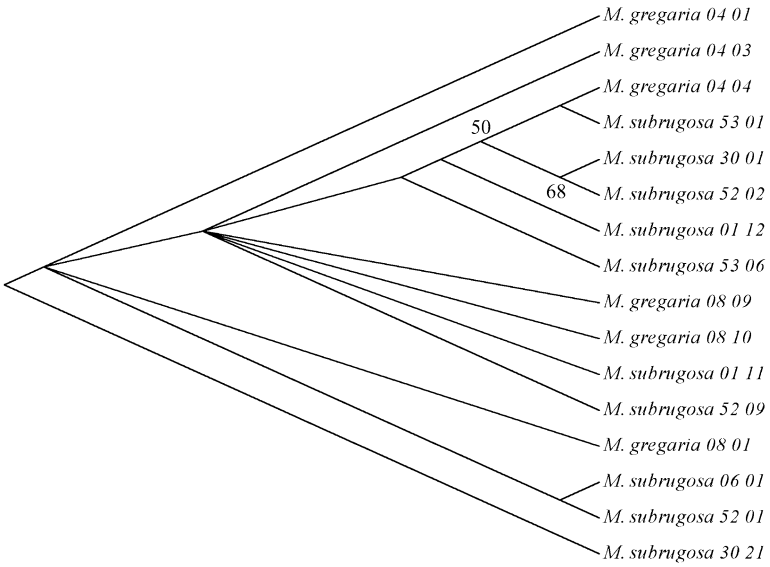

Figure 4. Unrooted strict consensus of 224 equally parsimonious trees (tree length = 50) generated from 16 internal transcribed spacer 1 (ITS1) clones of *Munida gregaria* and *Munida subrugosa*. Indels in the alignment were coded after the 'simple indel coding' procedure (Simmons & Ochoterena, 2000). Values at nodes represent bootstrap supports (> 50%).

(Sarver *et al.*, 1998), and (2) the results from molecular markers being in conflict with the morphological classification. For example, *Callinectes bocourti* A. Milne Edwards, 1879 and *Callinectes maracaiboensis* Taissoun, 1969 have been separated on the basis of minor features of coloration and structural characteristics, but have been found not to differ in their 16S mtDNA sequences (Schubart *et al.*, 2001). Other species, such as *Brachynotus gemmellari* (Rizza, 1839) and *Brachynotus sexdentatus* (Risso, 1827), have identical 16S rDNA sequences, suggesting a recent separation or continuing gene flow (Schubart, Cuesta & Rodríguez, 2001).

Mitochondrial molecular markers such as 16S and COI have been successfully used to reconstruct phylogenetic relations of closely-related species of *Munida*, even revealing the existence of three new cryptic species within the genus (Machordom & Macpherson, 2004; Macpherson & Machordom, 2005). The minimum interspecific divergences reported by Machordom & Macpherson (2004) for 16S and COI were 0.4% and 3.5%, respectively; values clearly

larger than the ones reported for *M. gregaria* and *M. subrugosa* in the present study (0% and 0.24% for 16S and COI, respectively). Furthermore, the molecular markers used in the present study have also proven to be successful in discerning between close species of other decapod genera closely related to *Munida*. For example, the use of COI sequences confirmed the morphologic classification of the species of *Raymunida* (Anomura: Galatheidae) and agreed with its differentiation from the genera *Munida* and *Agononida* (Anomura: Galatheidae) (Macpherson & Machordom, 2001). Likewise, a combined analysis of 12S, 16S, COI and COII sequences of species of *Aegla* (Anomura: Aeglidae) provided strong support for the monophyly of most species of currently recognized Chilean Aeglidae (Pérez-Losada *et al.*, 2002). Other phylogenetic studies of decapods less related to *Munida* such as *Potamonautes* (Brachyura: Potamonautidae) (Daniels *et al.*, 2003), *Panulirus* (Palinura: Palinuridae) (Sarver *et al.*, 1998), *Alpheus* (Caridea: Alpheidae) (Mathews *et al.*, 2002), and *Penaeus* (Dendrobranchiata: Penaeidae) (Gusmão *et al.*, 2000), made successful application of 16S and COI sequences for species identification.

Similarly, the nuclear rDNA ITS1 gene region was informative at resolving phylogenetic relationships between closely-related species of *Streptocephalus* (Branchiopoda: Streptocephalidae) (Daniels *et al.*, 2004), *Orconectes* (Harris & Crandall, 2000), and *Eriocheir* (Chu *et al.*, 2001), notwithstanding the existence of intragenomic variability in the two last cases.

Difficulties in sequencing ITS1 due to the presence of more than one template as a result of intragenomic variation have already been reported for other crustacean species (Harris & Crandall, 2000; Chu *et al.*, 2001). In the present study, such difficulty was due to the existence of insertions/deletions among the multiple copies of ITS1 present in a single genome. Percentage sequence divergence revealed that the within-individual sequence divergence found in *M. gregaria* and *M. subrugosa* is similar to that reported for crayfishes (0–0.5%) (Harris & Crandall, 2000), but lower than that found among clones of *Eriocheir formosa* (0.9–2.3%) (Chu *et al.*, 2001). Although it is generally assumed that concerted evolution homogenizes rDNA repeats, the presence of multiple ITS1 copies in a single genome can be maintained for periods of time sufficiently long to enable the differentiation of distinct subspecies (Vogler & DeSalle, 1994). In the present study, ITS1 sequences could not resolve the different morphspecies status of *M. gregaria* and *M. subrugosa*, suggesting the existence of gene flow among them, or incomplete lineage sorting of ancestral alleles.

Ecological controversy

Although Williams (1973) argued for the existence of an ontogenetic transformation from the 'gregaria' (pelagic) to the 'subrugosa' (benthic) morph, several ecological evidences in the Beagle Channel challenge her views arguing against such ontogenetic transformation. First, diagnostic characters at the megalopa larval stage allow for morphospecies identification in South America (Bacardit, 1986). Second, small benthic specimens of *M. subrugosa* and large benthic specimens of *M. gregaria* were found in the Beagle Channel, Argentina (Tapella, 2002). These facts contradict the prediction that arises from Williams' (1973, Williams, 1980) hypothesis, according to which small animals are pelagic with 'gregaria' morphology, whereas large ones are benthic with 'subrugosa' morphology. In addition, the low proportion of intermediate phenotypes found in the Beagle Channel (Tapella, 2002) cannot account for such morphological transition, and it would also count against an extensive interspecific hybridization, unless the hybrids retain the phenotype of one parental species.

Demographic and historical perspectives

The fact that the only sequence from a geographically more distant area (coast of Chile), rendered genetic differences in two genes (16S, and nuclear rDNA 18S; results not shown) that were otherwise invariant in the present dataset, suggests that genetic divergence is rather correlated to a geographical than a morphologic component. In spite of the apparent connection between South America and New Zealand through the West Wind Drift, there are no records of the occurrence of either morphospecies in between these two areas (e.g. Tristan da Cunha archipelago, Prince Edward, Bouvet and Kerguelen Islands). Therefore, we suggest that populations of *M. gregaria* and *M. subrugosa* from both areas could have undergone genetic differentiation due to the low feasibility of gene flow between these two geographically very distant areas.

The shallow star-like shape of COI and ND1 networks, the departure from neutrality expectations, and the high haplotype diversity accompanied by low nucleotide diversity are compatible either with a recent population expansion, high variance in female reproductive success, and/or selection (Avise, Neigel & Arnold, 1984; Hedgecock, 1994). Selective sweeps in unstable environments would be responsible for crashes and expansions, which were repeatedly reported for marine species (D'Amato & Carvalho, 2005). The Strait of Magellan, the Beagle Channel, and other parts of Tierra del Fuego were covered by glaciers during the Plio-Pleistocene glaciations which started around 3.5 Mya. At least five glacial advances

have been described for the Magellan Strait glaciar, from 1 Mya until the present (Rabassa *et al.*, 2000). The mean surface sea temperatures diminished significantly during the glaciations, lowering at least 5–6 °C near the polar zones (Rabassa, Coronato & Salemme, 2005). It has been proposed that, during quaternary glaciations, marine faunas would have retreated into refugia, either unglaciated parts of the continental shelf, or the deep sea (Gordillo, Coronato & Rabassa, 2005). We hypothesize that the distribution of the temperate marine fauna was restricted to lower latitudes than those at which they are found at present. Therefore, once sea water temperature started warming, a population expansion and recolonization probably occurred. This hypothesis would need to be tested by extending the analysis to other more variable nuclear markers. A demographic event like population growth would leave its signature in other loci, whereas selection would not necessarily affect all (Rand, 1996).

The presence of an apparently second ND1 lineage in the Burdwood Bank opens the possibility of isolation followed by range expansion and secondary contact. This hypothesis should be tested by studying the whole geographical range of this species, especially potential glacial refugia, such as the Atlantic continental shelf off southern South America (Burdwood Bank included), that have remained isolated during the last glacial period (cf. Thatje, Hillenbrand & Larter, 2005).

Conclusions

Taken together, our results do not support the recognition of the South American *M. gregaria* and *M. subrugosa* as separate species. *Munida gregaria* and *M. subrugosa* from southern South America may either be two morphs of a single species, or a case of incomplete lineage sorting. The fact that these two morphospecies cannot be consistently separated by even a single nucleotide position out of 1947 bp, including the variable COI, ND1, and ITS1 regions, reinforces the hypothesis of a single dimorphic species. This possibility could be investigated extending our study to the New Zealand species/forms. We predict that, if the single dimorphic species hypothesis holds, the genetic distances between continents (South America versus New Zealand) should be larger than within continents.

Finally, in contrast to the rapid speciation and phenotypic stasis proposed by Machordom & Macpherson (2004) to explain the evolution of this genus, the existence of phenotypically divergent, but genetically indistinguishable forms within this/these species may indicate that phenotypic stasis is not as widely extended in South American species as it is in its congeners from New Caledonia, Fiji, and Salomon Islands.

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