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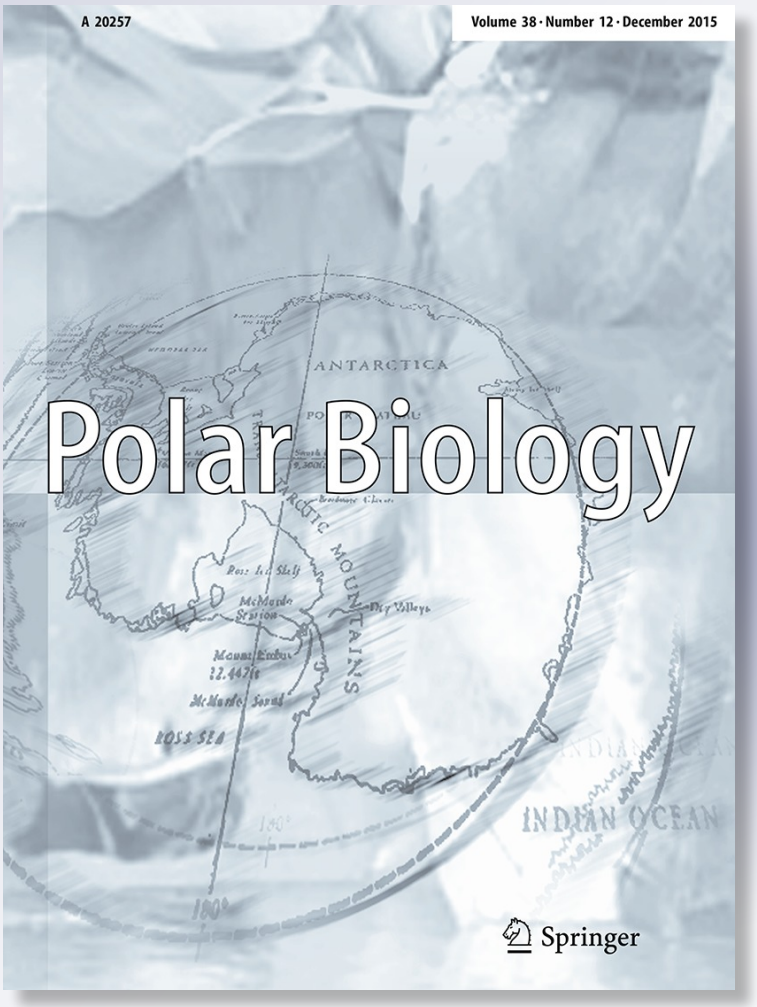

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ORIGINAL PAPER

Incongruence between molecular and morphological characters in the southern king crabs *Lithodes santolla* and *Lithodes confundens* (Decapoda: Anomura)

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Abstract The use of genetic tools has a relevant role in fishery resource management and conservation, for example, when used in species delimitation. Morphological variation can occur as an adaptative or plastic response to environmental variation, and therefore, be mistakenly used as a criterion to delimit species. Particularly, *Lithodes santolla* and *Lihodes confundens*, two commercially important lithodid species from sub-Antarctic South America, are mainly differentiated by the number and size of spines on the carapace and pereiopods. However, variability in the size of spines of *L. santolla* has been reported. We evaluated whether these two morphospecies constitute reciprocally monophyletic clades at the molecular level using mitochondrial markers, and performed a detailed morphological analysis of the carapace to search for correspondence between genetic and morphological differences. The Bayesian phylogenetic reconstruction showed that *L. santolla* and *L. confundens* belong to two sister clades. However, individuals identified as *L. santolla* and *L. confundens* did not resolve as reciprocally monophyletic groups. Instead, one clade was formed by individuals belonging to both morphospecies and was widely distributed, while the other one was exclusively formed by *L. santolla*, and its members were only found near Puerto Montt and in the Beagle Channel. No morphological characters were found on the carapace that could differentiate individuals belonging to each genetic clade. Either if the two genetic clades constitute species or remnants of two species that are merging through introgression, they represent two evolutionary significant lineages, and measures should be taken to preserve both. Our study suggests the need to revise the use of the number of spines as a relevant taxonomic character in the taxonomy of *Lithodes*, and to implement molecular genetic methods to control fisheries.

Keywords 16S · Burdwood Bank · Centolla · COI · Lithodidae · Species delimitation

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Introduction

The family Lithodidae Samouelle, 1819 includes ten genera and 122 species (McLaughlin 2014) of crab-like anomurans. Particularly, the genus *Lithodes* Latrielle, 1806 includes 29 species (McLaughlin 2014), three of which are distributed around the southern tip of South America: *Lithodes santolla* (Molina, 1782), *Lithodes turkayi* Macpherson 1988 and *Lithodes confundens* Macpherson

1988 (Macpherson 1988; Lovrich et al. 2002; Pérez-Barros et al. 2004; Boschi and Gavio 2005). *Lithodes confundens* was described by Macpherson (1988) on the basis of only ten specimens collected in the Burdwood Bank, south of Islas Malvinas, and the Straits of Magellan. However, because of its close resemblance to *L. santolla*, as well as their overlapping distribution (Hall 2010), both species have most likely been confused for a long time, principally in early prospective surveys and in fishery landing statistics (Lovrich et al. 2002). Previously, recorded specimens were collectively classified as the junior synonym of *L. santolla: Lithodes antarcticus.*

Lithodes santolla and *L. confundens* are mainly differentiated by the spines on the carapace and pereiopods, "which are always smaller and much more numerous in *L. confundens*" (Macpherson 1988). However, Macpherson (1988) recognized the existence of considerable variability in the size of the spines present in *L. santolla.* Vinuesa (1985) reported morphological variability among *L. santolla* caught in two different fishing grounds, i.e., Golfo San Jorge (ca. 46°S 65°W) versus Beagle Channel (ca. 55°S 67°W). Lovrich et al. (2002) further reported that individuals of *L. santolla* from the Golfo San Jorge were similar to *L. confundens* in that they had "pink coloration, shorter spines, and gastric and cardiac regions of the carapace flatter than those [*L. santolla*] from the Beagle Channel".

The geographic distribution of *L. confundens* and *L. santolla* only overlaps off the Atlantic coast between 38 and 40°S, near the continental slope (Lovrich et al. 2002; Sotelano et al. 2013). In the Golfo San Jorge, Beagle Channel and Staten Island the only occurring species is *L. santolla* whereas *L. confundens* distributes near the coast around the eastern entrance of the Straits of Magellan between 50 and 54°S, and offshore, i.e., in the Burdwood Bank and northern Scotia Ridge (Sotelano et al. 2013; Anosov et al. 2015). A single record of *L. confundens* is known from Punta Arenas, Chile (ca. 53°S; 71°W; Macpherson 1988), and was presumably caught by commercial fishers somewhere near this landing port.

Some members of the family Lithodidae have high commercial interest, e.g., *Paralithodes camtschaticus* (Tilesius, 1815), *Paralithodes brevipes* (H. Milne-Edwards & Lucas, 1841), *Paralithodes platypus* (Brandt, 1850) and *Lithodes aequispinus* Benedict, 1895, which is why important fisheries and biological studies have developed around the world (Zaklan 2002; Otto 2014). Around southern South America, only two species have been commercially harvested, and supported one of the most profitable fisheries: the southern king crab *L. santolla* and the stone crab *Paralomis granulosa* (Hombron & Jacquinot, 1846) (Lovrich and Tapella 2014). Nevertheless, given the potential for misidentification described before, it is probable that landings reported as *L. santolla* were actually *L. confundens*.

In this study, we used a phylogenetic approach to assess whether these two morphospecies constitute reciprocally monophyletic clades at the molecular level, and performed a detailed morphological study on the carapace to search for correspondence between genetic and morphological differences. Furthermore, we performed a phylogeographic analysis in order to propose hypothesis related to their evolutionary history.

Materials and methods

Sampling

Individuals of *L. santolla* and *L. confundens* were collected for molecular analysis between 2009 and 2012 from six localities: Golfo San Jorge (SJ, $N = 27$); Bahía Grande (BG, $N = 13$); off Río Grande (RG, $N = 20$); in the Namuncurá Marine Protected Area/Burdwood Bank (BB, $N = 11$); in the Beagle Channel (BC, $N = 20$) and near Puerto Montt (PM, $N = 10$) (Fig. 1). Crabs from the first four localities were sampled with trawl nets on board the RV "Puerto Deseado" whereas animals from BC and PM were obtained from commercial traps. Individuals were dissected, a portion of 2–3 g of pereiopod muscle was fixed in ethanol 96 % and the carapace fixed in 10 % formalin seawater. When dissection was not possible, the whole animal was fixed in ethanol 96 %.

DNA extraction, amplification, and sequencing

DNA was extracted from muscle using a salting out protocol (Reiss et al.1995). Fragments of two mitochondrial genes, 16SrDNA (16S) and cytochrome oxidase subunit I (COI) were amplified using primers, LitF1/LitR1 (Palero et al. 2010) and LCO1490/HCO2198 (Folmer et al. 1994), respectively. Polymerase chain reactions (PCR) were performed using Applied Biosystems, Primus and BioNeer thermal cyclers in 10, 20, and 50 µl reactions consisting of 20 ng of DNA, 0.2 mM of each dNTP, 2 mM/3 mM $MgCl_2$, 0.15 µM of each primer, 0.025 U μl^{-1} of Taq, the corresponding buffer and ddH_2O. For the amplification of 16S we used a touchdown (TD) protocol. Following 3 min of denaturation at 95 °C, samples were subjected to 15 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 1 min with a decrease in the annealing temperature of 1 °C every cycle (from 60 to 46 °C). After completion of the TD program, 20 final cycles with an annealing temperature of 50 °C were performed, ending with an extension at 72 °C for 10 min. Thermal cycling conditions for COI consisted of an initial denaturation step of 94 °C for 3 min followed

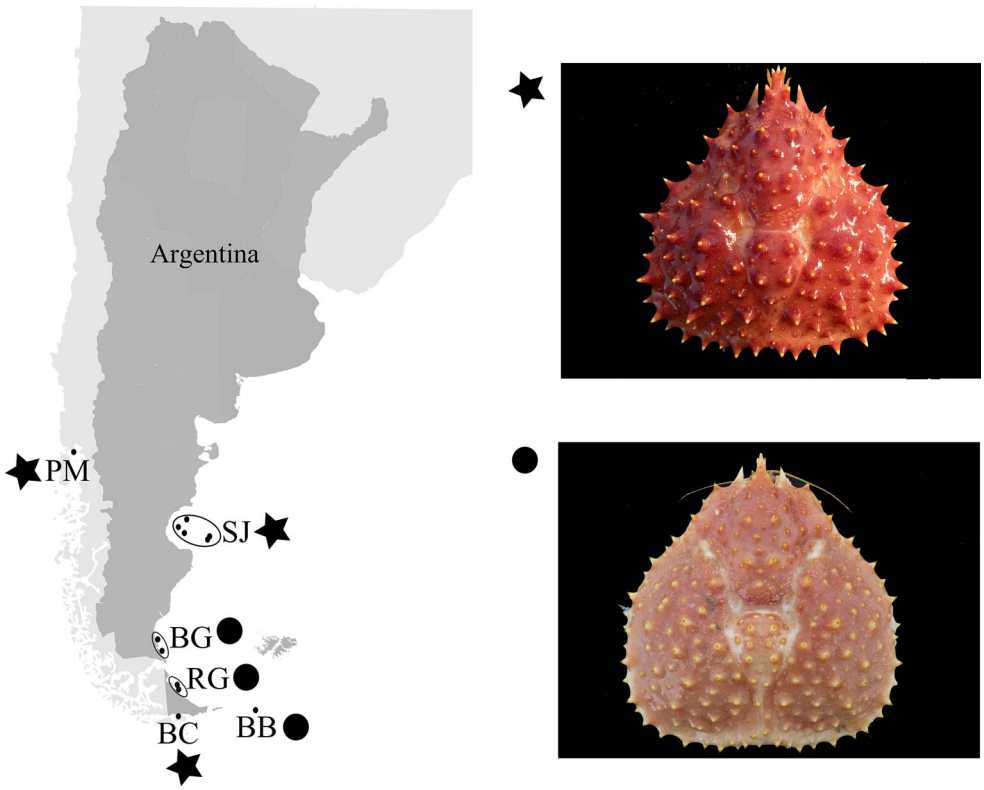

Fig. 1 Sampling locations of *Lithodes santolla* (*filled star*) and *Lithodes confundens* (*filled circle*) included in the present study. *Small dots* indicate sampling points in the different localities, i.e., *SJ* Golfo San Jorge ($N = 27$), *BG* Bahía Grande ($N = 13$), *RG* Río Grande ($N = 20$), *BB* Namuncurá Marine Protected Area/Burdwood Bank ($N = 11$), *BC* Beagle Channel ($N = 20$), *PM* Puerto Montt ($N = 10$). *Photos* above, a carapace of a *L. santolla* of 80.2 mm carapace length; and below, a *L. confundens* of 118.2 mm carapace length

by 35-38 cycles at 94 °C for 30 s, 40 °C to 50 °C for 50 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min.

Amplification products were cycle-sequenced in the sequencing facility of the Department of Ecology, Genetics and Evolution of the University of Buenos Aires.

16S and COI sequences were edited and aligned independently using BioEdit v5.0.9 with default gap-opening and gap-extension penalties (Hall 1999). COI sequences were translated into amino acids in MEGA v5.1 (Tamura et al. 2011) to check for the presence of pseudogenes. All sequences were deposited in GenBank (accession numbers: KM887436–KM887498). 16S and COI sequences belonging to the same individual were concatenated for further analysis.

Phylogenetic analyses

Phylogenetic reconstructions were conducted using Maximum Parsimony and Bayesian inference. 16S and COI sequences of *Lithodes maja* (Linnaeus, 1758) (AF425330, FJ581746), *L. ferox* Filhol, 1885 (HM020950, HM020903), *L. murrayi* Henderson, 1888 (HM020953, HM020899) and *Neolithodes brodiei* Dawson & Yaldwyn, 1985 (HM020943, HM020893) were obtained from GenBank to be used as outgroups in phylogenetic analyses. Parsimony analysis was performed in TNT v1.1 (Goloboff et al. 2003, 2008). Tree search was heuristic, with tree bisection and reconnection (TBR) branch swapping, and 100 random addition sequences. Gaps were coded as fifth state. Node support values were computed with standard bootstrapping (1000 replications). The Bayesian analysis was performed using Mr. Bayes v3.1.2 (Ronquist and Huelsenbeck 2003). Model selection was made using jModelTest (Guindon and Gascuel 2003; Posada 2008) which suggested a HKY (Hasegawa et al. 1985) and a TPM2uf+G model (TPM: three-parameter model = K81, Kimura 1981) for 16S and COI, respectively (see Posada 2008 for model specifications). However, Mr. Bayes does not implement the last model, so the next more complex model available in the program was selected as suggested by the software's manual. Therefore, two partitions were set, and a HKY and a GTR+G (GTR: general time reversible, Tavaré 1986) substitution model were specified for 16S and COI, respectively (see Posada 2008 for model specifications). The analysis was run for 5,000,000 generations. Trees were sampled every 100 generations. To determine convergence we examined the average standard deviation of split frequencies, and 12,500 trees were discarded as burn in. Tamura 3-parameter intra and interspecific genetic distances were calculated using MEGA5.1 (Tamura et al. 2011).

BEAST v1.7.4 (Drummond et al. 2012) was used to estimate the time to the most recent common ancestor (TMRCA) of clades of interest in the phylogeny. Unlinked HKY substitution models were selected for both partitions, 16S and COI, since when using the GTR model, low

effective sample sizes (ESSs) were obtained for the "prior" and "posterior" statistics, suggesting the GTR model was too complex (parameter-rich) for our data. We set a relaxed clock with uncorrelated lognormal distribution (Drummond et al. 2006) and a Yule speciation process as tree prior. The ucld.mean prior was specified taking into account the 16S and COI substitution rates published for other crabs (Schubart et al. 1998; 2000; Stillman and Reeb 2001; Sotelo et al. 2009). It was defined as a uniform distribution between 0.002 and 0.01 substitutions per site per lineage per myr. The midpoint of this interval, 0.006, was defined as the initial value. The analysis was run for 100 million generations, sampled every 10,000. ESS values of each parameter and convergence of the stationary distribution were checked using the software Tracer v1.5 (Rambaut and Drummond 2003).

Phylogeographic analyses

Genealogical relationships among haplotypes within each clade of *L. santolla*/*L. confundens* found in the present study were reconstructed as median joining networks using Network v4.6.1.1 (Bandelt et al. 1999). Departures from neutral expectations/constant population size were investigated by calculating Tajima's D (Tajima 1989a, b) and Fu's Fs (Fu 1997) statistics, and their significance was estimated by performing 1000 coalescent simulations. The level of genetic differentiation between individuals of different sampling localities was investigated by calculating pairwise F_{ST}s and their significance tested by 10,000 permutations, and by performing an analysis of molecular variance (AMOVA) among and within populations. The significance of the F_{ST} analogs of Excoffier et al. (1992) was estimated with 1023 permutations. The level of polymorphism of different sampling localities was determined by means of standard genetic diversity indices: haplotype number, number of segregating sites, haplotype diversity, and nucleotide diversity. Pairwise F_{ST}s, AMOVA, genetic diversity indexes, Tajima's D and Fu's Fs were calculated using Arlequin v3.5 (Excoffier and Lischer 2010).

Morphological analysis

A posteriori of the phylogenetic analysis, a detailed study of the number of spines on the carapace incorporating all carapace regions in the analysis, was performed to search for correspondence between genetic and morphological differences. Although the initial identification of crabs in *L. santolla* and *L. confundens* was based on the key to the species of the genus and descriptions made by Macpherson (1988), the morphological analysis was only performed on the carapace since due to logistic limitations found onboard during sampling, some king crabs could not be conserved complete, and only carapaces were fixed in formalin seawater.

The sizes of animals, i.e., carapace length (CL), were measured with a caliper to the nearest 0.1 mm. Carapaces were photographed, and spines, spinelets, and spiniform granules were counted in the following three carapace regions: left branchial, cardiac, and gastric. A hierarchical cluster analysis with the mean Euclidean linkage on average variables was performed using as variables the total number of spines, spinelets and spiniform granules in each of the three carapace regions analyzed. Clusters were defined at 50 % of the total distance to the root node. This analysis was performed in InfoStat v2013 (Di Rienzo et al. 2013).

Results

Phylogenetic analyses

Alignment lengths of 16S and COI gene fragments of the 105 sequences analyzed were 334 and 606 bp, respectively. When we analyzed the alignment only including samples of *L. santolla* and *L. confundens*, we found 57 variable sites (seven in 16S and 50 in COI), 43 of which were parsimony informative (four in 16S and 39 in COI). One parsimony informative gap, 1 bp in length, was inserted in some 16S sequences.

Both the maximum parsimony strict consensus and the Bayesian analysis recovered two monophyletic clades with high bootstrap supports and posterior probabilities (Fig. 2, Clades 1 and 2). Clade 1 was formed by 94 individuals, 50 of which conformed to the morphological description of *L. santolla* and 44 to the description of *L. confundens* (Figs. 2 and 3). Clade 2 was formed by seven individuals of *L. santolla* only (Fig. 2). These two clades were recovered as sister clades by the Bayesian analysis, and their interclade genetic distance was 1.2 ± 1.0 % for 16S and 11.0 ± 4.4 % for COI, similar to those observed between other species of lithodids (Online Resource 1), and two orders of magnitude higher than intraclade genetic distances (Online Resource 2). The time for the split between Clades 1 and 2 was estimated in 1.2 Myra (2.6–0.4 Myra, Pleistocene). Clade 1 was widely distributed (in all sampling localities) while Clade 2 was restricted to Puerto Montt and the Beagle Channel (Fig. 2).

Phylogeographic analyses

Due to the low number of individuals in Clade 2, most phylogeographic analyses were only performed on Clade 1.

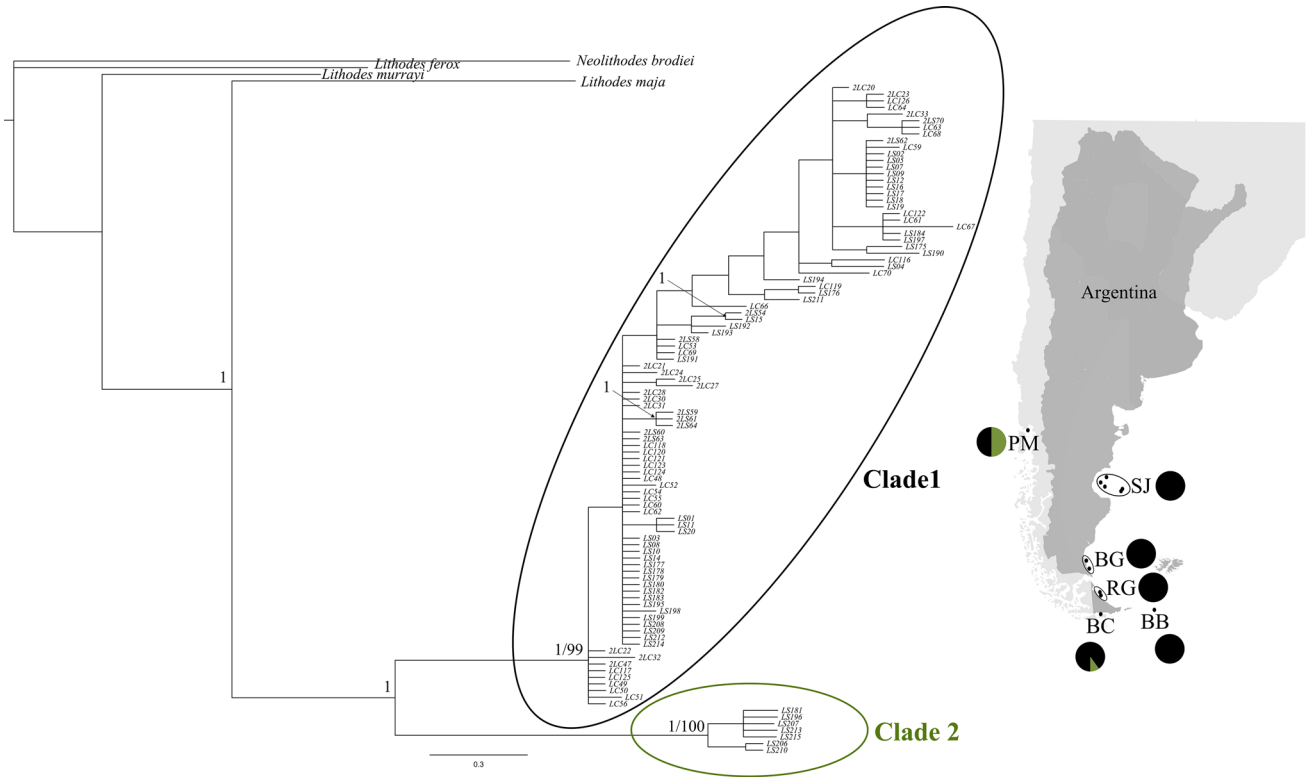

Fig. 2 Bayesian phylogenetic reconstruction based on 16S+COI sequences of *Lithodes santolla* (LS) and *Lithodes confundens* (LC). *Numbers on branches* indicate posterior probabilities (only values = 1 shown)/bootstrap supports for those clades that were also recovered by the maximum parsimony reconstruction and had more than 98 % bootstrap support. *Pie charts* next to each location indicate the proportion of individuals belonging to each clade captured in each sampling locality

Clade 1

Clade 1 was formed by 94 individuals, which presented 32 haplotypes determined by 35 variable sites (five in 16S and 30 in COI), 19 of which were parsimony informative (one in 16S and 18 in COI). The haplotype network revealed a most common haplotype present in 32 individuals, i.e., 18 *L. santolla* and 14 *L. confundens* (Fig. 3), distributed in all sampling localities (Fig. 4a), and several haplotypes which differed from the most common one in one or a few mutational steps (Figs. 3, 4a). The second most common haplotype was found in ten individuals from Golfo San Jorge (all *L. santolla*) and was separated from the most common one by seven mutational steps (Figs. 3, 4a). The third most common haplotype was found in seven individuals (all *L. confundens*), three of which were found in Río Grande, two in Burdwood Bank and the other two in Bahía Grande, and differed from the most common one by one mutational step (Figs. 3, 4a). In addition to the most common haplotype, there were other four haplotypes shared by both morphospecies three of which were derived haplotypes (Fig. 3).

The star-shaped pattern observed in the top part of the network (Figs. 3, 4a) and the occurrence of a large proportion of very low frequency mutations could be explained by a sudden population expansion (Schneider and Excoffier 1999) or a selective sweep (Nordborg 2007). Tajima's D and Fu's Fs neutrality tests performed on the entire dataset yielded different results. Tajima's D was negative but non-significant ($D = -1.19$, $p = 0.1$), whereas Fu's Fs was negative and highly significant ($Fs = -15.83$, $p < 0.01$). These results can be explained since Fs is a more sensitive indicator of population expansion and genetic hitchhiking than Tajima's D (Holsinger 2012).

Genetic diversity indices such as the haplotype and nucleotide diversities were similar between localities, except for the sample of Puerto Montt, which had the lowest haplotype and nucleotide diversity (Table 1). Haplotype diversity was similar among the rest of the analyzed localities, and was always higher than 0.80, while nucleotide diversity was very low, always lower than 0.005 (Table 1).

AMOVA results indicated no population genetic structure ($\Phi_{ST} = 0.01512$, $p = 0.2$) and evidenced that differentiation between samples was no larger than differentiation within a single locality (within population component of the variance explained 98.49 % of the

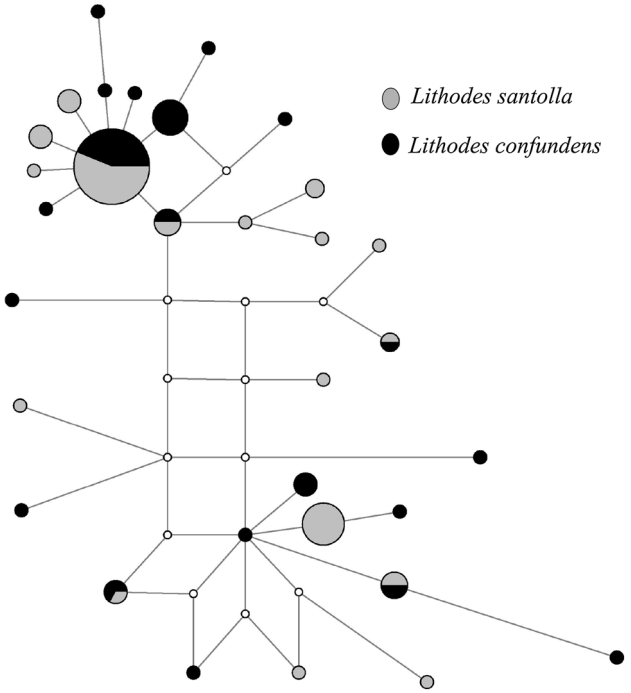

Fig. 3 Median-joining network of mitochondrial 16S+COI haplotypes of *Lithodes santolla* and *Lithodes confundens* belonging to Clade 1. Each *circle* represents a distinct haplotype; circle area and line length are proportional to haplotype frequency and number of mutational steps, respectively. The area of the *smallest full circle* corresponds to a frequency of one. *Small empty circles* are median vectors. *Colors* indicate morphospecies

variation in the samples). Pairwise F_{ST}s calculated to assess the existence of differentiation between sampling localities were not significant.

Clade 2

Clade 2 presented six haplotypes determined by seven variable sites (all in COI), two of which were parsimony informative (Fig. 4b). Clade 2 was formed by seven *L. santolla* individuals, of which five were found in Puerto Montt and two in the Beagle Channel (Fig. 4b).

Morphological analysis

No coincidence was found between morphological clustering and genetic separation in Clades 1 and 2 (Figs. 2, 5). Three morphological clusters were found (Fig. 5). Group A clustered all *L. santolla* from the three localities where they occurred, i.e., Golfo San Jorge, Beagle Channel and Puerto Montt, except for two from Golfo San Jorge (2LS70 and 2LS61), and was characterized by having the lowest average number of spines in each carapace region (Table 2). Group C was formed by seven *L. confundens* from the Burdwood Bank and had the highest average number of spines in each carapace region; and Group B clustered all the rest of *L. confundens* and 2LS70, and was characterized by having an intermediate average number of spines in each carapace region (Table 2). Specimen 2LS61 appeared in the cluster analysis as an outlier because it had fewer small granules in the gastric region and more granules in the cardiac region than individuals in group A. No characters were found on the carapace that could differentiate individuals belonging to each genetic clade.

Discussion

In the present study, the Bayesian phylogenetic reconstruction performed suggested that individuals of *L. santolla* and *L. confundens* from southern South America belong to two sister clades. However, individuals identified as *L. santolla* and *L. confundens* did not resolve as reciprocally monophyletic groups. Instead, Clade 1 was formed by individuals belonging to both morphospecies and was widely distributed, while Clade 2 was exclusively formed by *L. santolla*, and its members were only found near Puerto Montt and the Beagle Channel. These two clades would have diverged approximately 1.2 Myra. Furthermore, signs of population expansion were observed in Clade 1. The detailed morphological analysis evidenced the existence of three discrete clusters, one corresponding to the description of *L. santolla* (A), another to the description of *L. confundens* (B), and the third one formed by individuals with an extreme phenotype (C), i.e., numerous spines in all carapace regions. The incongruence observed between genetic clades and morphological identification and clustering, suggests the need to revise the use of the number of spines as a relevant taxonomic character in the taxonomy of *Lithodes*.

Two alternative hypotheses can explain the incongruence found. On the one hand, morphological variation could occur as a result of an adaptative or plastic response to environmental variation, and could be mistakenly used as a criterion to delimit species, consequently leading to the erroneous description of variants of a single species as different nominal species (Funk and Omland 2003; Camargo and Sites 2013), as in the case of *Munida gregaria* (Pérez-Barros et al. 2008; 2011), *Callinectes bocourti* (Schubart et al. 2001) and *Cyrtograpsus altimanus* (Spivak and Schubart 2003; Lezcano et al. 2012). Environmental conditions can produce morphological changes, especially in color and/or spine length, as in *Callinectes sapidus* (Davis et al. 2005) and *Paralithodes camtschaticus* (Westphal et al. 2014). Moreover, Dawson and Yaldwyn (1985), when publishing a key to the species of *Lithodes*, cautioned that some species may show considerable variation in carapace spinulation, which can lead to

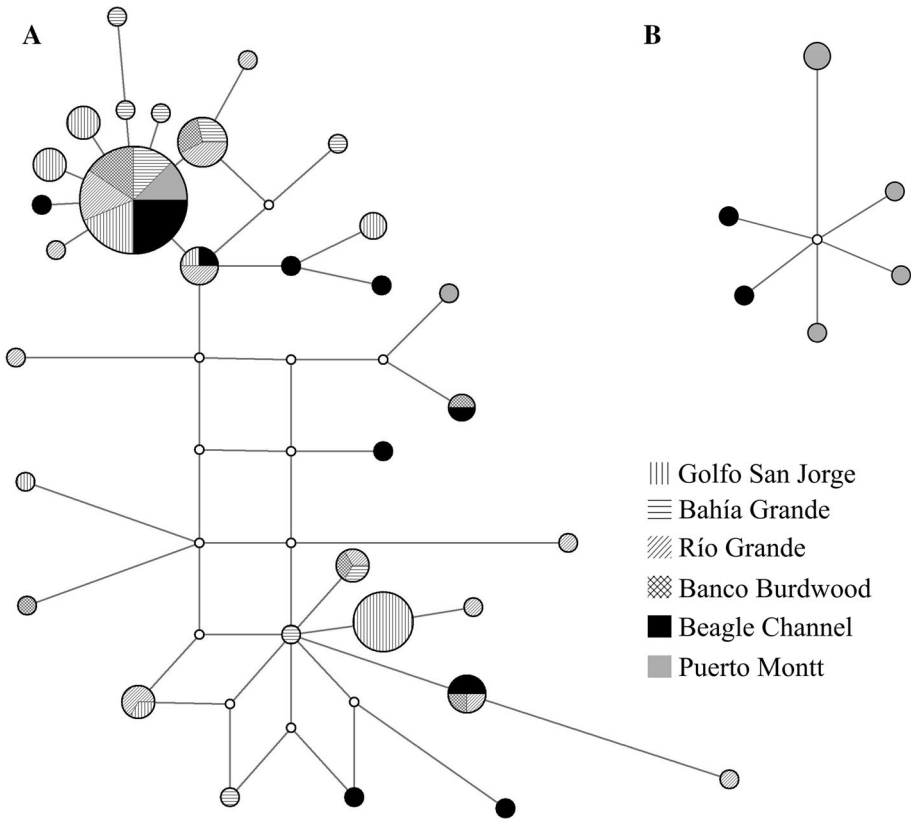

Fig. 4 Median-joining networks of mitochondrial 16S+COI haplotypes of *Lithodes santolla* and *Lithodes confundens* belonging to Clade 1 (**a**) and Clade 2 (**b**). Each *circle* represents a distinct haplotype; circle area and line length are proportional to haplotype frequency and number of mutational steps, respectively. The area of the *smallest full circle* corresponds to a frequency of one. *Small empty circles* are median vectors. Patterns indicate sampling locations

Table 1 Number of individuals in Clade 1 per sampling locality, and their respective diversity indices based on the combined 16S+COI mtDNA sequences

Sampling locality	*N*	*k*	*H*	*S*	*Π*	*π*
Golfo San Jorge (SJ)	27	8	0.81 ± 0.05	14	4.44 ± 2.26	0.0047 ± 0.0027
Bahía Grande (BG)	13	9	0.91 ± 0.07	13	3.90 ± 2.10	0.0042 ± 0.0025
Río Grande (RG)	20	12	0.92 ± 0.04	18	4.58 ± 2.35	0.0049 ± 0.0028
Burdwood Bank (BB)	11	6	0.80 ± 0.11	13	4.18 ± 2.25	0.0045 ± 0.0027
Beagle Channel (BC)	18	10	0.81 ± 0.09	16	4.07 ± 2.13	0.0043 ± 0.0025
Puerto Montt (PM)	5	2	0.40 ± 0.24	5	2.00 ± 1.34	0.0021 ± 0.0017
Total	94	32	0.87 ± 0.03	35	4.24 ± 2.12	0.0045 ± 0.0025

Abbreviations of localities correspond to those in Figs. 1, 2, and 5

N specimens analyzed, *k* haplotype number, *H* haplotypic diversity, *S* polymorphic sites, *Π* mean number of pairwise differences, *π* nucleotide diversity

misclassification. Macpherson (1988) named the species *L. confundens* in allusion to the Latin word "*confundo*", due to its resemblance with *L. santolla* and the confusion that could arise between them. If this hypothesis is true, Clades 1 and 2 found in the present study will constitute two species, however, not *L. santolla* and *L. confundens* as they have been described (Macpherson 1988), since the diagnostic characters used are variable and not useful to identify them. A more detailed morphological analysis including other parts of the animal is needed to search for new diagnostic characters e.g., relative length and length/width ratio of the merus in pereiopods (Ahyong and Chan 2010).

On the other hand, the fact that individuals of *L. santolla* and *L. confundens* did not resolve as reciprocally monophyletic groups for mitochondrial markers can be attributed to a recent mitochondrial introgression of *L. confundens* into *L. santolla*. This hypothesis entails populations that have diverged allopatrically into morphospecies (ca. 1.2 Myra), without attaining complete reproductive isolation, and recent secondary gene flow between them. A geographic scenario compatible with this hypothesis would be the independent evolution of *L. confundens* (originally Clade 1) and *L. santolla* (originally Clade 2) on both sides of South America, i.e., *L. confundens* in the Atlantic Ocean and *L. santolla* in the Pacific. This isolation could have

Table 2 Number of spines, spinelets, and spiniform granules in each of the three carapace regions analyzed, i.e., left branchial (LB), cardiac (CAR) and gastric (GAS), for each of the three morphological clusters obtained (A–C see Fig. 5)

Cluster		CL (mm)	LB	CAR	GAS
A	Min.	23.50	13	5	9
$N = 55$	Max.	139.39	37	12	25
	Average		19.53	6.58	16.71
	SD		5.28	1.76	4.33
B	Min.	21.48	31	10	20
$N = 38$	Max.	137.23	59	18	37
	Average		44.24	14.63	28.47
	SD		6.83	2.05	4.48
C	Min.	22.91	39	17	32
$N = 7$	Max.	32.51	70	24	59
	Average		59.71	21	45.71
	SD		10.16	2.31	8.14

N number of specimens, *CL* carapace length, *SD* standard deviation

been attained during the Great Patagonian Glaciation (GPG, ca. 1 Myra, Rabassa et al. 2011) or other Pleistocene glaciations, by a northward retraction of the present range of both species given by the decrease in water temperature experienced during these periods (Cavallotto et al. 2011; Rabassa et al. 2011) and the fact that temperature is a constraining factor in the distribution of lithodids (Hall and Thatje 2011). This retreat into warmer/unglaciated areas has been suggested for other sub-Antarctic marine organisms (Ceballos et al. 2012; Ocampo et al. 2013; Pérez-Barros et al. 2014). Once seawater temperature started warming *L. confundens* could have expanded its range, and hybridization between both morphospecies could have occurred.

Introgressive hybridization, i.e., the transfer of genes across species boundaries, has been reported between several species of fish and marine invertebrate, e.g., corals, sea urchins, sea stars and crabs (Avise and Saunders 1984; Bernatchez et al. 1995; Lessios and Pearse 1996; Harper and Hart 2007; Darling 2011; Ladner and Palumbi 2012). Contrary to what is generally assumed, hybrid individuals may be morphologically indistinguishable from one of the parental taxa, as in the case of Lake Alain brook trout *Salvelinus fontinalis* and sea stars *Asterias* spp. (Bernatchez et al. 1995; Allendorf et al. 2001; Harper and Hart

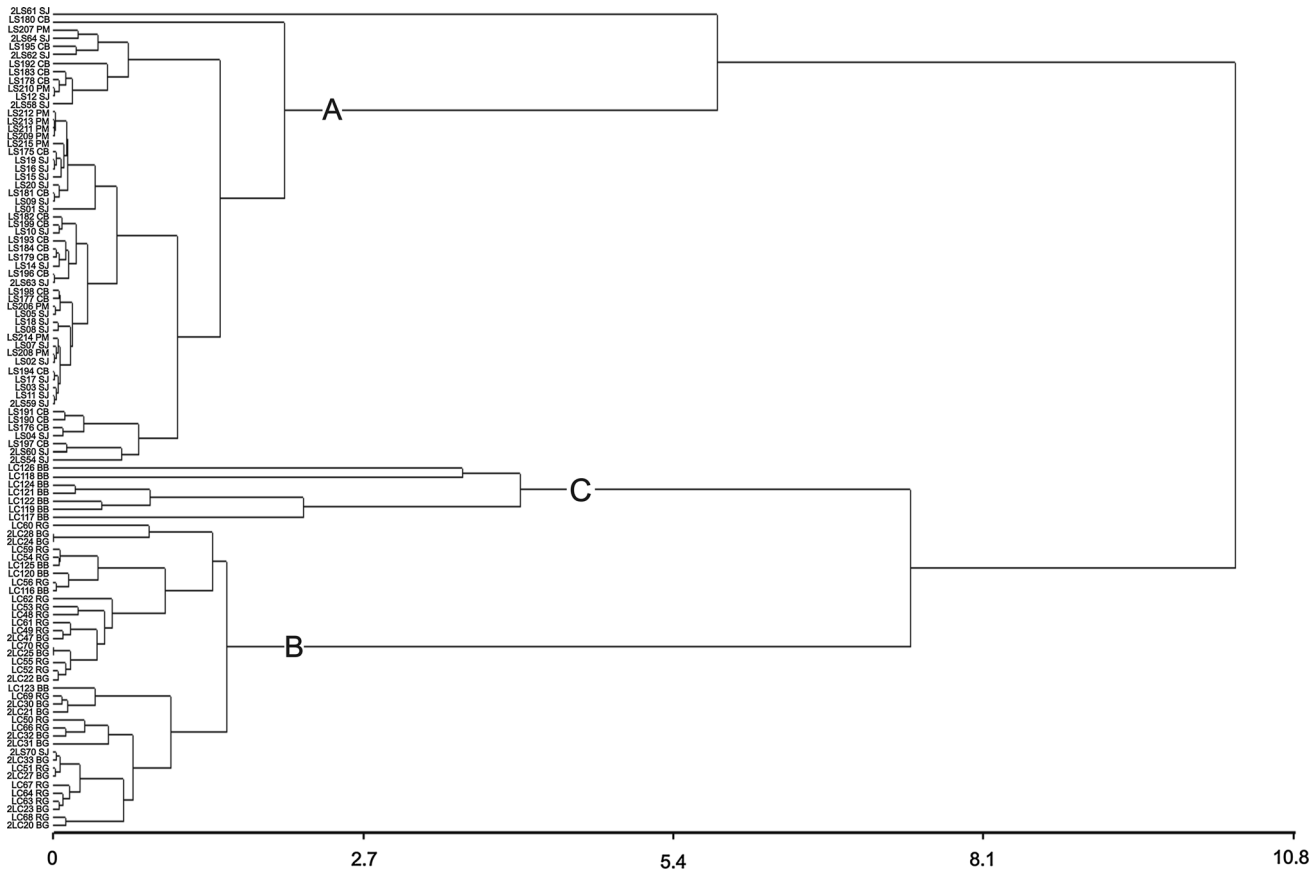

Fig. 5 Cluster analysis of three variables, i.e., total number of spines, spinelets, and spiniform granules in the left branchial, cardiac and gastric regions of the carapace of 101 specimens of *Lithodes santolla* (LS) and *Lithodes confundens* (LC) from different sampling localities (see Fig. 1 for labels). **A–C** Denote clusters at <50 % of the root distance

2007). Furthermore, in both cases, the reported mitochondrial introgressions were unidirectional, i.e., from Arctic char (*Salvelinus alpinus*) into brook trout (*S. fontinalis*) and from *Asterias rubens* into *Asterias forbesi*. If this hypothesis was true, introgression in this case would have occurred without the formation of morphologically intermediate specimens, since in our morphological analysis, despite having found three discrete clusters, there was no cluster formed by morphological intermediates between *L. confundens* and *L. santolla*. The third cluster found (C) constituted an extreme phenotype with its distribution restricted to the Burdwood Bank. Moreover, if the introgression hypothesis was true, it would have been unidirectional.

The analysis of other independent markers, i.e., nuclear markers, may help discern between these two hypotheses. Microsatellite markers have been used in a variety of species to assess the occurrence of nuclear introgression (Addison and Hart 2005; Pastorini et al. 2009; Darling 2011; Harris et al. 2013). Further sampling where both genetic clades and/or both morphospecies co-occur, i.e., Puerto Montt, the Beagle Channel, and off the Atlantic coast at ca. 38°S near the continental slope; as well as the analysis of nuclear markers could help elucidate which processes have been involved in the evolution of the southern species of *Lithodes*.

In any case, either if the two genetic clades found in this study constitute species, or remnants of two species that are merging through introgression, they represent two evolutionary significant lineages. Therefore, and because *Lithodes* spp. are being commercially exploited around southern South America, fisheries regulations should be modified, and measures should be taken to preserve both genetic clades. More importantly, since morphology is not a good indicator of genetic clades, molecular genetic methods should be implemented to control this fishery.

Finally, it is worth noting that our study is the first to address the issue on the identity of *L. santolla* and *L. confundens* with an intensive approach (more than 100 individuals analyzed from almost all the distribution range), and highlights the necessity of working with more than a few individuals when addressing such questions. Analyzing more individuals than are usually used to reconstruct phylogenies permits to uncover the morphological variability contained within a species and/or the processes involved in their evolution, which could be masked when dealing with a few individuals.

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