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Molecular identification by polymerase chain reaction - restriction fragment length polymorphism of commercially important lithodid species (Crustacea: Anomura) from southern South America

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1 Molecular identification by Polymerase Chain Reaction - Restriction Fragment Length
2 Polymorphism of commercially important lithodid species (Crustacea: Anomura) from
3 southern South America.

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18 Running title: Molecular identification of lithodids from southern South America.
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22 **Abstract**

23 King crab fisheries constitute a highly profitable marine business in southern South
24 America, where five fisheries for lithodids currently exist, two in Chile and three in
25 Argentina. These fisheries mainly target *Lithodes santolla*, however in some localities the
26 harvested morphospecies is *Lithodes confundens*, and in some others it constitutes a mixed
27 fishery since it also captures *Paralomis granulosa*. Fishery products can be commercialized
28 as frozen shredded or canned minced meat, making specific identification unfeasible since
29 the morphological characters used in the identification are on the carapace, and
30 consequently, not available in these processed products. Therefore, a potential for food
31 fraud exists, since southern king crab meat (*L. santolla/ L. confundens*) could be replaced
32 by the less expensive false southern king crab, i.e. *P. granulosa*. Furthermore, incongruence
33 between morphological and genetic characters rendered the taxonomic status of both
34 morphospecies of commercial *Lithodes* questionable, and stressed the need of
35 implementing molecular methods to monitor the abundance and distribution, as well as the
36 landings, of each genetic clade of *L. santolla/ L. confundens*. In the present study, we
37 developed rapid and cost-efficient Polymerase Chain Reaction - Restriction Fragment
38 Length Polymorphism (PCR-RFLP) tests that can aid in the identification of commercially
39 important lithodids that inhabit waters off southern South America in cases where
40 identification is not possible using morphological characters.

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43

44 Keywords: centolla; centollón; fisheries management; food fraud; southern king crab;
45 Namuncurá MPA/ Burdwood Bank

46 **1. Introduction**

47 Genetic species identification has several applications in fishery resource
48 management, e.g. detection of food fraud and regulation compliance (Sweijd et al., 2000;
49 Ogden, 2008). DNA markers that show variation between species but are conserved within
50 species are valuable markers for species identification (Ogden et al., 2009; Martisohn,
51 2011). Such markers have been used to develop methods to identify tissue samples to
52 species for a wide range of commercially important fish and crustaceans, e.g. anchovy and
53 sardine (Sebastio et al., 2001; Jérôme et al., 2003), gadoid species (Di Finizio et al., 2007),
54 salmon, trout and beam (Withler et al., 2004; Espiñeira et al., 2009), shark fins (Sebastian
55 et al., 2008), flatfish (Boukouvala et al., 2012), penaeid shrimps (Pascoal et al., 2008) and
56 spider crabs of the genus *Maja* (Guerao et al., 2011).

57 Fishing for southern king crabs constitutes one of the most profitable marine
58 business in southern South America (Stevens and Lovrich, 2014), where five fisheries for
59 lithodids currently exist. In the last decade, free on board (FOB) prices varied between 16
60 and 18 USD·kg^{-1} in Argentina and Chile (Anonymous, 2018; Molinet et al., 2019). On the
61 Southeastern Pacific coast, off the Chilean Regions Los Ríos, Los Lagos and Aysén, a
62 fishery for *Lithodes santolla* exists between 41º30´S and 46º30´S, with its main landing
63 port in Puerto Montt (Figure 1). Further south, in the numerous fjords and channels of the
64 Magallanes and Chilean Antarctic Region, between 49ºS and 56ºS, there is a mixed fishery
65 for *L. santolla* and *P. granulosa*, with Puerto Natales, Punta Arenas and Puerto Williams as
66 landing ports (Lovrich and Tapella, 2014). In Argentina, in the neighboring Beagle
67 Channel, there is another mixed fishery for the same two species, landing at Ushuaia. Off
68 the Atlantic Patagonian coast, south of 44ºS and over most of the continental shelf, there

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are two fisheries at different degrees of development. Off Golfo San Jorge the fishery harvests *L. santolla* and south of 48ºS the fishery targets *L. confundens*, which are mainly landed at Comodoro Rivadavia and Puerto Deseado, respectively (Wyngaard et al., 2016; Mauna et al., 2018; Figure 1).

Fisheries for lithodid crabs started in the southernmost locations of their distribution, around Tierra del Fuego, in the Strait of Magellan (Chile) and the Beagle Channel (Argentina), in the 1920s and 1930s. Since 1980s the interest for *P. granulosa*, originally considered as bycatch, increased and these fisheries became mixed, since both species were caught in the same trap. However in these southern fishing grounds, the main target species has always been *Lithodes santolla* due to its higher meat yield per crab, longer legs, and the possibility of obtaining large entire muscle pieces (Lovrich and Tapella, 2014; Figure 2). The collapse of the fishery in the Beagle Channel during the 1990s promoted the development of another fishery off Golfo San Jorge, first as an exploratory fishery and then fully exploited since 2008 (Lovrich and Tapella, 2014). Current annual yields are ca. 2300 t in the Golfo San Jorge. Latest signs of overexploitation of this fishery (Firpo et al., 2017) are promoting the experimental fishery for *L. confundens* further south (Mauna et al., 2018). The fishery of the Beagle Channel is still collapsed and exploited at a small scale by artisan fishers, ca. <80 t (Lovrich and Tapella, 2017). In Chile, recent increases in FOB prices of *L. santolla* have triggered the growth of the fishery near Puerto Montt, with recent landings of ca. 1000 t (Molinet et al., 2019). The fishery of the Magallanes and Chilean Antarctic Region has always been the most productive one, with landings of 6000 t in 2016. Yields of *P. granulosa* are quite variable and dependent on other crab markets in the world. Landings in Chile have been ca. 2000 t per year during the

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last decade, with previous peaks of ca. 6000 t in 2001 and 2005 (Daza et al., 2016; FAO, 2019). In Argentina, landings of *P. granulosa* have been modest, i.e. <20 t per year during the last decade (https://www.agroindustria.gob.ar/sitio/areas/pesca_maritima/desembarques/, last accessed 12/09/2019).

Fishery products can be commercialized either as clusters of four whole legs, as fancy (frozen shredded meat), or canned minced flesh (Lovrich and Tapella, 2014). In these last two cases, specific identification is unfeasible since the morphological characters used in the identification are on the carapace (Figure 2), and consequently, not available in processed products. Therefore, and due to its high commercial value, a potential for food fraud exists, since southern king crab meat (*L. santolla/ L. confundens*) could be replaced by other low-priced products, e.g. the false southern king crab *P. granulosa*.

Furthermore, in 2015, in an attempt to clarify the taxonomic status of *L. santolla* and *L. confundens*, we published a mitochondrial phylogeny that evidenced that individuals identified as *L. santolla* and *L. confundens* did not resolve as reciprocally monophyletic groups (Pérez-Barros et al., 2015). Instead, one clade was formed by individuals belonging to both morphospecies and was widely distributed, i.e. throughout the continental shelf in the SW Atlantic, Beagle Channel and SE Pacific (hereafter Clade 1), while the other one was exclusively formed by *L. santolla*, and its members were only found in the SE Pacific (i.e. near Puerto Montt) and in the Beagle Channel (hereafter Clade 2) (Figure 1). This matter needs to be further investigated (i.e. with nuclear markers) in order to clarify species boundaries, and renders both morphospecies provisionally as a species complex (hereafter *L. santolla/ L. confundens*) (Sigovini et al., 2016). So far, no morphological characters on

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115 the carapace have been found that could differentiate individuals belonging to each
116 mitochondrial clade. This highlights the need to implement molecular genetic methods to
117 manage and control southern king crab fisheries, since although no Clade 2 individuals
118 were found in the SW Atlantic, both clades co-occur in the Beagle Channel and near Puerto
119 Montt (Pérez-Barros et al., 2015).
120 In the present study, PCR-RFLP assays were developed to differentiate
121 commercially important lithodids that inhabit waters off southern South America.
122

123 **2. Material and Methods**

124 *2.1. PCR-RFLP method development*

125 *2.1.1. Lithodes and Paralomis species and populations considered*

126 Sequences of the mitochondrial cytochrome c oxidase subunit I (COI) of a total of
127 101 specimens of *L. santolla/ L. confundens* (Clades 1 and 2, see Pérez-Barros et al., 2015)
128 captured in waters off southern South America, i.e. Golfo San Jorge (N=27); Bahía Grande
129 (N=13); off Río Grande (N=20); the Namuncurá Marine Protected Area/Burdwood Bank
130 (NMPA/BB, N=11); the Beagle Channel (N=20) and near Puerto Montt (N=10) were
131 obtained in a previous study (Pérez-Barros et al., 2015). Sequences of *L. santolla/ L.*
132 *confundens*, as well as those of the sympatric *L. turkayi* sequenced by other authors were
133 downloaded from GenBank. Sequences of *Paralomis* spp., i.e. *P. granulosa*, *P. anamerae*,
134 *P. formosa*, *P. spinosissima* that inhabit waters off southern South America were also
135 obtained from GenBank (see accession numbers in Table 1).
136 Individuals of *P. granulosa* were collected between 2009 and 2013 from five
137 localities: off Puerto Deseado (N=16); Bahía Grande (N=1); off Río Grande (N=1); in the
138 NMPA/BB (N=2); and in the Beagle Channel (N=25) (Figure 1). Crabs from the first four

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139 localities were sampled with trawl nets on board the RV "Puerto Deseado" whereas
140 specimens from Beagle Channel were obtained from commercial traps. Individuals were
141 dissected, a portion of 2-3 g of pereiopod muscle was fixed in 96% ethanol and the
142 carapace fixed in 10% formalin seawater. When dissection was not possible, the whole
143 animal was fixed in 96% ethanol.

144 *2.1.2. DNA extraction, amplification and sequencing*

145 DNA was extracted from muscle of *P. granulosa* specimens using a salting out protocol
146 (Reiss et al., 1995). DNA integrity was checked with 1% agarose gel electrophoresis in 1X
147 TAE buffer for 60 minutes at 110v, stained with GelRed (Biotium) and visualized in an
148 Image Quant 350 (GE Healthcare) under UV light. DNA was also quantified and A280/260
149 checked in a Nanodrop 1000. The COI fragment was amplified using primers
150 LCO1490/HCO2198 (Folmer et al., 1994). Polymerase chain reactions (PCR) were
151 performed using Primus, BioNeer and MJ Research thermal cyclers in 10, 20 and 50 µl
152 reactions consisting of 20 ng of DNA, 0.2 mM of each dNTP, 2 mM $MgCl_2$, 0.15 µM of
153 each primer, 0.25 U of Taq (Invitrogen Taq DNA polymerase, recombinant), the
154 corresponding buffer and ddH_2O. Thermal cycling conditions consisted of an initial
155 denaturation step of 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 40°C to 50°C
156 for 50 s, 72°C for 1 min, and a final extension at 72°C for 7 min. The presence of
157 amplification products was checked with gel electrophoresis (same conditions as for
158 genomic DNA). Amplification products were cycle-sequenced in the sequencing facility of
159 the Department of Ecology, Genetics and Evolution of the University of Buenos Aires
160 (ABI3130xl Genetic Analyzer, Applied Biosystems) or in Macrogen (ABI3730xl DNA
161 Analyzer, Applied Biosystems).

162 *2.1.3. Data analysis*

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163 COI sequences were edited and aligned using BioEdit v7.1.3 with default gap-opening and
164 gap-extension penalties (Hall, 1999). COI sequences were translated into amino acids in
165 MEGA v5.1 (Tamura et al., 2011) to check for the presence of pseudogenes. All sequences
166 were deposited in GenBank (MK619312-MK619356).

167 The Restriction Enzyme Picker software (REPK, online v.1.3, Collins and Rocap,
168 2007) was used to find restriction endonucleases that could uniquely differentiate
169 designated sequence groups, i.e. genera and species. For the first analysis, i.e.
170 discrimination of *Lithodes* from *Paralomis* species (*Lithodes* vs. *Paralomis*), an alignment
171 of all the sequences of *Lithodes* and *Paralomis* that could co-occur with *P. granulosa* and
172 *L. santolla*/ *L. confundens* available in GenBank, and those produced in the present and a
173 previous study (Pérez-Barros et al., 2015) was entered in the program. For the second
174 analysis, i.e. the separation of Clades 1 and 2 of *L. santolla/ L. confundens* (*L. santolla/L.*
175 *confundens* Clade 1 vs. Clade 2), the same rationale was used. An alignment with
176 sequences of *L. santolla/ L. confundens* produced in a previous study (Pérez-Barros et al.,
177 2015) and those of *L. turkayi* available in Genbank was entered in the program.
178 Subsequently, the R package seqRFLP v.1.0.1 (Ding and Zhang, 2013) was run in R v3.0.2
179 to simulate and visualize the restriction enzyme cutting pattern of the enzymes selected by
180 REPK.

181 2.1.3.1. *Lithodes* vs. *Paralomis*

182 REPK indicated that the enzymes Eco24I and XmnI were able to produce genus-specific
183 restriction patterns (Supplementary material Figure S1). The COI amplification product of
184 individuals of *Paralomis* does not have the recognition site for either of the enzymes
185 mentioned and therefore yields one fragment after restriction, i.e. the complete

186 amplification product, whereas the *Lithodes* sequences have the restriction site yielding two
187 fragments after restriction (Supplementary material Table S1).

188 To test these *in silico* results and to determine restriction conditions, COI was
189 amplified *de novo* from:

190 - up to five specimens of *P. granulosa* and *L. santolla/ L. confundens* used to develop the
191 method from each of the localities mentioned above (see section 2.1.1.), and from 15 *P.*
192 *granulosa,* five *P. spinosissima* and 29 *L. santolla/ L. confundens* not previously used
193 (Tables 1 and 2).

194 The same conditions as in section *2.1.2. DNA extraction, amplification and sequencing*
195 were used. Amplification products were subjected to the following digestion conditions *in*
196 *vitro*:

197 Eco24I: The reaction mixture was prepared with 18 µL water, 10 µL PCR reaction mixture,
198 2 µL 10X Buffer Tango and 1 µL (10U) Eco24I (Thermo Scientific).

199 XmnI: The reaction mixture was prepared with 34.5µL water, 10µL PCR reaction mixture,
200 5µL NEBuffer and 0.5µL (10U) XmnI enzyme (New England BioLabs).

201 Mixtures were incubated at 37ºC overnight in an incubator (ID-Incubator 37 SI,
202 DiaMed-ID Micro Typing System). Afterwards, incubation mixtures were inactivated at
203 65ºC for 20 minutes. Restriction fragments were electrophoretically separated in 1.5%
204 agarose gels in 1X TAE buffer for 75 minutes at 90v, stained with GelRed (Biotium) and
205 visualized in an Image Quant 350 (GE Healthcare) under UV light.

206 2.1.3.2. *Lithodes santolla/ L. confundens* Clade 1 vs. Clade 2

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207 REPK indicated that the enzyme TscAI was able to produce clade specific restriction
208 patterns (Supplementary material Figure S2). The COI amplification product of individuals
209 of *L. santolla*/ *L. confundens* belonging to Clade 1 does not have the recognition site for
210 TscAI and therefore yields one fragment after restriction, i.e. the complete amplification
211 product, whereas the *L. santolla*/ *L. confundens* Clade 2 sequences have one restriction site
212 yielding two fragments after the *in silico* digestion (Supplementary material Table S2). The
213 *Lithodes turkayi* sequences used also have a TscAI restriction site. However, it is not
214 located in the same position as the one of *L. santolla*/ *L. confundens* Clade 2, therefore
215 yielding two fragments but of a different size than those generated after digestion of *L.*
216 *santolla*/ *L. confundens* Clade 2 (Supplementary material Figure S2 and Table S2).

217 To test these *in silico* results and to determine restriction conditions, COI was
218 amplified *de novo* from:

219 - up to five specimens of the *L. santolla/L. confundens* Clade 1 and Clade 2 used to develop
220 the method from each of the localities from which they were available (Table 3)

221 and subjected to the following digestion conditions *in vitro*. The reaction mixture was
222 prepared with 17 µL water, 10 µL PCR reaction mixture, 2 µL 10X FastDigest Green
223 Buffer and 1 µL (10U) TscAI FastDigest enzyme (Thermo Scientific). The mixture was
224 incubated at 65ºC for 30 minutes in a Veriti Thermal Cycler (Applied Biosystems).
225 Restriction fragments were electrophoretically separated on 1.5% agarose gels in 1X TAE
226 buffer for 60 minutes at 90v, stained with GelRed (Biotium) and visualized in an Image
227 Quant 350 (GE Healthcare) under UV light.

228 **3. Results**

229 3.1. *Lithodes* vs. *Paralomis*

230 All analyzed *Lithodes* specimens yielded three fragments after the digestion of the
231 amplified COI with either enzyme, since although several digestion conditions were tested,
232 a remnant of the undigested PCR product was always registered (i.e. 750bp, and two bands
233 resulting from the digestion, i.e. at 500bp and 250bp for Eco24I and at 450bp and 300bp
234 approximately for XmnI) (Figure 3). All analyzed *Paralomis* specimens yielded one
235 fragment after the digestion of the amplified COI with either enzyme (Figure 3).

236 3.2. *Lithodes santolla/ L. confundens* Clade 1 vs. Clade 2

237 All analyzed Clade 2 specimens yielded three fragments after the digestion of the
238 amplified COI with TscAI, since although several digestion conditions were tested, a
239 remnant of the undigested PCR product was always registered (i.e. at 750bp, and two bands
240 resulting from the digestion, i.e. at 450bp and 300bp approximately) (Figure 4). All
241 analyzed Clade 1 specimens yielded one fragment after the digestion of the amplified COI
242 with TscAI, although some negligible digestion of the PCR product was observed, yet it did
243 not interfere with the possibility of differentiating both clades (Figure 4).

244 **4. Discussion**

245 In the present study, we developed rapid and cost-efficient tests that can aid in the
246 identification of commercially important lithodids that inhabit waters off southern South
247 America in cases where identification is not possible using morphological characters. We
248 developed methods to differentiate southern king crabs from false southern king crabs, and
249 both clades of *L. santolla/ L. confundens* commercially exploited around the southern tip of
250 South America (see Pérez-Barros et al., 2015) without the need of sequencing. These

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251 methods could be used as a first approximation to detect potential food fraud (i.e. *Lithodes*
252 vs. *Paralomis*) and to monitor resources, e.g. abundance and distribution, and landings of
253 each lineage of *L. santolla/ L. confundens*. However, if unambiguous identification is
254 required, the specific identity should be corroborated by sequencing the COI fragment.

255 With respect to the presence of incomplete digestions, several RFLP conditions were
256 tested for a set of individuals in order to minimize this issue. In the first case (*Lithodes* vs.
257 *Paralomis*) in order to achieve the full digestion of the PCR product, different digestion
258 times (1, 2, 3 and 16 h), initial PCR product volumes (5 and 10 µl) and enzyme
259 concentrations (10 and 20U) were tested. We finally decided to digest 10 µl (< 100ng) of the
260 PCR product overnight with 10U enzyme in 30/50 µl final volume (as suggested by the
261 manufacturer for Eco24I and XmnI, respectively) since digesting a smaller volume of PCR
262 product resulted in low intensity of bands, and higher enzymatic concentrations resulted in
263 blurred bands in the gel. However, some undigested product was still observed after 16 h of
264 incubation at 37ºC. Notwithstanding, although the PCR product was not fully digested, all
265 *Lithodes santolla/confundens* individuals showed three bands, and all *Paralomis granulosa*
266 individuals showed one band after overnight digestion. In the second case, *Lithodes*
267 *santolla/confundens* Clade 1 vs. Clade 2, 10µl of PCR products were incubated with 10U
268 TscAI FastDigest enzyme at 65ºC in a final volume of 30 µl (as suggested by the
269 manufacturer) for 5, 15, 30, 60 minutes, and 6 h. In all cases some undigested product was
270 still observed (for Clade 2 individuals), even with the longest incubation time. Additional
271 conditions were tested, i.e. less PCR product (5 µl), more enzyme (20U), however this did
272 not change our results.

273 Regarding the possibility of applying these protocols to processed products, a
274 preliminary analysis (unpublished own data) showed that COI could be amplified with
275 Folmer´s primers from five out of six processed samples (two from ravioli filling, two
276 scalded and frozen and two in brine) corroborating their potential utility. Nevertheless, in
277 two out of the five samples that amplified, more than one PCR product was obtained
278 probably deriving from different species mixed in the processed product. In such cases,
279 PCR-RFLP methods may not be suitable.

280 In relation to the utility of the method developed to differentiate *L. santolla*/ *L.*
281 *confundens* Clade 1 from Clade 2, the procedure could be used to study each lineage´s
282 abundance and distribution, as well as to monitor the landings of king crabs from each
283 lineage. This is especially relevant in the Chilean fishery where Clade 2 seems to be more
284 abundant (Pérez-Barros et al., 2015). However, due to the fact that both *L. santolla/ L.*
285 *confundens* genetic clades cannot be morphologically differentiated, the method developed
286 to discriminate both lineages could not be further validated (i.e. with specimens not
287 previously used for the *in silico* development) without incurring in the cost of sequencing
288 to find new individuals pertaining to each genetic clade. Nevertheless, this should be done
289 in order to corroborate the restriction pattern observed so far.

290

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405 Table 1: *Lithodes* vs. *Paralomis*. Number of sequences used in the *in silico* development
406 and number of individuals for which the digestion was performed in the laboratory. The
407 numbers in parentheses correspond to individuals whose sequences had already been used
408 for the *in silico* development.

Species	*In silico*	In the laboratory	Total	GenBank accession numbers
Lithodes santolla/*L. confundens*[1]	123	66 (37)	152	KM887436-KM887471, KM887487-KM887497, HM020897, HM020898, HM020900 - HM020902, KC196532, KC196535- KC196538
Lithodes turkayi	4	0	4	KC196529, KC196531, KC196539, KC196540
Paralomis granulosa	47	39 (24)	62	HM020925, HM020926, MK619312-MK619356
Paralomis spinosissima	7	5	12	HM020927, HM020928, HM020929, HM020931, HM020932, HM020933, KC196534
Paralomis formosa	14	0	14	HM020918 - HM020924, EU493265, EU493262, KC196525 - KC196527, KC196530, KC196533
Paralomis anamerae	2	0	2	HM020905, HM020906

409 [1]Species complex.

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410 Table 2: *Lithodes* vs. *Paralomis*. Number of individuals used discriminated by locality.
411 The numbers in parentheses correspond to individuals whose sequences were digested in
412 the lab. Numbers in bold correspond to sequences with unknown locality (see accession
413 numbers in Table 1).

Locality	*Lithodes santolla/ L. confundens*[1]	*Lithodes turkayi*	*Paralomis granulosa*	*Paralomis spinosissima*
Golfo San Jorge	40 (12)			
Puerto Deseado			11 (5)	
Off Puerto Deseado	1 (1)		5 (5)	
Bahía Grande	13 (5)		1 (1)	
Río Grande	33 (14)		1 (1)	
NMPA/BB	24 (16)		10 (10)	5 (5)
Beagle Channel	21 (8)		32 (17)	
Puerto Montt	10 (10)			
Total	142 (66) + **10**	**4**	60 (39) + **2**	5 (5) + **7**

414 [1]Species complex.

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Table 3. *Lithodes santolla*/ *L. confundens* Clade 1 vs. Clade 2. Number of individuals used discriminated by locality. Numbers in parentheses correspond to individuals whose sequence was digested in the lab. Numbers in bold correspond to sequences with unknown locality (see accession numbers in Table 1).

Locality	*Lithodes santolla*/ *L. confundens*[1] Clade 1	*Lithodes santolla*/ *L. confundens*[1] Clade 2	*Lithodes turkayi*
Golfo San Jorge	33 (5)		
Bahía Grande	13 (5)		
Río Grande	24 (5)		
NMPA/BB	13 (5)		
Beagle Channel	18 (5)	2 (2)	
Puerto Montt	5 (5)	5 (5)	
Total	106 (30) + **7**	7 (7)	**4**

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422 Figure legends

423 Figure 1: Locations mentioned in the present study and geographic distributions of *Lithodes*
424 *santolla* (LS), *Lithodes confundens* (LC) and *Paralomis granulosa* (PG) (Sotelano et al.,
425 2013; Spivak et al., 2019).

426 Figure 2: Dorsal view of commercial specimens of (A) *Lithodes santolla*, (B) *Lithodes*
427 *confundens* and (C) *Paralomis granulosa*. Scale bar: 20mm.

428 Figure 3: Agarose gel electrophoresis of the amplified COI Folmer fragment of *Lithodes*
429 *santolla/ Lithodes confundens* and *Paralomis granulosa* after digestion with: (A) Eco24I
430 and (B) XmnI. Lanes 1 and 20: 100bp molecular weight standard, lanes 2 to 10: *L. santolla/*
431 *L. confundens*, lanes 11 to 19: *P. granulosa*.

432 Figure 4: Agarose gel electrophoresis of the amplified COI Folmer fragment of *Lithodes*
433 *santolla/ Lithodes confundens* Clade 1 and Clade 2 after digestion with TscAI. Lanes 1 and
434 20: 100bp molecular weight standard, lanes 2 to 8: *L. santolla/ L. confundens* Clade 2, lanes
435 9 to 19: *L. santolla/ L. confundens* Clade 1.

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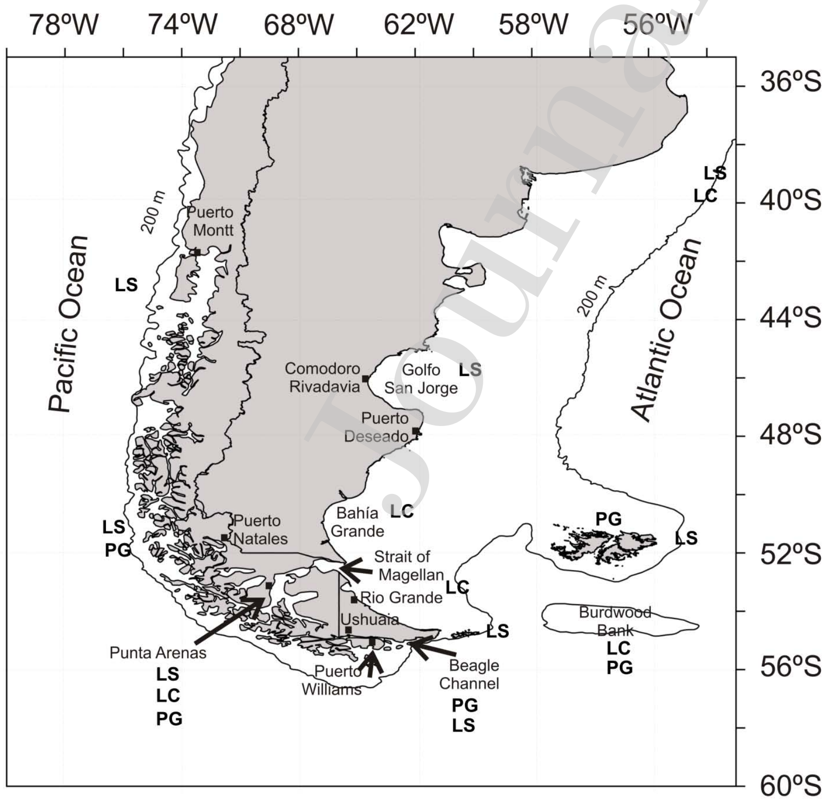

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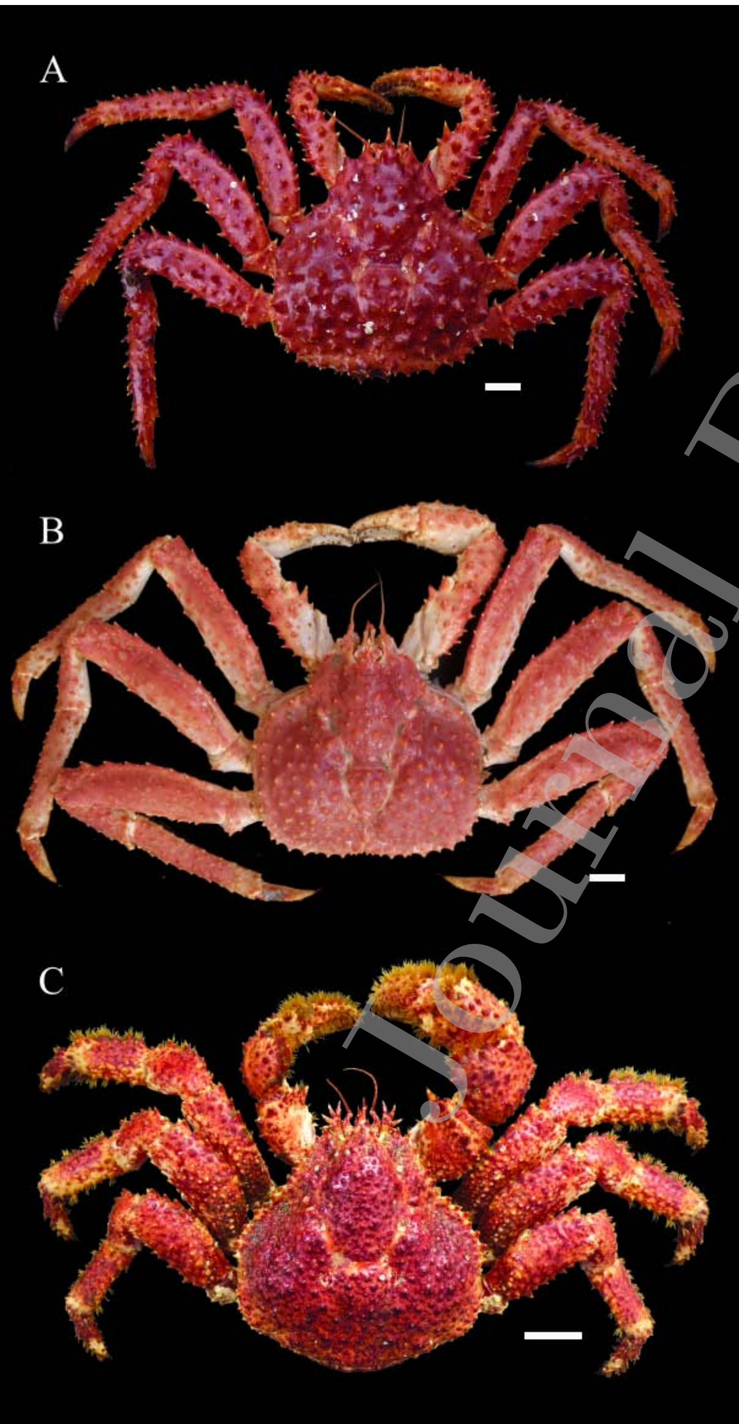

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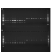

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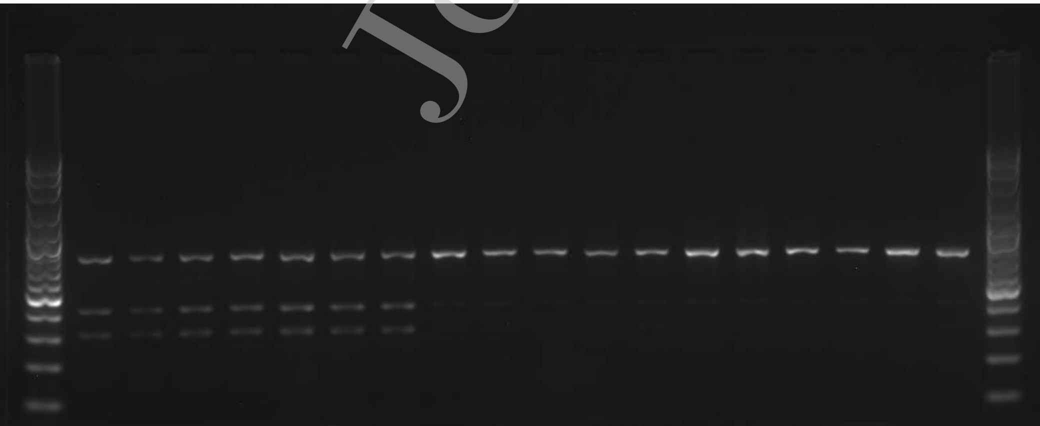

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Declaration of interests

✕ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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