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Short Communication

Genetic composition of *Mytilus* species in mussel populations from southern Chile

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ABSTRACT. Mussels are one of the most cultivated and commercialized bivalves worldwide and in southern Chile its culture represent an important economic activity. The species identification within the *Mytilus* genera, by morphological features, is unreliable, so we used a polymorphism RFLP in the gene encoding the polyphenolic adhesive protein as a species-specific genetic marker to describe *Mytilus* species diversity in southern Chile, and evaluate possible applications in traceability, food quality and safety. Using Me 15-16 marker most mussels were *M. chilensis*, finding no other pure individuals; however, putative hybrids of *M. chilensis* x *M. trossulus* and *M. chilensis* x *M. galloprovincialis* were detected. There was no evidence of *M. edulis*. The presence of the *M. trossulus* allele, faraway from its distribution area, demands further analysis with different genetic markers to allow a better understanding of its origin. In addition, the correspondence between markers that distinguishes northern from southern hemisphere *M. galloprovincialis*, with those who discriminates between *M. chilensis* and *M. galloprovincialis* would contribute to the taxonomic status of Chilean blue mussels. In Chile, the genetic composition of *Mytilus* indicates that geographical origin of mussels and its traceability cannot be established merely from the identification of the species. The use of other markers would be required.

Keywords: *Mytilus*, mussels, genetic identification, Me 15-16, PCR-RFLP, southern Chile.

Composición genética de especies de *Mytilus* en poblaciones de mejillón del sur de Chile

RESUMEN. Los mejillones son una de las especies de bivalvos más cultivadas y comercializadas, en el sur de Chile donde su cultivo representa una actividad económica importante. La identificación de la especie dentro del género *Mytilus*, basada en las características morfológicas no es confiable por lo que se utilizó un polimorfismo RFLP en el gen que codifica la proteína adhesiva polifenólica como marcador genético específico de la especie para describir la diversidad de especies *Mytilus* en el sur de Chile, y evaluar posibles aplicaciones en trazabilidad, calidad e inocuidad de los alimentos. Usando el marcador Me 15-16, la mayoría de los mejillones fueron *M. chilensis*, no se encontraron individuos puros de otras especies. Sin embargo, se detectaron híbridos putativos *M. chilensis x M. trossulus* y *M. chilensis x M. galloprovincialis*. No se encontró evidencia de *M. edulis*. La presencia del alelo de *M. trossulus*, lejos de su área de distribución, requiere de análisis posteriores con diferentes marcadores genéticos para entender su origen. Además, conocer la correspondencia entre marcadores que distinguen *M. galloprovincialis* del hemisferio norte y sur con aquellos que discriminan entre *M. chilensis* y *M. galloprovincialis* contribuirá al estatus taxonómico del mítilido chileno. La composición genética de *Mytilus* en Chile no permite establecer su origen geográfico ni la trazabilidad, basándose solo en la identificación de la especie y se requiere el uso de otros marcadores.

Palabras clave: *Mytilus*, mejillones, identificación de especies, Me 15-16, PCR-RFLP, sur de Chile.

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Marine mussels are one of the most cultivated and marketed bivalves, being the genus *Mytilus* widely used in prepared food products. There are various *Mytilus* species at higher latitudes in all the oceans and major seas of the world, in temperate zones of both hemispheres, but not reported in equatorial regions (Hilbish *et al.*, 2000). In addition, many genetic studies describe their ability to generate hybrid individuals when two species are in contact (Dias *et al.*, 2009; Kijewski *et al.*, 2009; Gardner & Westfall, 2012). Mussels develop for weeks, even months, in planktonic larval stages and thereby potentially dispersed over large geographical areas by marine currents or human-mediated activities (Hilbish *et al.*, 2002; Toro *et al.*, 2004). Biochemical and molecular characterization divided the morphologically classified *Mytilus edulis* Linnaeus, 1758 into a complex of three sibling species termed the *Mytilus edulis* species complex: *M. edulis, M. trossulus* Gould, 1850 and *M. galloprovincialis* Lamarck, 1819 (Westfall & Gardner, 2010). The genetic composition of these three taxa, now globally well defined, in the northern hemisphere (Wonham, 2004; Gérard *et al.*, 2008); in contrast, in southern hemisphere the composition is still under analysis.

The taxonomic status of Chilean blue mussel *M. chilensis* has been controversial for decades because of its phenotypic and genetic proximity to other species of the genus *Mytilus* from both hemispheres. Toro (1998), suggested the taxonomical status of *M. edulis chilensis;* then, Cárcamo *et al*. (2005) proposed *M. galloprovincialis chilensis* based on 30 allozyme loci. Current reports suggest that *M. chilensis* is a valid, distinct species within the genus *Mytilus*. Ouagajjou *et al.* (2011 based on nine microsatellite developed for *M. chilensis*, suggest that this is a distinct valid species within the genus, but recently other authors (Borsa *et al.*, 2012) proposed that native Chilean smooth-shelled *Mytilus*, should be named *M. edulis platensis* D'Orbigny, 1846.

In southern Chile, mussel culture is an important economic activity that yielded a 12.1% of the world's mitylid production in 2009 (FAO, 2011). Irrespective of the taxonomic status of Chilean mussels, regulatory and commercial interest to differentiate them from northern hemisphere *M. galloprovincialis* is a major concern to prevent improperly labeling [Regulations (CE)104/2000 and 2065/2001] ENREF 20, to protect consumers rights, to achieve food traceability, and to fulfill other quality objectives, such as designation of origin. To differentiate both species, Santaclara *et al.* (2006) developed a PCR-RFLP assay in polyphenolic adhesive protein cutting only *M. galloprovincialis* PCR product into two, allowing such discrimination. The interest in this differentiation has led to the recent developing of alternative PCR-RFLP methods for mussel *Mytilus* species identification (Fernández-Tajes *et al.*, 2011).

The objective of this study was to identify the species of *Mytilus* individuals using polyphenolic adhesive protein locus and RFLP–*Aci* I to describe *Mytilus* species diversity in southern Chile and assess potential applications in traceability and food quality and safety.

Samples of mussels (n = 50) were collected in southern Chile covering 11 sites from 41º31'S, 72º20'W to 50º50'S, 74º00'W (Fig. 1): one wild population, five seed collection centers and five on-growing centers, these last ones were supplied with seed from three collection centers. The samples were collected sub tidally. Shell size of 15-25 mm was considered seed and higher sizes as adult (Table 1). Control samples of *M. chilensis* and *M. galloprovincialis* were obtained from the hatchery of Universidad de Concepción in Dichato and commercial samples of *M. galloprovincialis* from Galicia (Spain). Up to 24 h after collection, mussels were dissected and a small piece of mantle edge tissue (approximately 200 mg) was removed, placed in a 1.5 mL Eppendorf tube, fixed with 95% ethanol and stored at -20ºC. DNA extraction was based on the simplified protocol of Taggart *et al.* (1992), approximately 50-100 mg tissue was coarsely chopped and digested overnight in 385 µL of lysis buffer (50 mM Tris HCl pH 8.0; 100 mM EDTA (ethylendiaminetetraacetic acid) pH 8.0; 100 mM NaCl; 1% SDS (sodium dodecyl sulphate) with 10 µL proteinase K (US Biologicals®) at 37ºC. The solution was digested for 1 h with 200 µg RNAse (US Biologicals®) at 37ºC. A precipitation step with 130 µL of saturated NaCl solution (6.1 M) followed by centrifugation (13,400 *g* x 10 min) was performed to remove mucopolysaccharides. The solution transferred to a clean tube, and 400 µL phenol was added and vigorously stirred for 10 s and then gently stirred during 20 min afterwards, 400 µL of chloroform–isoamyl alcohol (24:1) was added following the same agitation procedure. After centrifugation (9,300 *g* x 5 min), 380 µL of upper phase was taken to a clean tube and was ethanol precipitated. DNA extracted was resuspended in 100 µL of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) until complete dissolution. DNA integrity verified by 0.7% agarose gel electrophoresis, showed no degradation. Concentration and purity controls were done in spectrophotometer (NanoDrop, ND-1000), using samples with absorbance ratio 260 280^{-1} nm higher than 1.5. Concentration was adjusted to 20 ng μL^{-1} with 0.1 TE buffer. All DNA samples were stored at -18ºC.

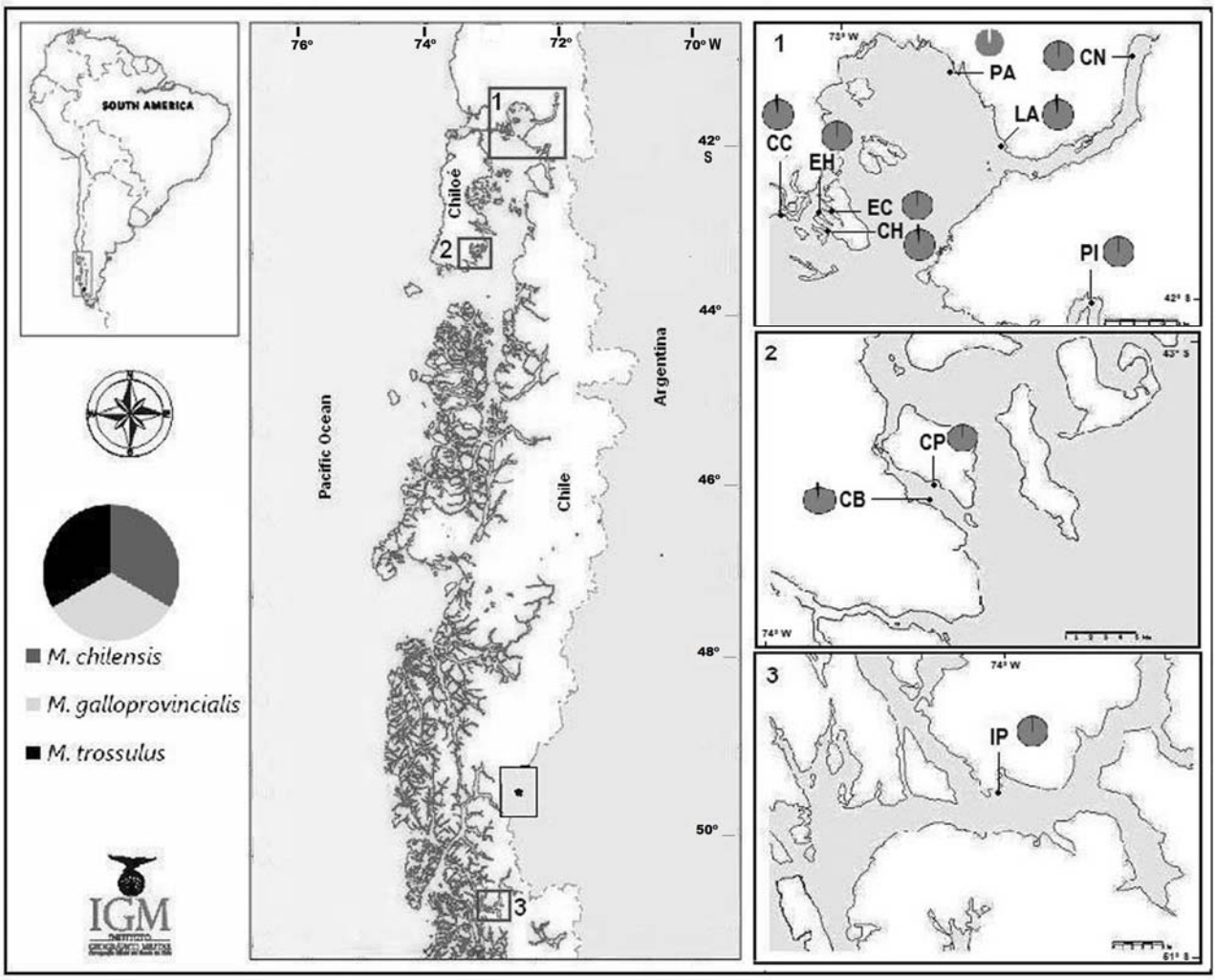

Figure 1. Location of sampling sites in southern Chile. Allele composition of *Mytilus* samples are shown as frequencies of alleles for the nuclear DNA marker Me 15-16 - RFLP *Aci* I.

Figura 1. Ubicación de los sitios de muestreo en el sur de Chile. Composición de alelos de las muestras de *Mytilus* se muestran como frecuencia de alelos para el marcador de ADN nuclear Me 15-16 - RFLP *Aci* I.

To prevent in the samples the unintended presence of individuals from other genera inhabiting the same geographical region -Cholga (*Aulacomya ater*) and Choro zapato (*Choromytilus chorus*)- a genus assignment was made. PCR-RFLP technique was accomplished according to Santaclara *et al.* (2006), using *MusRFLP* F and R primers to amplify a fragment of the gene encoding the small subunit rDNA (18S rDNA) and later digestion with endonuclease *Bsa* HI (New England Biolabs®).

Species identification was performed by PCR using the primers Me 15-16 of Inoue *et al.* (1995). RFLP-*Aci* I (Fermentas®) was used to differentiate between *M. galloprovincialis* and *M. chilensis* (Santaclara *et al.*, 2006). Each PCR was undertaken in 25 µL. A negative control without DNA template added and a positive control with DNA from control samples of *M. chilensis* and *M. galloprovincialis* were included in all PCR assays.

Currently DNA amplifications were performed in a thermocycler Techne TC-412 (Bibby Scientific Ltd®, UK), with high quality recombinant Taq DNA polymerase (RBC Bioscience®), and PCR-grade water. PCR products were visualized in agarose gels (1.8%) in TBE buffer with 10 mg mL^{-1} of ethidium bromide for band detection under ultraviolet light and in Polyacrylamide gels (8%), with silver staining. For every gel, the size of amplified fragments was estimated from 10 bp DNA ladder (Invitrogen®) or Hyperladder V (BioLine®).

Amplicons of 168 bp and 126 bp from CB-Canal Coldita-Piedra Blanca, were isolated from the gel,

Table 1. Data of mussel samples (n = 50): sampling location, source, sampling date and stage of development. Besides, for on-growing centers, seed origin (in brackets) and beginning of culture date.

Tabla 1. Datos de las muestras de mejillón (n = 50): lugar de muestreo, origen, fecha de toma de muestra y etapa de desarrollo. Además, para centros de engorda, origen de la semilla (entre paréntesis) y fecha de comienzo del cultivo.

Code	Sampling location South latitude / West longitude	Source (seed origin)	Beginning of culture date	Sampling date	Stage of development
IP	Isla Peel 50º 50' 29,83" / 74º 00' 41,27"	Wild population	-	16.02.2009	Adult
PA	Piedra Azul 41º 32' 55, 35" / 72º 46' 14,35"	Seed collection center	-	24.06.2009	Seed
PI	Pichicolo 42º 02' 23,76" / 72º 35' 27,17"	Seed collection center	-	24.06.2009	Seed
LA	Caleta La Arena 41º 41' 00,00" / 72º 40' 18,92"	Seed collection center	-	24.06.2009	Seed
CN	Canutillar 41º 31' 13,9" / 72º 20' 15,69"	Seed collection center	-	24.06.2009	Seed
CB	Canal Coldita - Piedra Blanca 43º 14' 48,82" / 73º 41' 42,77"'	Seed collection center	-	25.06.2009	Seed
CP	Canal Coldita - Patagonia 43º 14' 02,98" / 73º 41' 37,64"	On growing center (CB)	2008	25.06.2009	Adult
CC	Canal Caicaén 41º 47' 47,41" / 73º 10' 5,55"	On growing center (LA)	29.09.2008	27.06.2009	Adult
EC	Estero Chauquiar 41º 47' 29,0" / 73º 04' 55"	On growing center (LA)	19.05.2009	27.06.2009	Seed
CH	Canal Chidguapi 41º 49' 10,39" / 73º 06' 4,29"	On growing center (CN)	07.08.2008	27.06.2009	Adult
EH	Estero Chope 41º 48' 31,23" / 73º 05' 27,08"	On growing center (CN)	06.06.2009	27.06.2009	Seed

reamplified, purified with FavorPrep® Gel purification kit (Favorgen®) and sent to be sequenced in both directions with primers Me 15-16. Consensus sequences were obtained using Multalin (http://multalin.toulouse.inra.fr/multalin/multalin.html), and in order to verify their identity, alignments with sequences of polyphenolic adhesive protein of *M. trossulus* (DQ640589.1) and *Mytilus* sp. (AF489933.1) were performed with BLAST (bl2seq) (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Scoring with the Me 15-16 primers was possible for all 550 *Mytilus* genera individuals sampled from the 11 localities. After the RFLP–*Aci* I digestion of all individuals showing a single 126 bp PCR product, *M. chilensis* alleles were the most (98-100%) in all collection places, as expected from this native species in commercial production in Chile. In six sites (IP-Isla Peel, PI-Pichicolo, CN-Canutillar, CP-Canal Coldita–Patagonia, EC-Estero Chauquiar y EH-Estero Chope) only *M. chilensis* alleles (one fragment 126 bp and no restriction site for *Aci* I) were found. Only homozygous individuals were identified for *M. chilensis* alleles, no other *Mytilus* homozygous were found in this study (Table 2).

M. galloprovincialis allele (126 bp and restriction site for *Aci* I) was identified only in PA-Piedra Azul collection center, in a single individual (Figs. 2 and 3) that after the digestion showed three fragments of 126, 75 and 51 bp and was considered a putative hybrid *M. chilensis x M. galloprovincialis.* The same restriction pattern size (75 and 51 bp) was found in control samples of *M. galloprovincialis* from Dichato-Chile, and commercial samples from Galicia-Spain (Fig. 3). The difference in the restriction fragment size described by Santaclara *et al.* (2006) and the one observed in our study was probably due to the different methods employed to estimate size (sequencing *vs* 8% polyacrylamide gel electrophoresis). Pure *M. galloprovincialis* individuals were not found in the studied area, as was the case in

Table 2. Number of individuals of each genotype -*Mytilus chilensis (Mch), M. chilensis x M. galloprovincialis* apparent hybrids *(Mch x Mg), M. chilensis x M. trossulus* apparent hybrids *(Mch x Mt)*- found in samples (n = 50) from the 11 sites studied.

Tabla 2. Número de individuos de cada genotipo -*Mytilus chilensis (Mch)*, hibridos aparentes *M. chilensis x M. galloprovincialis (Mch x Mg)*, híbridos aparentes *M. chilensis x M. trossulus (Mch x Mt)*- encontrados en las muestras (n = 50) de los 11 lugares estudiados.

Code	Sampling location	*Mch*	*Mch x Mg*	*Mch x Mt*
	Wild population			
IP	Isla Peel	50	0	0
	Seed collection center			
PA	Piedra Azul	49	1	0
PI	Pichicolo	50	0	0
LA	Caleta La Arena	48	0	2
CN	Canutillar	50	0	0
CB	Canal Coldita - Piedra Blanca	48	0	2
	On growing center			
CP	Canal Coldita - Patagonia	50	0	0
EC	Estero Chauquiar	50	0	0
EH	Estero Chope	50	0	0
CH	Canal Chidguapi	48	0	2
CC	Canal Caicaén	48	0	2

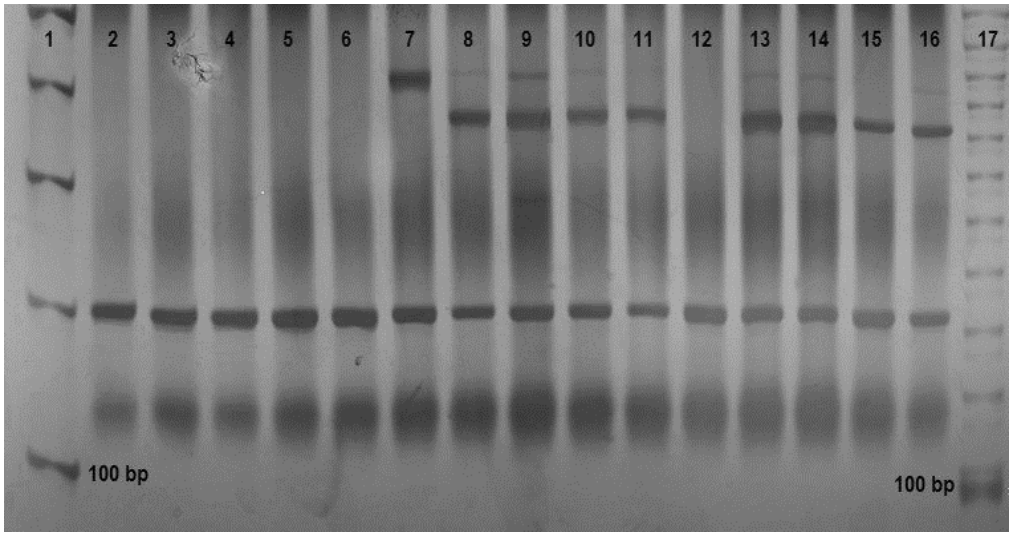

Figure 2. Polyacrylamide gel (8%) with Me 15-16 PCR products: 1. Molecular size standard (25 bp) Lanes 2-6. Control individuals, 2. *M. chilensis* from Dichato-Chile 3-4. *M. galloprovincialis* from Dichato-Chile 5-6. *M. galloprovincialis* from Galicia-Spain 7. Heterozygous mussel with 126 bp and 180 bp alleles from Galicia-Spain 8-11 and 13-16. Heterozygous mussel with 126 bp and 168 bp allele from southern Chile 12. Heterozygous mussel with 126 bp allele and restriction site for *Aci* I from southern Chile 17. Molecular size standard (10 bp).

Figura 2. Gel de poliacrilamida (8%) con los productos de PCR Me 15-16: 1. Estándar de tamaño molecular (25 pb) Lineas 2-6. Individuos control 2. *M. chilensis* de Dichato-Chile 3-4. *M. galloprovincialis* de Dichato-Chile 5-6. *M. galloprovincialis* de Galicia-España 7. Mejillón heterocigoto de Galicia-España con alelos de 126 pb y 180 pb 8-11 and 13-16. Mejillones heterocigotos del sur de Chile con alelos de 126 pb y 168 pb 12. Mejillón heterocigoto del sur de Chile con alelo de 126 pb y sitio de restricción para *Aci* I 17. Estándar de tamaño molecular (10 pb).

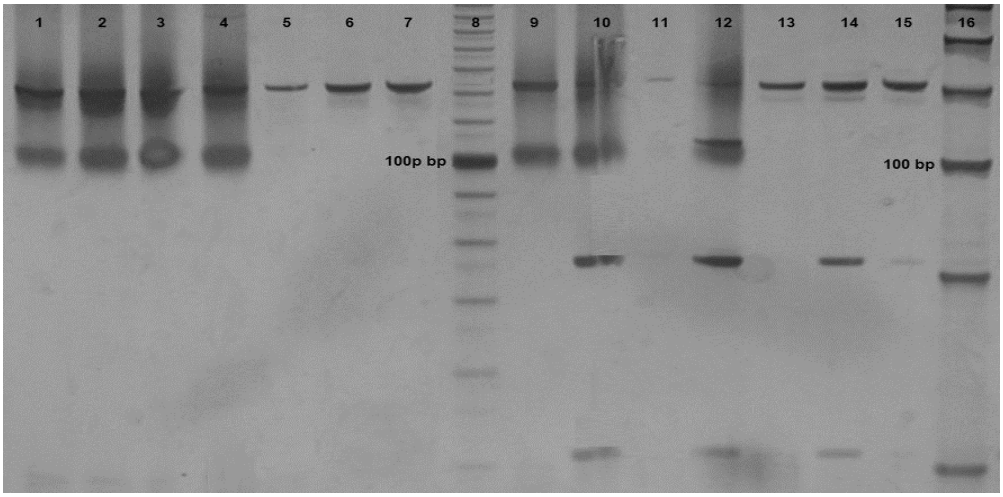

Figure 3. Polyacrylamide gel (8%) of PCR Me 15-16 -RFLP *Aci* I analysis: Lanes 1-4. Control individuals 1. *M.chilensis* from Dichato-Chile 2. *M. galloprovincialis* from Dichato-Chile 3. *M. galloprovincialis* from Galicia-Spain 4. Heterozygous mussel with 126 bp and 180 bp alleles from Galicia-Spain 5-7. Mussels with 126 bp allele from southern Chile 8. Molecular size standard (10 bp). 9-16. After digestion with *Aci* I. Lanes 9-12. Control individuals 9. *M. chilensis* from Dichato-Chile 10. *M. galloprovincialis* from Dichato-Chile 11. *M. galloprovincialis* from Galicia-Spain 12. Heterozygous mussel with 126 bp and 180 bp alleles from Galicia-Spain 13 and 15. Mussels with 126 bp allele from southern Chile 14. Heterozygous mussel with 126 bp allele and restriction site for *Ac i*I from southern Chile 16. Molecular size standard (25 bp).

Figure 3. Gel de polyacrylamida (8%) del análisis PCR Me 15-16 -RFLP *Aci* I: Lineas 1-4. Individuos control 1. *Mytilus chilensis* de Dichato-Chile 2. *M. galloprovincialis* de Dichato-Chile 3. *M. galloprovincialis* de Galicia-España 4. Mejillón heterocigoto de Galicia-España con alelos 126 pb y 180 pb 5-7. Mejillones del sur de Chile con alelos 126 pb 8. Estándar de tamaño molecular (10 pb). 9-16. Después de digestión con *Aci* I. Lineas 9-12. Individuos control 9. *M. chilensis* de Dichato-Chile 10. *M. galloprovincialis* de Dichato-Chile 11. *M. galloprovincialis* de Galicia-España 12. Mejillón heterocigoto con alelos de 126 pb y 180 pb de Galicia-España 13 y 15. Mejillones del sur de Chile con alelo de 126 pb 14. Mejillón heterocigoto del sur de Chile con alelo 126 pb y sitio de restricción para *Aci* I 16. Estándar de tamaño molecular (25 pb).

Scottish shellfish farms and in the Oostershelde estuary in the Netherlands (Dias *et al.*, 2009; Kijewski *et al.*, 2009). The low frequency of the *M. galloprovincialis* allele found in our survey and the limited sample size obtained in each location (n = 50), could cause underreport of *M. galloprovincialis* in southern Chile. There might be a wider distribution of this species within the sampled locations.

We did not find *M. edulis* alleles (180 bp). A maximum of two alleles from different species were found per sampling location. In this study, no site presented the three alleles altogether (Table 2).

M. trossulus alleles (168 bp) were detected together with *M. chilensis* alleles in 4% of individuals from collection centers (LA-Caleta La Arena and CB-Canal Coldita-Piedra Blanca) and on-growing centers (CC-Canal Caicaén and CH-Canal Chidguapi); these apparent hybrids exhibited amplicons of 126 bp and 168 bp. There was no clear relationship between on-growing centers and seed collection centers regarding the presence of the 168 bp allele. The aligned sequences of amplicons of 168 bp and 126 bp obtained from CB-Canal Coldita-Piedra Blanca, supposedly *M. trossulus* and *M. chilensis* alleles respectively (Table 3), showed high similitude with the polyphenolic protein gene sequences from GenBank, permitting to verify their identity.

M. trossulus is circumpolar in the northern hemisphere, but ambiguously been identified in the southern hemisphere (Wonham, 2004). Previous information (McDonald *et al.*, 1991), described that South American blue mussels contain iso-enzymatic alleles of the three *Mytilus* species and recently, Fernández-Tajes *et al.* (2011), found two individuals with *M. chilensis* and *M. trossulus* alleles in canned samples of *M. chilensis*, and confirmed their identity by sequencing the *M. trossulus* amplicon. Faraway from *M. trossulus* distribution area, the presence of the 168 bp allele is quite surprising. Further analysis with different genetic markers on larger number of

Table 3. Score, E value and Maximum identities resulting from the alignments of amplicons of 168 bp and 126 bp obtained from CB-Canal Coldita-Piedra Blanca with sequences of polyphenolic adhesive protein of *M. trossulus* and *Mytilus* sp. from Gene Bank.

Tabla 3. Score, valor E y máxima identidad resultantes de alinear los amplicones de 168 pb y 126 pb obtenidos de CB-Canal Coldita-Piedra Blanca con secuencias de la proteína adhesiva polifenólica de *M. trossulus* y *Mytilus* sp. de Gene Bank.

	Polyphenolic adhesive protein				
Fragment size [pb]	Species	NCBI Accesion nº	Score [bits]	E value	Max identity [%]
168	*M. trossulus*	DQ640589.1	208	5,E-45	96
126	*Mytilus* sp.	AF 489933.1	150	2,E-29	93

specimens and sequencing of the Me 15-16 fragment would allow a better understanding of the origin of the 168 bp allele in the studied region.

Testing correspondence between the 16s rRNA RFLP marker, that distinguishes the northern from southern hemisphere *M. galloprovincialis*, and markers that allowed the discrimination between *M. chilensis* and *M. galloprovincialis*, Me 15-16 RFLP *Aci* I and the COIXbarF/R RFLP *Xba* I recently developed by Fernández-Tajes *et al.* (2011), in a wider range of southern hemisphere *Mytilus* samples would contribute to the taxonomic status of Chilean blue mussel. Our results, and the previously discussed studies, about the presence of *M. galloprovincialis* in Chile indicated that the geographical origin of mussels and its traceability, cannot be established merely from the identification of the species but would require the use of other markers such as microsatellites (Presa & Diz, 2002; Gardeström *et al.*, 2007).

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