

# Native and invasive taxa on the Pacific coast of South America: Impacts on aquaculture, traceability and biodiversity of blue mussels (*Mytilus* spp.)

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## Abstract

Gaining new knowledge of the native distributions of species (phylogeography) is more and more difficult in a world affected by anthropogenic disturbance, in particular by species translocations. Increasingly, molecular markers are required to support decisions about the taxonomy of native vs. introduced species, and the existence of their hybrids, to answer phylogeographic questions. In many fields, including aquaculture, traceability and food security, taxonomic and phylogeographic knowledge is key to the successful management and conservation of biodiversity. The Pacific coast of Chile is one of the last regions without a clear and agreed understanding of the taxonomy and systematics of smooth-shelled blue mussels of the genus *Mytilus*. A panel of 49 bi-allelic single nucleotide polymorphisms (SNPs) was genotyped in 338 *Mytilus* individuals collected from nine Chilean and five reference populations. All analyses confirmed the hypothesis that the native Chilean blue mussel is genetically distinct from the reference species *M. edulis*, *M. galloprovincialis* and *M. trossulus*. These results support the hypothesis of a unique evolutionary history of the native Chilean blue mussel on the Pacific coast of South America. It is therefore concluded that the native blue mussel from Chile should be recognized as *M. chilensis* Hupé 1854. We confirmed a recent Mediterranean origin of introduced *M. galloprovincialis* on the coast of Chile. This knowledge advances the understanding of global phylogeography of blue mussels and their bioinvasions and harmonizes taxonomy in the context of aquaculture production, seafood traceability, labelling and trade.

## KEYWORDS

aquaculture, conservation genetics, food security, invasive species, *Mytilus chilensis*, *Mytilus galloprovincialis*

## 1 | INTRODUCTION

Determining the native distributions of species and thereby establishing baseline areas of natural ranges in the absence of anthropogenic

disturbance is a key component of phylogeography (Avice, 2000). Such research is now often underpinned by taxonomic classifications and phylogenies that are based on molecular differentiation at one or more marker types (Morin, Luikart, & Wayne, 2004; Pereira, Carneiro,

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& Amorim, 2008). The determination of distributions of native species is not only important in its own right, but is applied in a variety of different management aspects, such as aquaculture, food security, traceability and labelling. Food fraud is now a bigger problem than ever, including dilution, mislabelling, ingredient substitution and tampered with products. This problem is addressed in recent international legislation (E.U. 2013; FDA 2011). Related to aquaculture, marine bioinvasions that may affect cultivated species are now being detected in many production areas and also in remote regions including offshore islands and Antarctica (Gardner, Zbawicka, Westfall, & Wenne, 2016; Lee & Chown, 2007; Shaw, Terauds, Riddle, Possingham, & Chown, 2014). It has been suggested that we are no longer in a position to wait for the establishment of native species baselines and that immediate management action is required (Ojaveer et al., 2015). Thus, taxonomy has a key role to play in the protection and sustainable exploitation of species (Mace, 2004; Seddon, Parker, Ostrander, & Ellegren, 2005).

Blue mussels of the genus *Mytilus* exhibit an antitropical distribution (Hilbish et al., 2000). They are an important source of protein for many coastal communities and are farmed commercially in many countries (FAO 2016; Ferreira & Bricker, 2016). Smooth-shelled *Mytilus* species are characterized by a high degree of phenotypic plasticity that has limited power to discriminate amongst the taxa, but they can now be identified with much greater certainty using genetic molecular markers (Gardner et al., 2016; Jilberto, Araneda, & Larraín, 2017; Wenne, Bach, Zbawicka, Strand, & McDonald, 2016; Wennerstrom et al., 2013; Zbawicka, Drywa, Śmietanka, & Wenne, 2012; Zbawicka, Sanko, Strand, & Wenne, 2014). Traditionally, biochemical and molecular characterization has divided the *Mytilus edulis* species complex into three sibling species: *M. edulis* (Linnaeus, 1758), *M. trossulus* (Gould, 1850) and *M. galloprovincialis* (Lamarck, 1819) (Koehn, 1991; McDonald, Seed, & Koehn, 1991). Although the distributions of these taxa have been studied extensively in the Northern Hemisphere, in the Southern Hemisphere taxon distributions do not always fit the classical "*Mytilus edulis* complex" scheme. Whilst we have a better (but still not completely resolved) understanding of the situation in Australia and New Zealand (Ab Rahim et al., 2016; Gardner et al., 2016; Westfall and Gardner, 2010, 2013), the situation in South America still requires resolution (Gaitán-Espitia, Quintero-Galvis, Mesas, & D'Elía, 2016; Oyarzún, Toro, Cañete, & Gardner, 2016).

The taxonomic status of native smooth-shelled blue mussels inhabiting the coast of Chile has been unclear and disputed for a number of years, with authors advancing different suggestions for its nomenclature, including *M. edulis*-like (Koehn, 1991; McDonald et al., 1991), *M. edulis chilensis* (Toro, 1998), *M. galloprovincialis chilensis* (Cárcamo, Comesaña, Winkler, & Sanjuan, 2005), *M. galloprovincialis* (Toro, Ojeda, Vergara, Castro, & Alcapán, 2005), *M. edulis platensis* (Borsa, Rolland, & Daguin-Thiébaud, 2012), *M. galloprovincialis* lineage of Southern Hemisphere origin (Westfall & Gardner, 2013), *M. planulatus* and *M. platensis* (Astorga, Cardenas, & Vargas, 2015). These different taxonomic designations may have arisen from a lack of clarity about which mussel species occur at specific sites and because the number and type of markers used in each research project are different (Borsa et al., 2012; Larraín et al., 2015). Despite the lack of agreement

about the taxonomic status of the native Chilean mussel, the term *M. chilensis* has long been employed on food product labels and in scientific articles (Araneda, Larraín, Hecht, & Shawn, 2016; Astorga, 2014; Larraín, Díaz, Lamas, Uribe, & Araneda, 2014; Ouagajjou, Presa, Astorga, & Pérez, 2011; Oyarzún et al., 2016). The name is also used in aquaculture production statistics (FAO 2016) and on good aquaculture practice certifications (GAA 2013). However, as several authors have pointed out, the name has historically had no formal taxonomic standing. This controversy and the history of the discussion are reflected in changes listed in the Word Register of Marine Species (Horton et al., 2017).

*Mytilus* species in Chile, both native and introduced, have been studied extensively using nuclear (RFLP-PCR *Me 15-16*, *mac-1*, *Glu-5*, *ITS*) and/or mitochondrial (*16s rDNA* RFLP, *COI*, *COIII*) molecular markers (Borsa et al., 2012; Fernandez-Tajes et al., 2011; Gérard, Bierne, Borsa, Chenuil, & Féral, 2008; Larraín, Díaz, Lamas, Vargas, & Araneda, 2012; Tarifeño et al., 2012; Toro, 1998; Toro et al., 2005; Westfall & Gardner, 2010, 2013). The primary problem has been that these markers target different regions of the genome that have different evolutionary rates, giving nonequivalent results when a few are used simultaneously (Kijewski et al., 2011; Rawson, Agrawal, & Hilbish, 1999; Zbawicka et al., 2012). Whilst the monolocus approach has the advantage of being easy to apply, it has the drawback that one locus represents a low power approach to delimit a species. A secondary problem arises from the fact that all smooth-shelled *Mytilus* taxa interbreed extensively (Michalek, Ventura, & Sanders, 2016). Such hybridization may complicate taxonomic resolution by blurring species boundaries and is not reflected in maternally inherited mtDNA markers that are often used for species identification, and also the evolutionary history of extensive interbreeding and introgression is not well represented by only a few nuclear DNA markers. What has been missing until recently is nuclear DNA markers that are species-specific and that can resolve ancestry when hybridization occurs either naturally or as a result of anthropogenic transfer of mussel types to non-native regions. Recent advances in next-generation sequencing methods, along with the increase in sequence data deposited in public databases, now permit the development of genomewide single nucleotide polymorphism markers (SNPs) that may be applied in mussel studies to resolve many of these issues because they cover multiple regions of the genome (Araneda et al., 2016; Mathiesen et al., 2017; Saarmann & Pogson, 2015; Zbawicka et al., 2012; Zbawicka, Sanko et al., 2014).

Blue mussels have long been farmed in many regions of the world (Kijewski, Wijsman, Hummel, & Wenne, 2009; Molinet et al., 2015). Chile is now the world's second largest mussel (family Mytilidae) aquaculture producer (FAO 2016). Production is concentrated in the Gulf of Reloncaví and along the coastline of Chiloé Island (Los Lagos region) and is based on the native blue mussel, nominally *Mytilus chilensis*. However, other species in Chile have also been reported, including *M. galloprovincialis* in the Gulf of Arauco-Bío-Bío region (Astorga et al., 2015; Daguin & Borsa, 2000; Tarifeño et al., 2012; Westfall & Gardner, 2010) and *M. edulis* in the Strait of Magellan-Magallanes region (Oyarzún et al., 2016), although this last species is

named as *M. platensis* by Gaitán-Espitia et al. (2016). In addition, at a limited number of locations along the Chilean coast, allele characteristics of *M. trossulus* (but not *M. trossulus* mussels themselves) have also been reported (Larraín et al., 2012; Oyarzún et al., 2016). Because accurate identification of mussel species produced by aquaculture is necessary for labelling, traceability, food security and marketing purposes (Larraín et al., 2012, 2014), it is important to understand which species is being grown where, which are native mussels, if native and introduced mussels interbreed, and if/how invasive mussels may affect aquaculture.

Hybridization between *Mytilus* taxa has been reported in all geographic areas where two or more species coexist (Gardner, 1997) and hybridization patterns are complicated additionally by the occurrence of two mitochondrial lineages, female and male, and their recombination and introgression (Filipowicz, Burzyński, Śmietanka, & Wenne, 2008; Zbawicka, Wenne, & Skibinski, 2003; Zbawicka, Wenne et al., 2014). Significantly, a number of studies have now demonstrated that hybridization of *Mytilus* taxa in aquaculture can cause unwanted or harmful effects to the industry and/or to native mussel populations (Crego-Prieto et al., 2015; Dias, Fotedar, & Snow, 2014; Michalek et al., 2016).

In this article, we describe molecular genetic analyses of blue mussels from the coast of Chile, one of the last biogeographic regions without a clear and universally agreed understanding of its mytilid taxonomy. Using a 49 SNP panel against blue mussels from multiple locations in Chile and reference species, we test the hypothesis that the native Chilean blue mussel (*M. chilensis*) is an endemic species, with its own unique evolutionary history in the Southern Hemisphere. Our aim was to contribute to the understanding of the taxonomic status of native smooth-shelled blue mussels and to test for the presence of other *Mytilus* taxa in Chile. This knowledge has practical applications by providing tools to solve issues related to aquaculture policy and management and highlights the potential threats to native mussel populations and aquaculture posed by introduced taxa along the Chilean coast. The SNP panel has direct global uses in seafood labelling, traceability and food security, and regionally by setting the basis for a protected origin designation for native Chilean mussels.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample collection and DNA extraction

Samples ( $n = 338$ ) of *Mytilus* spp. were collected from nine locations in Chile spanning almost all of the native mussel's distributional range (~2,500 km) and from five regions as reference samples: Pacific coast of Canada, Northern Ireland, Italy, New Zealand and Spain (Table 1, Figure 1). Provisional species identification of each individual was determined using PCR of the nonrepetitive region of the polyphenolic adhesive protein gene with the RFLP *Acil* method with primers Me15-16 (Santacarla et al., 2006) or directly by genotyping the equivalent SNP locus BM151A (Gardner et al., 2016). Reference samples of *M. edulis*, *M. trossulus* and *M. galloprovincialis*

were previously genotyped using SNPs (Zbawicka et al., 2012; Zbawicka, Sanko et al., 2014).

### 2.2 | SNP genotyping

In total, 338 *Mytilus* samples were genotyped using the Sequenom MassARRAY iPLEX genotyping platform (Gabriel, Ziaugra, & Tabbaa, 2009). Assays were designed for 79 candidate SNPs, selected from 385 putative SNPs, which were tested on 300 specimens of *Mytilus* collected from geographic regions including Europe, North and South America and New Zealand. Mussel SNPs were genotyped as previously described and following earlier testing of their reproducibility (Gardner et al., 2016; Zbawicka et al., 2012; Zbawicka, Sanko et al., 2014).

### 2.3 | Data analysis

Observed ( $H_O$ ) and expected ( $H_E$ ) heterozygosities and exact tests of departure from Hardy-Weinberg equilibrium (HWE) were determined using the R package adegenet 3.1-1 (Jombart, 2008). Significance was determined by Markov chain Monte Carlo with 100,000 simulations, and the Benjamini and Yekutieli false discovery rate (FDR-BY) was used to correct significance ( $p$ ) values after multiple testing (Benjamini & Yekutieli, 2011). Genetic differentiation amongst populations was determined using global and pairwise  $F_{ST}$  (theta) values (Weir & Cockerham, 1984), and the 95% confidence intervals for  $F_{ST}$  were estimated by bootstrapping with 10,000 replicates using the R package diveRcity (Keenan, McGinnity, Cross, Crozier, & Prodöhl, 2013). The  $F_{ST}$  distance matrix was used to construct a neighbour-joining (NJ) tree illustrating the genetic relations of all populations, using POPTREEW (Takezaki, Nei, & Tamura, 2014).

Cluster analysis was performed using three unsupervised methods: (i) discriminant analysis of principal component (DAPC) performed with adegenet 3.1-1 (Jombart, Devillard, & Balloux, 2010) where the number of clusters ( $K$ ) was identified using the Bayesian information criteria (BIC). DAPC variation was plotted using a  $K = 14$  to match mussel populations with clusters; (ii) the nonparametric method implemented in AWclust (Gao & Starmer, 2008) that identifies the number of clusters based on a gap statistic; and (iii) the Bayesian-based method implemented in STRUCTURE with no prior information about the origin of individuals (Pritchard, Stephens, & Donnelly, 2000) assuming admixture and allowing for the correlation of allele frequencies between clusters. The tested number of clusters ( $K$ ) ranged from 1 to the number of sampling locations plus 1. The length of burn-in period and the number of MCMC cycles after burn-in was 1,000,000 iterations each. Six runs were carried out for each  $K$ , and we used the Evanno, Regnaut, and Goudet (2005) method to identify the single value of  $K$  which captures the uppermost level of structure in Structure Harvester (Earl, 2012).

Genetic assignment was performed to assign or exclude sampled populations as possible origins of individuals, using the frequency criteria of Paetkau, Calvert, Stirling, and Strobeck (1995) in a self-assignment test with the leave-one-out (LOO) procedure, implemented

**TABLE 1** Sample location, genotype designation based on Me 15-16 and SNP BM151A assays, number of individuals, sampling date and stage of development

Code	Sampling location—Country Latitude/Longitude	Genotype Me 15-16	Genotype SNP BM151A	No.° of individuals	Sampling date	Stage of development
Chilean samples						
COCL	Cocholgue—Chile S: 35°7'38.62"/W: 73°11'25.56"	126/126	GG	20	2007	Adult
QICL	Quillaípe—Chile S: 41°32'55.35"/W: 72°46'14.35"	126/126	TT	20	2009	Seed
LACL	Caleta La Arena—Chile S: 41°41'00.00"/W: 72°40'18.92"	126/126	TT	27	2009	Seed
PICL	Pichicolo—Chile S: 42°02'23.76"/W: 72°35'27.17"	126/126	TT	30	2009	Seed
ABCL	Abtao—Chile S: 42°24'0.54"/W: 74°10'48.49"	126/126	TT	17	2013	Adult
CBCL	Canal Coldita—Piedra blanca—Chile S: 43°14'48.82"/W: 73°41'42.77"	126/126	TT	29	2009	Seed
GTCL	Golfo Trinidad—Chile S: 49°57'59.69"/W: 75°11'12.99"	126/126	TT	5	2002	Adult
IPCL	Isla Peel—Chile S: 50°50'29.83"/W: 74°00'41.27"	126/126	TT	28	2009	Adult
PUCL	Punta Arenas—Chile S: 53°9'16.12"/W: 70°54'59.31"	126/126	TT	33	2012	Adult
Reference samples						
ORIT <sup>a</sup>	Oristano—Italy N: 39°47'59.88"/E: 8°31'9.72"	126/126	GG	29	2004	Adult
CAES	Punta Camarinal—Spain N: 36°4'48.01"/W: 5°47'58.00"	126/126	GG	29	2004	Adult
WENZ	Wellington—New Zealand S: 41°22'12.19"/E: 174°47'39.89"	126/126	TT	27	2005	Adult
LFGB	Lough Foyle – Northern Ireland N: 55°5'35.50"/W: 7°4'48.92"	180/180	GG	27	2006	Adult
VACA <sup>a</sup>	Vancouver—Canada N: 49°18'33.75"/W: 123°49'15.41"	168/168	GG	17	2006	Adult
Total				338		

<sup>a</sup>These samples were also used by Gardner et al. (2016).

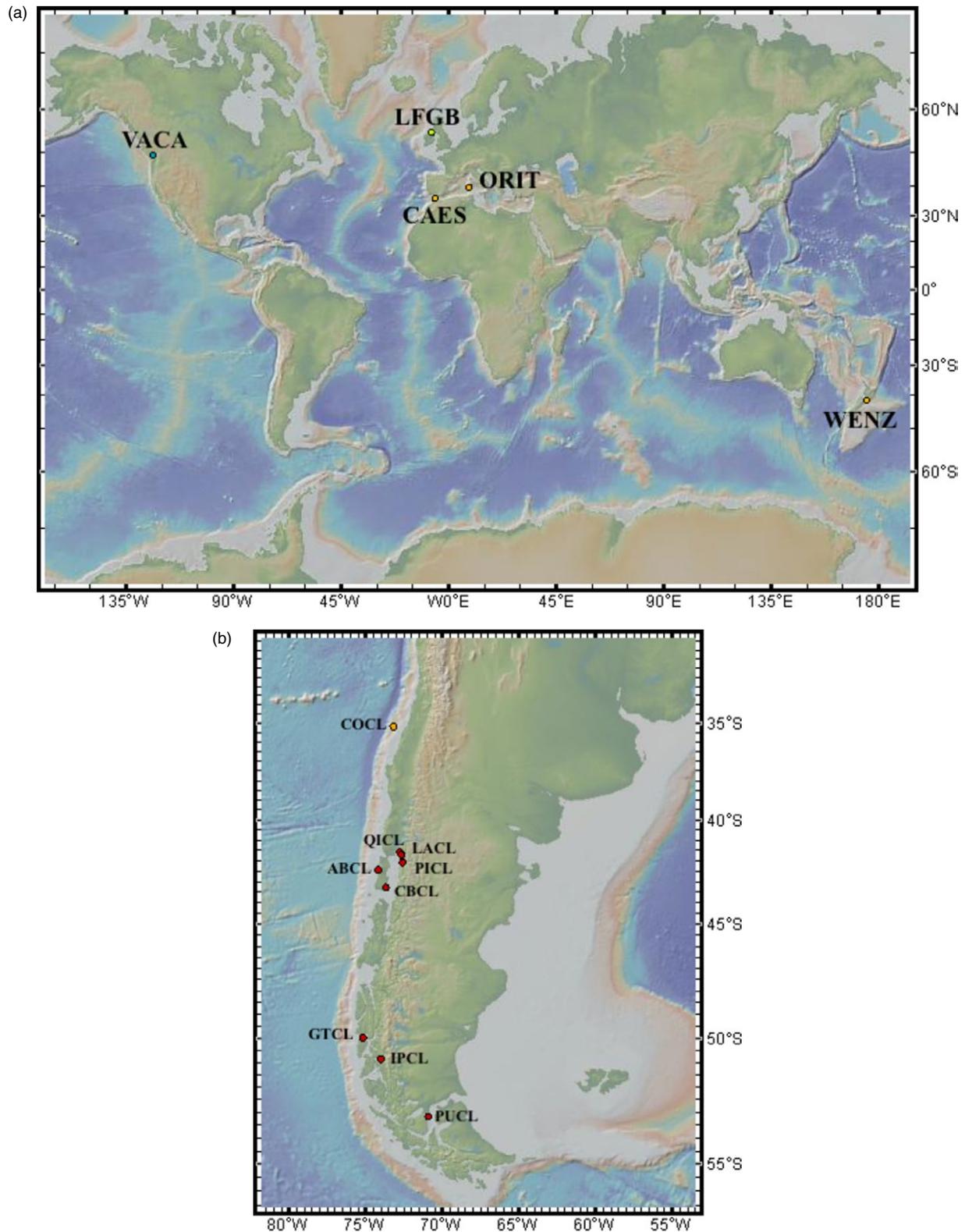
in GeneClass2.0 (Piry et al., 2004). In this supervised method, individuals were considered to be correctly assigned to their location of origin if the assignment probability to that group was higher than any other assignment probability to any other group.

To identify loci with high information content for individual assignment to species, different ranking criteria were tested as follows: (i)  $F_{ST}$  outlier loci above the upper limit of the 95% confidence interval (CI 0.95) were identified by use of LOSITAN (Antao, Lopes, Lopes, Beja-Pereira, & Luikart, 2008) with 1,000,000 simulations, a false discovery rate of 0.1 and a subsample size of 50; (ii) loci with minor allele frequency (MAF) above 0.1, 0.2, 0.3 and 0.4 (Hess, Matala, & Narum, 2011); and (iii) the most informative loci selected with backward elimination locus selection (BELS) version 1.0 (Bromaghin, 2008). This program assesses the power of all loci and sequentially eliminates the locus that makes the smallest contribution to individual assignment performance, thereby providing a ranking order for all loci.

### 3 | RESULTS

#### 3.1 | SNP markers, genetic diversity and Hardy-Weinberg equilibrium

Initially, 79 SNPs were used to genotype 338 *Mytilus* individuals from the 14 locations. Of these, 24 did not provide an acceptable quality score, four were monomorphic in all samples, and two were tri-allelic. These 30 loci were removed from further analysis. The remaining 49 loci were bi-allelic, of which seven loci (BM11A, BM151A, BM202A, BM202B, BM203D, BM2G and BM92B) were monomorphic across all locations but for alternative alleles, eight loci (BM103B, BM10B, BM201B, BM30A, BM30C, BM44B, BM50B and BM75C) were polymorphic only in the *M. trossulus* (VACA) reference population, and three loci (BM32A, BM38B and BM9B) were polymorphic in mussels from all locations (Tables S1 and S2). MAF by locus ranged from 0.003 (BM75C) to 0.491 (BM101A) with a mean



**FIGURE 1** Locations and codes of the 14 *Mytilus* sampling sites. Four letter code of locations can be found in Table 1. Colour indicates species according to the PCR-RFLP Me15-16, *Acil* assay. (●) *Mytilus chilensis*, (●) *Mytilus galloprovincialis*, (●) *Mytilus edulis*, (●) *Mytilus trossulus*. Background topographic map from GeoMapApp (<http://www.geomapapp.org>)

( $\pm$ SD) across all loci of  $0.181 \pm 0.144$ . In total, 60% of the SNP loci exhibited MAF values of  $<0.2$ . Considering all populations globally, 19 of 49 loci departed from HWE (Table S1). The average observed

heterozygosity ( $H_o$ ) by locus ranged from  $<0.001$  (BM106B, BM11A, BM151A, BM201B, BM202A, BM202B, BM203D, BM2G, BM92B) to 0.434 (BM32A).

### 3.2 | Genetic differentiation amongst populations

Global genetic differentiation amongst the 14 populations was high,  $F_{ST} = 0.517$  (95% CI of 0.505–0.523). All loci, with the exception of BM75C, had  $F_{ST}$  values significantly different from zero, and seven loci had  $F_{ST} = 1.000$  (Table S1). Only the pairwise  $F_{ST}$  values amongst populations within the putative *M. chilensis* group and between the Mediterranean *M. galloprovincialis* sample (ORIT) and the Chilean Cocholgue sample (COCL) were not significantly different from zero according to their 95% CIs (Table S3). All other pairwise  $F_{ST}$  values were significantly different from zero, reaching a maximum value of 0.830 (VACA–WENZ). Very large genetic differentiation was observed amongst species (estimated as the arithmetical mean of pairwise  $F_{ST}$  values from Table S3) between native mussels from Chile (QICL, LACL, PICL, ABCL, CBCL, GTCL, IPCL and PUCL locations) and the reference mussels *M. edulis* (LFGB), *M. galloprovincialis* (COCL, ORIT and CAES) and *M. trossulus* (VACA), 0.538, 0.555 and 0.805, respectively.

The NJ tree based on the pairwise  $F_{ST}$  distance matrix (Figure 2) revealed four groups, showing high coincidence with species: (i) *M. trossulus* from Canada (VACA), (ii) *M. edulis* from Northern Ireland (LFGB), (iii) *M. galloprovincialis* including the New Zealand sample (WENZ), the Atlantic (CAES) and Mediterranean (ORIT) individuals and also the Chilean sample from Cocholgue (COCL) and (4) a group with 100% bootstrap support and with short internal branches that included all the other Chilean samples (here after the CLMch-mix group).

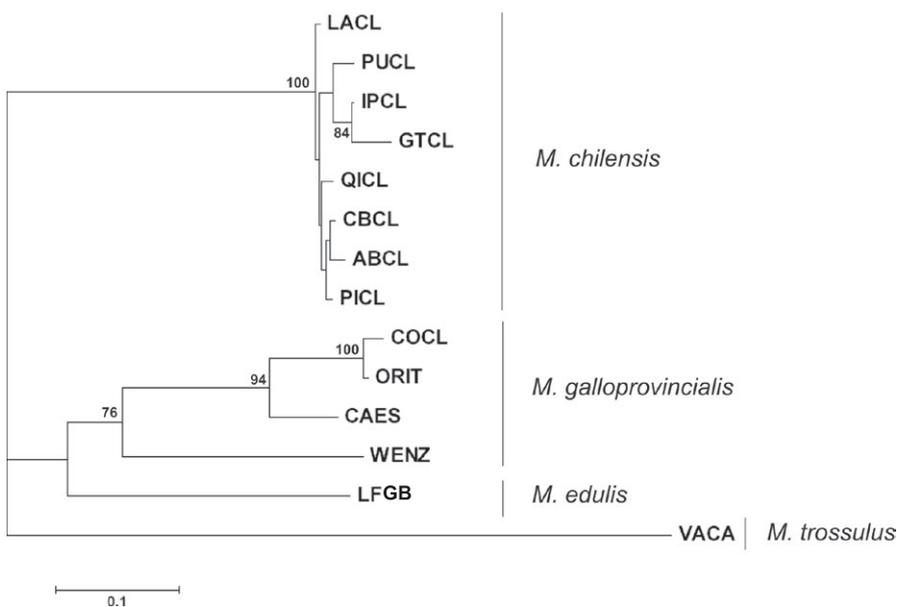
### 3.3 | Population genetic structure

The DAPC identified three clusters ( $K = 3$ ) as shown by the elbow in the curve of BIC values vs.  $K$  (Fig. S1a). In this scenario, all the VACA samples (*M. trossulus*) were clearly separated from the second group formed by the CLMch-mix (*M. chilensis*) and from the third cluster including the LFGB (*M. edulis*), CAES, ORIT, COCL and WENZ

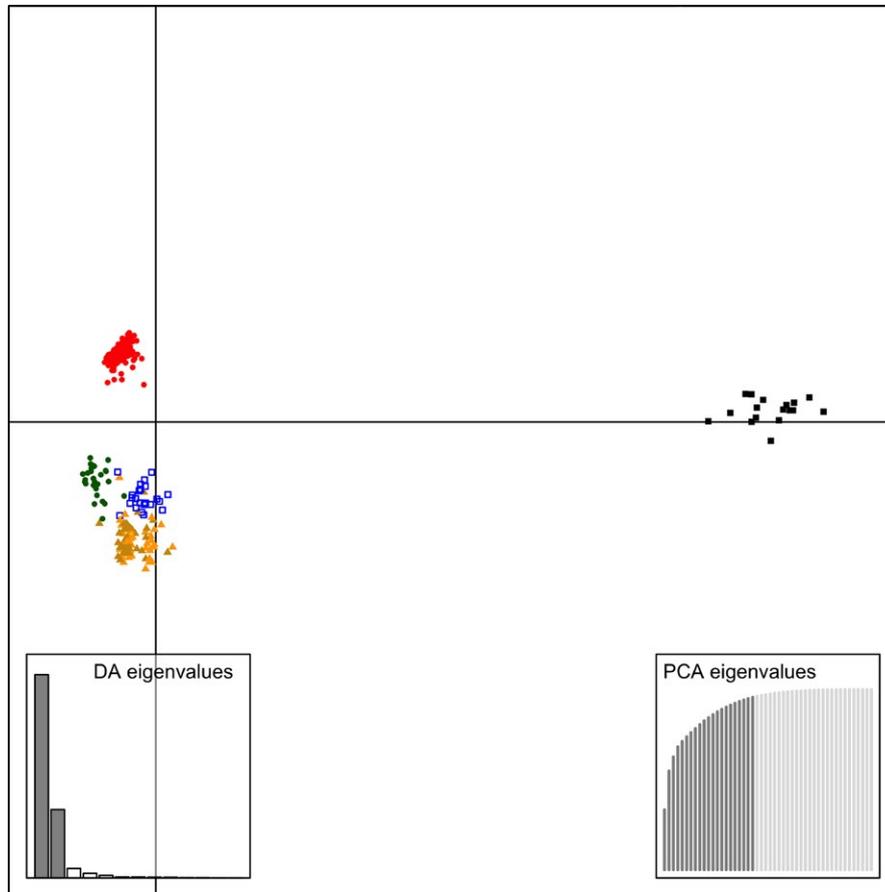
(*M. galloprovincialis*) samples. Only a single QICL individual was included in the *M. galloprovincialis*–*M. edulis* group. Exactly the same clustering results were apparent from the AWclust analysis, with  $K = 3$  determined from the gap statistic (Fig. S1b, Table S4). Both methods correctly matched 99.7% of the individuals (337 of 338 mussels) to the three groups.

The DAPC plot of variation considering all locations ( $K = 14$ ) also revealed the three major groups as previously detected using  $K = 3$  (Figure 3). As clusters are abstract objects that are not necessarily coincident with sampling sites, to draw the plot, the colour and shape of the symbols used for all individuals included in the cluster were given by the location that contributed highest number of individuals to that cluster. The number of individuals from each location in each of the 14 clusters is presented in Table 2. Seven clusters included exclusively CLMch-mix (*M. chilensis*) individuals represented by red filled circles in the plot; these were grouped together in Figure 3. All VACA samples (*M. trossulus*) were clearly separated into one cluster, represented by filled black squares. A third group included individuals from WENZ plotted as circles, as well as LFGB (*M. edulis*) individuals identified by squares and CAES, ORIT, COCL (*M. galloprovincialis*) represented as triangles. In this last group, the mussels from Spain to Atlantic (CAES) were divided into two clusters, the first one containing only CAES individuals and the second one containing mussels from CAES but also from ORIT (Mediterranean *M. galloprovincialis*) and Northern Ireland (LFGB) individuals (*M. edulis*). The Cocholgue (COCL) mussels were mainly clustered with the ORIT individuals, but because they were few in number in each cluster, no red triangles are shown in Figure 3. Overall, these results indicate that *Mytilus* in Chile are composed of two groups: (i) a northern group located in the Gulf of Arauco (Bío-Bío region) represented here by the COCL population, presumptively being introduced *M. galloprovincialis* and (ii) a southern group of native Chilean blue mussels (*M. chilensis*).

The Bayesian clustering algorithm STRUCTURE identified three clusters ( $K = 3$ ) as capturing the highest level of structure, separating



**FIGURE 2** Neighbour-joining tree of *Mytilus* populations based on  $F_{ST}$  distance matrix



**FIGURE 3** Clusters obtained by discriminant analysis of principal components of 338 *Mytilus* individuals from 14 locations ( $K = 14$ ) along with discriminant analysis eigenvalues retained (left) and PCA eigenvalues retained (right). Different shapes represent species according to their genotype assays using Me15-16 or SNP BM151A (● Southern Hemisphere mussels, including *M. chilensis* [Chile] and Southern Hemisphere *M. galloprovincialis* WENZ [New Zealand] samples, ▲ Northern Hemisphere *M. galloprovincialis*, □ *M. edulis*, ■ *M. trossulus*). Each symbol in the graph represents an individual. Colours represent the sampling location: in red—Chile (CLMch-mix), in black—Pacific coast of Canada (VACA), in blue—Northern Ireland (LFGB), in yellow—Italy (Mediterranean—ORIT), in gold—Spain (Atlantic—CAES), in green—New Zealand (WENZ). As clusters are abstract objects that are not necessarily coincident with sampling sites, the colour of all individuals included in the cluster was given by the location that contributes the highest number of individuals. Detailed composition of individuals in each cluster is shown in Table 2

*M. trossulus*, *M. chilensis* and placing together in a single group the reference *M. edulis* population (LFGB) with the two European reference *M. galloprovincialis* populations of Italy (ORIT) and Spain (CAES), as well as with the populations of Cocholgue in Chile (COCL) and Wellington in New Zealand (WENZ) (Fig. S1c and Figure 4a). For 337 individuals, the  $Q$ -values (cluster membership assignment estimates) were greatest in the cluster that coincides with the species, with the exception of one QICL individual that showed a higher  $Q$  value in the *M. edulis*–*M. galloprovincialis* cluster (Table S4). The maximum  $Q$ -values per individual were  $>0.8$ , so they may be considered as group members, with the exception of two individuals, one from QICL and the other from LFGB, that were considered potentially admixed. A second peak in the Delta  $K$  plot identified seven clusters ( $K = 7$ ) that separated into five groups, *M. trossulus* (VACA), *M. chilensis* individuals (CLMch-mix—composed of three clusters), *M. edulis* (LFGB), *M. galloprovincialis* (ORIT, CAES and COCL) and New Zealand (WENZ) (Fig. S1c and Figure 4b).

For the 49 SNP panel, the LOO method (Piry et al., 2004) correctly assigned 337 of 338 individuals (99.7%) to species. In total,

100% of the COCL, ORIT, CAES and WENZ individuals were assigned to the *M. galloprovincialis* group (Table 3a). As well, 100% of the LFGB and VACA individuals were assigned correctly to the *M. edulis* and *M. trossulus* groups, respectively. Only one individual (0.5%) from the CLMch-mix group was assigned to the *M. galloprovincialis* group, indicating a low rate of hybridization (*M. chilensis*  $\times$  *M. galloprovincialis*).

Assignment success to region of origin was 100% for the VACA and WENZ locations (Table 3b). Not one mussel of the Chilean COCL samples was assigned to the MchCL-All (Chilean) group, but instead all were assigned to the ORIT (90%) Italian (Mediterranean Sea) and the CAES (10%) Spain (Atlantic Ocean) locations. In addition, 7.4% of the LFGB samples were also assigned to CAES, whilst the rest of the LFGB mussels were assigned to the Northern Ireland location. The ORIT and CAES samples were assigned to both locations, with 93.1% and 86.2% of assignment success, respectively, showing the mixture of individuals between these two European locations. The mussels from VACA and WENZ were assigned with 100% accuracy to their sampling locations (Table 3b).

**TABLE 2** Clusters obtained by discriminant analysis of principal components of SNPs variation amongst 338 *Mytilus* individuals from 14 locations

	●	●	●	●	●	●	●	□	■	▲	▲	▲	▲	●	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Location	CL-mix	LFGB	VACA	ORIT-COCL- others	ORIT- COCL	CAES- others	CAES	WENZ	Total						
COCL	0	0	0	0	0	0	0	0	0	13	6	1	0	0	20
QICL	0	1	3	5	5	1	4	0	0	0	1	0	0	0	20
LACL	3	4	7	3	2	4	4	0	0	0	0	0	0	0	27
PICL	6	2	4	1	6	9	2	0	0	0	0	0	0	0	30
ABCL	2	3	2	3	3	3	1	0	0	0	0	0	0	0	17
CBCL	2	4	8	7	2	5	1	0	0	0	0	0	0	0	29
GTCL	2	0	0	1	0	2	0	0	0	0	0	0	0	0	5
IPCL	6	1	1	4	0	7	9	0	0	0	0	0	0	0	28
PUCL	9	8	3	5	3	2	3	0	0	0	0	0	0	0	33
LFGB	0	0	0	0	0	0	0	25	0	0	0	2	0	0	27
VACA	0	0	0	0	0	0	0	0	17	0	0	0	0	0	17
ORIT	0	0	0	0	0	0	0	0	0	14	13	2	0	0	29
CAES	0	0	0	0	0	0	0	0	0	4	0	10	15	0	29
WENZ	0	0	0	0	0	0	0	0	0	0	0	0	0	27	27
	30	23	28	29	21	33	24	25	17	31	20	15	15	27	338

Different shape symbols represent species according to their genotype assays using Me15-16 or SNP BM151A (● *M. chilensis* and WENZ sample (Southern Hemisphere *M. galloprovincialis*) ▲ Northern Hemisphere *M. galloprovincialis*, □ *M. edulis*, ■ *M. trossulus*). Each symbol in the graph represents an individual. Colours represent the sampling location: in red: Chile (CLMch-mix), in black: Canada (VACA), in blue: Northern Ireland (LFGB), in yellow: Italy (Mediterranean—ORIT), in gold: Spain (Atlantic—CAES), in green: New Zealand (WENZ). As clusters are abstract objects that are not necessarily coincident with sampling sites, the colour of all individuals included in the cluster was given by the location that contributes with the high number of individuals.

### 3.4 | Highly informative locus panel

The ranking criteria selected different numbers of most informative loci, ranging from 6 to 25 (MAF > 0.4 to MAF > 0.1, respectively). The panel that performed best at assigning individuals to species was selected using the ranking criterion of MAF > 0.2 and included 19 highly informative SNP loci that correctly assigned 336 (99.4%) individuals to species (Table S5 and Table 4). Considering this MAF criterion along with  $F_{ST}$  outlier loci and results from BELS, three loci (BM106B, BM151A and BM6C) were included in the group of most informative loci (Fig. S2). Two of these three loci have known mRNA functions, with the polyphenolic adhesive foot protein (BM151A) and the elongation factor G (BM6C), whilst the function of BM106B is presently unknown.

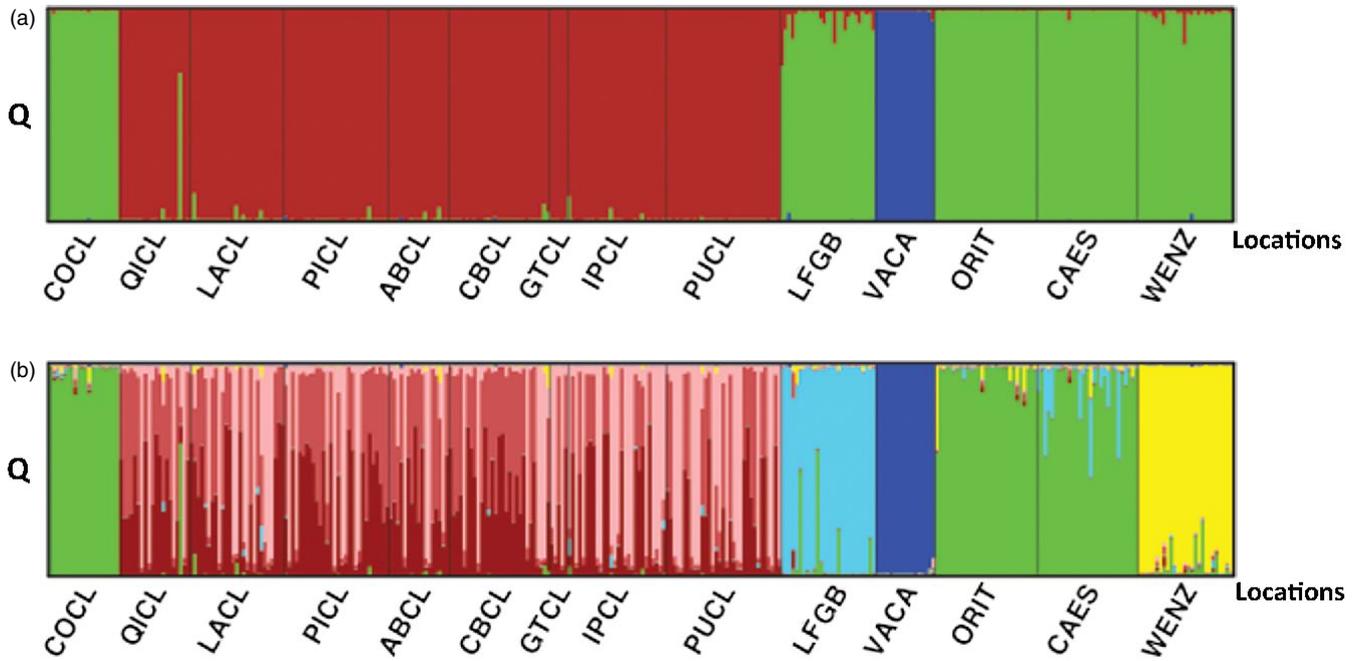
## 4 | DISCUSSION

Single nucleotide polymorphisms (SNPs) are powerful markers to monitor organisms at the individual, population and species levels. They have been developed recently for mussels of the genus *Mytilus* to study hybrid zones, introgression and adaptive genetic variation with traceability purposes (Araneda et al., 2016; Saarman & Pogson, 2015; Zbawicka, Sanko et al., 2014). The availability of a SNP panel now allows for a multi-locus scan of the genome, adding more confidence to *Mytilus* species identification. Because not all SNP loci are

equally informative based on their performance answering a specific research question, different ranking criteria have been employed to identify loci with high information content (Hess et al., 2011; Storer et al., 2012). These criteria can be used to create minimum panels that maximize individual assignment success to test hypotheses about species identification.

### 4.1 | The taxonomic status of the native blue mussel in Chile

The question of the taxonomic status of the native Chilean blue mussel goes back more than 150 years. In the present study, the taxonomic priority and also the validity of the descriptions of the two South American species (*M. platensis* on the Atlantic coast, by d'Orbigny in 1846 and *M. chilensis* on the Pacific coast, by Hupé in 1854) are critical in assigning species designations. Based on morphological traits, d'Orbigny (1846) described the native blue mussel collected at Maldonado, Uruguay (Rio de la Plata) on the Atlantic coast of South America as *M. platensis*. In total, d'Orbigny described 17 *Mytilus* species from South America from his journey (1826–1833) via the Atlantic to the Pacific coast of South America. Significantly, d'Orbigny did not refer to or describe *M. platensis* (the Atlantic species) from Chile, despite the fact that he sampled in Chile. Subsequently, based on morphology, Hupé (1854) described *M. chilensis* from Concepcion, Chile on the Pacific coast. Thus, in accordance with the rules of taxonomic



**FIGURE 4** Proportional membership ( $Q$ ) of *Mytilus* individuals to each of the: (a) three clusters ( $K = 3$ ) and (b) seven clusters ( $K = 7$ ) inferred by STRUCTURE

priority, *M. platensis* d'Orbigny (1846) holds for the native Atlantic smooth-shelled blue mussels, whereas *M. chilensis* Hupé (1854) holds for native Pacific smooth-shelled blue mussels. Interestingly, this situation is often not reflected in current taxonomic websites such as WoRMS and ITIS. Subsequently, but much later on, protein-based (allozyme) and morphometric assessments of blue mussels led McDonald et al. (1991) to conclude that mussels from South America (both coasts), the Falkland Islands and the Kerguelen Islands should be included tentatively in *M. edulis*. More recently, Borsa et al. (2012), in their review of allozyme and morphometric variation of Chilean blue mussels, confirmed that the Southern Hemisphere form of *M. edulis* occurs “along the shores from the North Patagonia region of Chile to the southern tip of the South American continent” (p. 52, Borsa et al., 2012) and concluded that native Chilean blue mussels should be assigned subspecific rank and named *M. edulis platensis* d'Orbigny 1846. Numerous authors, using a range of different molecular markers, have reported molecular genetic differences between the native Chilean blue mussel and reference Northern Hemisphere *M. edulis* and/or *M. galloprovincialis* (e.g., Astorga et al., 2015; Gérard et al., 2008; Śmietanka & Burzyński, 2017; Westfall & Gardner, 2010). Regardless of the taxonomic recommendation made by these (and several other) research groups, the common theme is that native *Mytilus* from South America, and often specifically from Chile, are genetically different from other smooth-shelled blue mussels anywhere in the world. These results challenge the interpretation of native Chilean mussels as being *M. edulis*-like and also of being like South American Atlantic mussels.

As our SNPs data reveal, there are pronounced nuclear genetic differences between the native blue mussels of Chile and all other mussels that we tested, including reference Northern Hemisphere *M. edulis* (e.g., as employed by McDonald et al., 1991 and also by Borsa et al.,

2012). Our analyses confirmed the hypothesis that the native Chilean blue mussel is genetically distinct from reference *M. edulis*, *M. galloprovincialis* and *M. trossulus*, to the extent that this mussel now warrants recognition as a separate and geographically isolated taxon within the genus *Mytilus*. We therefore conclude, based on taxonomic priority as discussed above, that the genetically distinct native blue mussel from Chile should be recognized as *M. chilensis* Hupé 1854. In addition, the SNPs results support the hypothesis that the native Chilean blue mussel has a unique evolutionary history in the Southern Hemisphere. Similarly, Śmietanka and Burzyński (2017), who analysed the complete sequence of the female mitogenome of the native Chilean blue mussel, reported that the genetic distance between *M. edulis* and *M. galloprovincialis* (~2.5%) was half the distance that separates *M. chilensis* from either of these two species (5%), a result that indicates that the native Chilean mussel is a separate taxon within the genus *Mytilus*.

The use of SNPs in the present study has revealed that *M. trossulus* is the most differentiated of the taxa examined here, consistent with the suggestion that it is the oldest (ancestral) form (Chichvarkhin, Kartavtsev, & Kafanov, 2000; Kafanov, 1987; Vermeij, 1992). Whilst the SNPs are able to differentiate amongst reference *M. edulis* (LFGB), *M. galloprovincialis* (Northern Hemisphere—CAES, ORIT) and *M. galloprovincialis* (Southern Hemisphere—WENZ), these three distinct taxa form a group more similar to one another than any of these are to *M. chilensis*. This highlights the power of a multilocus approach in comparison with a single marker approach, when multiple loci are scattered throughout the genome to more accurately reflect lineage and/or species evolutionary histories. As noted elsewhere (e.g., Gardner et al., 2016; Zbawicka et al., 2012; Zbawicka, Sanko et al., 2014), the application of SNPs to global questions of taxonomy and hybridization for mussels of the genus *Mytilus* will greatly enhance our knowledge of

**TABLE 3** Number of individuals (%) correctly reassigned to (A) species and (B) region of origin by the leave-one-out procedure implemented in GeneClass2.0 (Piry et al., 2004) using the frequency criteria of Paetkau et al. (1995)

(A) Assigned to species						
Location	<i>M. chilensis</i>	<i>M. galloprovincialis</i>	<i>M. edulis</i>	<i>M. trossulus</i>		
COCL	-	20 (100.0)	-	-		
QICL	19 (95.0)	1 (5.0)	-	-		
LACL	27 (100.0)	-	-	-		
PICL	30 (100.0)	-	-	-		
ABCL	17 (100.0)	-	-	-		
CBCL	29 (100.0)	-	-	-		
GTCL	5 (100.0)	-	-	-		
IPCL	28 (100.0)	-	-	-		
PUCL	33 (100.0)	-	-	-		
ORIT	-	29 (100.0)	-	-		
CAES	-	29 (100.0)	-	-		
WENZ	-	27 (100.0)	-	-		
LFGB	-	-	27 (100.0)	-		
VACA	-	-	-	17 (100.0)		
(B) Assigned to region of origin						
Location	CLMch-mix	ORIT	CAES	WENZ	LFGB	VACA
COCL	-	18 (90.0)	2 (10.0)	-	-	-
QICL	19 (95.0)	1 (5.0)	-	-	-	-
LACL	27 (100.0)	-	-	-	-	-
PICL	30 (100.0)	-	-	-	-	-
ABCL	17 (100.0)	-	-	-	-	-
CBCL	29 (100.0)	-	-	-	-	-
GTCL	5 (100.0)	-	-	-	-	-
IPCL	28 (100.0)	-	-	-	-	-
PUCL	33 (100.0)	-	-	-	-	-
ORIT	-	27 (93.1)	2 (6.9)	-	-	-
CAES	-	4 (13.8)	25 (86.2)	-	-	-
WENZ	-	-	-	27 (100.0)	-	-
LFGB	-	-	2 (7.4)	-	25 (92.6)	-
VACA	-	-	-	-	-	17 (100.0)

**TABLE 4** Number of individuals (%) correctly reassigned to species and region of origin by panels composed of highly informative SNP loci selected by different ranking criteria

Ranking criterion	All	$F_{ST}$ outlier loci <sup>a</sup>	Minor allele frequency (MAF)				BELS <sup>b</sup>
			>0.1	>0.2	>0.3	>0.4	
No.° of selected loci	49	11	25	19	13	6	19
To species	337 (99.7)	262 (77.5)	336 (99.4)	336 (99.4)	335 (99.1)	331 (97.9)	317 (93.8)
To region of origin	309 (91.4)	267 (79.0)	307 (90.8)	307 (90.8)	297 (87.9)	269 (79.6)	300 (88.8)

<sup>a</sup> $F_{ST}$  outlier loci (CI > 0.95).<sup>b</sup>Loci whose removal caused the assignment performance measure to drop down 0.55 in the BELS software program (Bromaghin, 2008).

how many species exist, where they are found, and how they interact in terms of their interbreeding and introgression.

Beside *M. chilensis* and *M. galloprovincialis*, no other *Mytilus* taxa were detected at Chilean sites in this study. Elsewhere, Oyarzún et al. (2016) reported *M. edulis* individuals in the Strait of Magellan (based on genotyping of the *Me15-16* locus), consistent with the western-most natural occurrence of *M. edulis*-like mussels or *M. edulis platensis* on the Atlantic coast of South America (e.g., Borsa et al., 2012; Hilbish et al., 2000; McDonald et al., 1991). In addition, *M. trossulus* alleles have been described in Chile in very low frequencies using the *Me15-16* marker (Larraín et al., 2012; Oyarzún et al., 2016). We found no evidence of either *M. edulis* or *M. trossulus* in our samples from Chile using the 49 SNP panel. To address the question of the taxonomic status of native blue mussels from the Atlantic Ocean coast of South America (*M. edulis*, *M. platensis* or another species), additional studies analysing mussels from this area (i.e., Argentina, Uruguay, Falkland Islands) will be needed.

## 4.2 | The role of taxonomy in protecting and exploiting blue mussels

Systematics and taxonomy are important to delimit species, the fundamental units of biodiversity. The newly revealed SNPs-based difference between *M. chilensis* and all other studied taxa within the genus highlights the key role that molecular approaches to support taxonomy may play in disciplines such as conservation, aquaculture and food security.

From an ecological point of view, our results highlight the need for policies to mitigate the impact of or to prevent further expansion of nonindigenous species (NIS) along the Chilean coast. Specifically, the SNPs panel has confirmed several previous reports (e.g., Astorga et al., 2015; Daguin & Borsa, 2000; Tarifeño et al., 2012; Westfall & Gardner, 2010) of the presence of the highly invasive *M. galloprovincialis* in the Gulf of Arauco, but shows that the COCL sample is genetically more similar to the ORIT (Italy, Mediterranean Sea) *M. galloprovincialis* sample than to the CAES (Spain, Atlantic Ocean) *M. galloprovincialis* individuals. This strongly suggests a possible recent introduction from the Mediterranean Sea via human-mediated activities.

Introductions of alien species are one of the most important environmental issues today (Ojaveer et al., 2015). Considering the differences between *M. chilensis* and the other commercial blue mussel species revealed in this study, the potential biosecurity risk posed by the anthropogenic introduction and spread of *M. galloprovincialis* needs further attention. Given that the Mediterranean mussel is listed amongst the hundred worst invasive species, and taking into account the dispersal capacity of this invasive mussel (Branch & Steffani, 2004; McQuaid & Phillips, 2000), for the Chilean aquaculture sector the spread of *M. galloprovincialis* to other mussel production area poses a threat to the native Chilean blue mussel. This has led local producers to express concerns about a negative effect on production of the native *M. chilensis*. In Chile, protection and control measures to avoid the introduction of marine species that constitute pests, to isolate their presence on occurrence and to prevent

their spread and promote their eradication are regulated by the general law of fisheries and aquaculture (Chilean law N° 18892, www.leychile.cl/Navegar?idNorma=30265). Although *M. galloprovincialis* is not considered to be a pest under Chilean law, this species cannot be imported live into the country and can be cultivated only at an experimental scale in the Bio-Bío region (regulation N° 96 2015, Ministry of Economy, Development and Tourism, www.leychile.cl/Navegar?idNorma=256174). As with all such cases worldwide, once established in Chile it is highly unlikely that an invasive mussel such as *M. galloprovincialis* can be eradicated. The only future option appears to be monitoring its distribution with molecular tools, such as those used here, and to take action directed to containing it within limited areas.

Hybridization between the NIS and native taxa may also occur, incorporating exotic DNA/genes into local populations and thereby modifying localized gene pools by introgression with unpredictable consequences over time, which may vary from cryptic to substantial changes in ecosystem structure and function. Only one individual (0.53%) from the Mch-mix group was assigned to *M. galloprovincialis*, indicating that at the time of sample collection (2009) only a limited dispersal of this species had occurred from the Gulf of Arauco to the *M. chilensis* aquaculture zone in the south. Detailed investigation and ongoing monitoring is required to check for further spread and interaction between the two taxa. From an economic point of view, in addition to the unpredictable consequences that introgression can have on productivity of mussel farms (Michalek et al., 2016), the recognition of the name *M. chilensis* to designate the native Chilean blue mussel will contribute to traceability, authenticity and compliance with seafood labelling regulations, promoting transparency in the seafood trade. This recognition also provides an opportunity to the local mussel industry to apply for a geographically protected origin indication for the Chilean blue mussel.

## 4.3 | Highly informative loci

The best ranking criterion to select the most informative loci for species identification was minor allele frequency (MAF) > 0.2. This selection criterion includes 19 loci that showed a power comparable to the total panel of 49 SNPs for species identification. In practical terms, these 19 loci constitute a reduced multilocus SNP panel with high performance that permits identification of commercial *Mytilus* taxa without the inconsistencies associated with the use of a single or a few molecular markers. The performance of the reduced SNP panels selected with the MAF and BELS criteria, to identify mussel geographic origin region, was always lower than for species identification and shows a small increment only when  $F_{ST}$  outlier loci were included (Table 4). This finding is probably explained by the fact that loci used to perform species identification must ideally be variable amongst species but fixed amongst populations within species. On the other hand, loci for identification of geographic origin must be more variable amongst populations within species, because they can reflect adaptation to local conditions, as demonstrated recently by Araneda et al. (2016).

## 5 | CONCLUSIONS

A new multilocus SNP marker panel reveals a clear separation between *M. chilensis* and all other *Mytilus* taxa, a degree of separation that is greater than that between *M. edulis* and *M. galloprovincialis*. Because *M. edulis* and *M. galloprovincialis* are already considered different species, this finding provides conclusive evidence of the status of smooth-shelled blue mussels native to the Chilean coast as a species within the *Mytilus* genus. Taxonomic priority indicates that these mussels should be recognized as *Mytilus chilensis* Hupé, 1854. The SNP markers also corroborate the previously reported presence of the highly invasive *M. galloprovincialis* in the Gulf of Arauco, revealing its probable recent introduction from the Mediterranean Sea. Recognition of this invasion poses challenges to avoid environmental and economic damage in Chile. The 49 SNP panel was able to assign 99.7% of individuals correctly to species, whilst a comparable (99.4%) assignment success was obtained with a reduced panel of the 19 most informative SNP loci. These SNPs are a particularly valuable tool in terms of increasing our understanding of (i) *Mytilus* phylogeography, population genetics and connectivity, (ii) elucidating evolutionary processes such as natural hybridization and introgression, (iii) helping to enforce aquaculture and conservation policies, and (iv) increasing transparency in seafood labelling, authenticity and traceability field.

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## DATA ARCHIVING STATEMENT

The sequences of SNP originated mRNA fragments are available from GenBank (accession numbers KJ871032-74, KT713368-88). Allele frequencies of SNP loci and clusters identified for samples of *Mytilus* are presented in Supporting information.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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