

PCR-based nuclear and mtDNA markers and shell morphology as an approach to study the taxonomic status of the Chilean blue mussel, *Mytilus chilensis* (Bivalvia)

Jorge E. Toro (*)

Instituto de Biología Marina, Universidad Austral de Chile, Casilla 567, Valdivia, Chile.

Received January 26, 1998; accepted June 26, 1998.

Abstract – Most recent authors have called the Chilean blue mussel (formerly *Mytilus chilensis*) *Mytilus edulis*. Mussels from four nominal species (*Mytilus edulis*, *M. trossulus*, *M. galloprovincialis* and *M. chilensis*) were collected from the eastern coast of Newfoundland, Canada, New Zealand and the southern coast of Chile. Canonical discriminant analysis of selected shell morphometrical characters showed that although there is some overlap in canonical variates, the three nominal species, *M. edulis*, *M. trossulus* and *M. chilensis* can be morphologically divided into three distinct groups. The two nuclear-DNA markers (*ITS* and *Glu-5*) and the mtDNA marker studied indicate that the nuclear and mitochondrial genomes of *M. edulis* and *M. trossulus* are different. *M. edulis*, *M. galloprovincialis* and *M. chilensis* share similar restriction fragment patterns for the mtDNA and for the *ITS* nuclear-DNA marker, while the *Glu-5* polymerase chain reaction (PCR) assay showed that the *M. edulis* and *M. chilensis* genotypes disagree, producing species-specific banding patterns that can be used as a diagnostic marker between the two nominal species. The molecular and morphometric data reported here give more evidence to support the view of other authors that the Chilean blue mussel should be included as *M. edulis* subspecies: *Mytilus edulis chilensis*. © Ifremer/Elsevier, Paris

PCR / nuclear markers / mitochondrial DNA / *Mytilus chilensis* / *Mytilus edulis* / *Mytilus trossulus* / *Mytilus galloprovincialis*

Résumé – PCR et marqueurs d'ADN nucléaires et mitochondriales et morphologie de la coquille en tant qu'approche taxonomique des moules du Chili, *Mytilus chilensis* (Bivalves). Récemment, des auteurs ont dénommé la moule du Chili : *Mytilus edulis* (précédemment appelée *Mytilus chilensis*). Des moules (*Mytilus edulis*, *M. trossulus*, *M. galloprovincialis* et *M. chilensis*) ont été collectées sur la côte est de Terre-Neuve (Canada), de Nouvelle Zélande et de la côte sud du Chili. Une analyse canonique discriminante de quelques caractères morphologiques de la coquille a montré que trois espèces *M. edulis*, *M. trossulus* et *M. chilensis* peuvent être divisées morphologiquement en trois groupes distincts. Les deux marqueurs d'ADN (*ITS* et *Glu-5*) et l'ADN mitochondrial étudiés indiquent que les génomes du noyau et des mitochondries de *M. edulis* et *M. trossulus* sont différents. En revanche, *M. edulis*, *M. galloprovincialis* et *M. chilensis* partagent les mêmes types d'ADN mitochondrial et d'*ITS* marqueur d'ADN nucléaire, tandis que l'amplification en chaîne par polymérase de *Glu-5* montre que les génotypes de *M. edulis* et *M. chilensis* ne correspondent pas, produisant des chromatogrammes spécifiques ; celui-ci peut donc être utilisé comme marqueur pour distinguer les deux espèces. Les données moléculaires et morphométriques apportent davantage d'arguments pour considérer les moules du Chili en tant que sous-espèces de *M. edulis* : *Mytilus edulis chilensis*. © Ifremer/Elsevier, Paris

PCR / marqueurs nucléaires / ADN mitochondrial / *Mytilus chilensis* / *Mytilus edulis* / *Mytilus trossulus* / *Mytilus galloprovincialis*

1. INTRODUCTION

Marine mussels belonging to the genus *Mytilus* are widely distributed throughout temperate and boreal waters of both hemispheres [9, 10, 16, 26]. They repre-

sent an important component of the intertidal and subtidal communities in terms of the number of individuals and of biomass and production, and are of considerable economic importance to aquaculture in many regions of the world [1, 9, 10, 26]. Early system-

* Present address: Department of Biology, Memorial University of Newfoundland, St. John's, Newfoundland, A1B 3X9, Canada.
e-mail: jtoro@morgan.ucs.mun.ca

atic taxonomical studies in mussels of the genus *Mytilus* have been largely based on shell morphological and morphometrical characters [9, 26]. The enormous environmental plasticity of shell morphology [24, 25] and the availability of routine molecular techniques have led recently to the use of a combination of morphological attributes of the shell and allozyme genetic markers [2, 3, 6, 8, 14, 16, 20, 22, 23], nuclear and/or mtDNA markers [12, 13, 19, 21, 28, 29] or a combination of allozyme and DNA markers [4, 18].

The Chilean blue mussel is an economically important resource in southern Chile [17]. Its culture began in 1943 in the Chiloé Island, southern Chile, and the aquaculture production of this species in 1993 was 3 864 t [1]. The Chilean mussels formerly *Mytilus chilensis* Hupe, 1854, have been recently subjected to a taxonomic revision using electrophoretically generated genetic data [14, 16]. The authors [16] used eighteen enzyme loci to discriminate between mussels from the Northern and Southern hemisphere, concluding that mussels from South America (formerly *M. chilensis*), the Falkland Islands (formerly *M. platensis*) and the Kerguelen Islands (formerly *M. desolationis*) should be included tentatively in *Mytilus edulis*.

With the advent of modern molecular biological techniques, such as the polymerase chain reaction (PCR), we have the opportunity to apply these techniques at any life stage of the mussels, using newly developed genetic markers [5, 11, 12, 13, 18]. The present study deals with *M. edulis* and *M. trossulus* from the eastern coast of Newfoundland, a sample of New Zealand blue mussel *M. galloprovincialis* as well as the Chilean blue mussel from southern Chile. Canonical discriminant analysis of morphometric variation and three PCR-based nuclear and mitochondrial DNA markers were used as another approach to study the taxonomic status of the Chilean blue mussel. We show that Chilean mussels are not *M. trossulus* but it is not possible, with this data, to determine whether they are more closely related to *M. edulis* or *M. galloprovincialis*.

2. MATERIALS AND METHODS

2.1. Study sites and sampling

Chilean mussels (shell length range of sampled individuals: 57–73.30 mm) were collected subtidally by scuba diving at the Bay of Corral, southern Chile. Each mussel ($n = 30$) was dissected and a small piece of the mantle border was placed in a 1.5-mL eppendorf tube and fixed with 95 % ethanol. These samples were kept at -20°C for a week before being brought to Canada for analysis. In addition, 120 Chilean mussel shells were brought for morphometric analysis. In Newfoundland, adult mussels were also collected subtidally by scuba diving at Bellevue ($n = 100$) and Chance Cove ($n = 100$), located in Trinity Bay, eastern coast of Newfoundland, sites where *M. edulis* and *M. trossulus*

coexist [2], and brought alive to the laboratory. A piece of mantle edge tissue was dissected, fixed in 95 % ethanol and kept at -20°C until analysis. A sample of mantle border tissue from 8 New Zealand mussels, *M. galloprovincialis* [9, 16, 22, 26] fixed in 95 % ethanol as described above, were also sent for analysis. All samples were analysed at Memorial University of Newfoundland, Canada.

2.2. Morphometric analysis

The 8 morphometric shell characters used to distinguish between three different forms of *Mytilus* (*M. edulis*, *M. trossulus* and Chilean mussels) were as follows: (i) shell height; (ii) shell width; (iii) length of anterior adductor muscle scar; (iv) length of hinge plate; (v) distance between the anterior edge of the posterior adductor muscle scar and the posterior margin of the shell; (vi) distance between the ventral edge of the posterior adductor muscle scar and ventral shell margin; (vii) distance between the pallial line and the ventral shell margin midway along the shell; and (viii) distance between umbo and posterior end of the ligament (*figure 1*) [15, 16]. Each character was standardized using \log_{10} and divided by the \log_{10} of shell length [15]. A canonical discriminant analysis was used to derive a canonical function that separated the three mussel types, using Systat V5.1 [30].

2.3. DNA extraction

Approximately 50–100 mg of mantle-edge tissue was removed from each mussel, coarsely chopped and digested in 500 μL of lysis buffer (50 mM Tris-HCL (pH 8.0); 1.0 % SDS; 25 mM EDTA) with 200 μg proteinase K at 37°C overnight. The solution was then

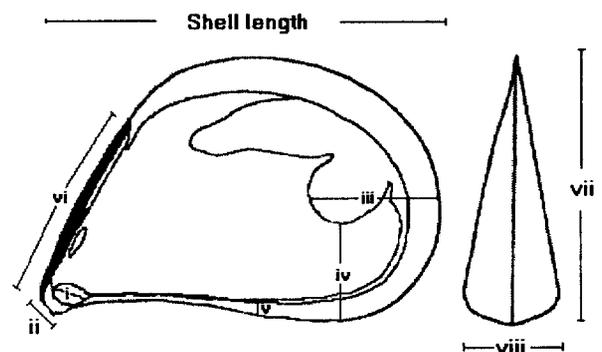


Figure 1. The 8 morphometric mussel shell characters used in the canonical discriminant analysis: i) length of anterior adductor muscle scar; ii) length of hinge plate; iii) distance between the anterior edge of the posterior adductor muscle scar and the posterior margin of the shell; iv) distance between the ventral edge of the posterior adductor muscle scar and ventral shell margin; v) distance between the pallial line and the ventral shell margin midway along the shell; vi) distance between the umbo and the posterior end of the ligament; vii) shell height; and viii) shell width (modified from McDonald et al. [16]).

extracted once with 500 μL of an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) followed by ethanol precipitation. The extracted DNA was resuspended in 200 μL of ultra-pure sterile distilled water.

2.4. Species markers

2.4.1. Nuclear-DNA markers

– *ITS*: A polymerase chain reaction (PCR) of nuclear species marker based on the internal transcribed spacer (*ITS*) regions between the 18S and 28S nuclear rDNA coding regions was applied in the present study [11]. The primers used were ITS1 5'-GTTTCCGTAGGTGAACCTG-3' and ITS2 5'-CTCGTCTGATCTGAGGTCG-3', with an expected PCR gene fragment size of 1 250 bp. Standard PCR amplifications were carried out in 25- μL reaction mixtures containing DNA template, 0.2 mM each of the four deoxyribonucleotide triphosphates (dNTPs), 2.0 mM MgCl_2 , primers at 0.4 mM, 1 unit of Taq DNA polymerase (Promega), the manufacturer-supplied PCR buffer and sterile distilled water. The reaction mixtures were overlaid with a drop of mineral oil to prevent evaporation, and were then placed in a programmable thermocycler (MJ Research, Inc.). The thermal cycler protocol consisted of an initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 2 min.

– Restriction analysis: An amount of 5 μL of each amplified PCR-products was digested for 12 h at 37 °C with 0.5 unit of the restriction enzyme *Haemophilus haemolyticus* (*Hha*I) in a total volume of 15 μL , including 3 μL of buffer supplied by the manufacturer (Pharmacia) and 6.5 μL of ultra-pure distilled water. The digested products were electrophoresed for 30 min at 112 V on 3 % agarose gels in 0.5 \times Tris-borate-EDTA (TBE) buffer. The random fragment length polymorphisms (RFLPs) were visualized by placing the gels in a solution of ethidium bromide and photographed under ultraviolet illumination. Individual mussels were scored for genotype at each marker locus on the basis of diagnostic RFLPs.

– *Glue-5*: A second nuclear-DNA marker targets the gene encoding the mussel polyphenolic adhesive protein [18]. This protein is produced by the endocrine gland in the foot of mussels and is used for its attachment to the substrate. The primers used were JH5 5'-GTAGGAACAAAGCATGAACCA-3' and the reverse primer JH54 5'-GGGGGATAAGTTTTCTTAGG-3'. PCR amplifications were carried out in 25- μL reaction mixtures containing approximately 50 ng of DNA template, 2.5 nmoles of dNTPs, 2.0 mmoles MgCl_2 , 50 pmol of each primer and 1 unit of Taq DNA polymerase (Promega), the manufacturer-supplied PCR buffer and sterile distilled water. The reaction mixtures were overlaid with a drop of mineral oil to prevent evaporation, and were then placed in a programmable thermocycler. The thermal cycler protocol consisted of an initial denaturation at 94 °C for

3 min, followed by 30 cycles of 94 °C for 20 s, 53 °C for 20 s and 72 °C for 45 s. PCR products were then directly resolved on 3 % agarose gels stained with ethidium bromide and scored for species using Polaroid photos taken under UV light.

2.4.2. mtDNA marker

– This marker is based on a DNA sequence of a region of 860 bp fragment of the *cytochrome c oxidase subunit III* gene (*COIII*) [19]. Total DNA from the mussels was amplified using the following primers for the *COIII* gene: FOR1 5'-TATGTACCAGGTC-CAAGTCCGTG-3' and REV1 5'-ATGCTCTTCT-TGAATATAAGCGTACC-3'. This primer pair amplifies the corresponding mtDNA fragment from the F and M types of *Mytilus edulis* and *Mytilus trossulus*.

PCR amplifications were carried out in 25- μL reaction mixtures containing approximately 0.2 μg of DNA template, 10 mM Tris-HCL, pH 8.3, 5 mM NH_4Cl , 50 mM KCl, 2 mM MgCl_2 , 0.2 mM each of the four deoxyribonucleotide triphosphates (dNTPs), primers at 0.4 mM and 1 unit of Taq DNA polymerase. The reaction mixtures were overlaid with a drop of mineral oil to prevent evaporation, and were then placed in a programmable thermocycler. The thermal cycler protocol consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 54 °C for 10 s and 72 °C for 1 min. An amount of 15 μL of each amplified sample was resolved by electrophoresis in a 1.5 % agarose gel, stained with ethidium bromide and photographed under ultraviolet illumination.

– Restriction analysis: An amount of 5 μL of the amplified PCR-products were digested with the endonuclease *Eco*RI. The digested products were electrophoresed for 30 min at 95 V on 3 % agarose gels (2 % Sigma agarose and 1 % NuSieve GTG low melting temperature agarose) in 0.5 \times TBE buffer. The DNA-bands were visualized by placing the gels in a solution of ethidium bromide and photographed under ultraviolet illumination. Individual mussels were scored for various mtDNA genomes to establish the gender and species. The mitotypes for *Eco*RI were found to be highly correlated with multilocus allozyme genotypes for 'pure' *M. edulis* and *M. trossulus* [28]. There are also studies [19, 28] that reported that no *M. edulis* mtDNA type was found in *M. trossulus* and vice versa.

3. RESULTS

3.1. Morphometric discrimination

The 8 shell characters used in the discriminant function were able to clearly distinguish between the three nominal species of mussels (*M. edulis*, *M. trossulus* and *M. chilensis*) previously identified using the PCR-based markers (Wilk's lambda: $P < 0.0001$) (figure 2). The standardized canonical coefficients showed that

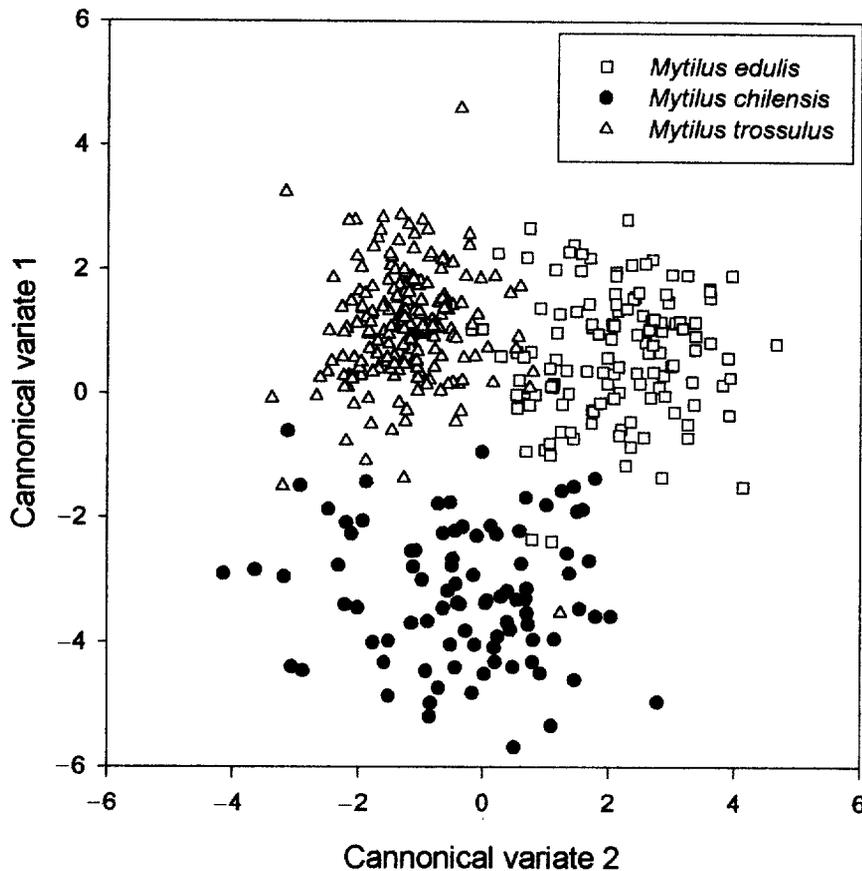


Figure 2. First and second canonical variates of morphometric data from mussel populations sampled in southern Chile and Eastern Newfoundland.

the characters length of hinge plate, shell width, distance between the umbo and the posterior end of the ligament and distance between the pallial line and the ventral shell margin contributed the most to discriminating among the three *Mytilus* forms (table I). Standardized canonical coefficients (table I) represent the amount by which canonical variates change for each change of one standard deviation in the individual character, then the character with the highest standardized coefficients contributes the most to the canonical function. Canonical discriminant analysis of morphometric variation (figure 2) indicate that there is some

overlap in canonical variates, but, most individuals do provide non-overlapping, discrete clusters for each mussel taxon, which is what is observed when allopatric populations on a world-wide scale are compared.

3.2. Species markers

3.2.1. Nuclear-DNA markers

– *ITS*: The restriction digestion of the 1 250-bp *ITS* PCR amplified product with *Hha*I produced two spe-

Table I. Standardized canonical coefficients of the eight morphometric characters used in the discriminant analysis. Standardized coefficient for the first (St. can 1) and second (St. can 2) canonical variate and the probability from F statistic (*P*), involving the three group analyzed (*M. edulis*, *M. trossulus* and *M. chilensis*). ** *P* < 0.001.

Character	St. can 1	St. can 2	<i>P</i>
Shell height	0.353	0.343	**
Shell width	-0.544	0.448	**
Length of anterior muscle scar	0.250	0.382	**
Length of hinge plate	0.641	-0.062	**
Distance between anterior edge of the posterior adductor muscle scar and posterior margin of the shell	0.107	0.081	**
Distance between ventral edge of the posterior adductor muscle scar and ventral shell margin	-0.198	-0.044	**
Distance between pallial line and the ventral shell margin	0.406	0.119	**
Distance between the umbo and the posterior end of the ligament	-0.533	0.280	**

cific RFLPs. In *M. edulis*, the Chilean mussel and *M. galloprovincialis*, the 1 250-bp fragment was cut into two 450-bp fragments and two 180-bp fragments. In *M. trossulus*, the *ITS*-PCR product was cut into two 280-bp fragments, two 180-bp fragments and several < 100-bp fragments (figure 3). This assay was able to separate *M. trossulus* from *M. edulis*; however, the banding pattern produced after restriction analysis was similar in *M. edulis*, *M. galloprovincialis* and the Chilean mussels.

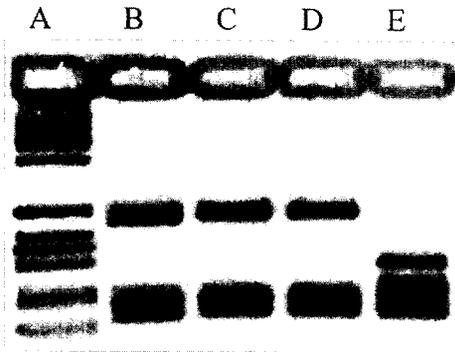


Figure 3. Scanned photonegative of an ethidium bromide stained 3 % agarose gel transilluminated with ultraviolet light showing the RFLP (random fragment length polymorphism) patterns produced by the *ITS* (internal transcribed spacer) nuclear marker for *Mytilus edulis* (lane B), *M. chilensis* (lane C), *M. galloprovincialis* (lane D) and *M. trossulus* (lane E). Molecular weight marker (Gibco BRL 1 kb ladder) (lane A).

– *Glu-5*: This PCR assay produced species-specific banding patterns. In *M. edulis*, two different banding patterns were found, 90 % of *M. edulis* mussels produced a single 350-bp band and 8 % produced one 350-bp band and one 380-bp band. In *M. trossulus*, this PCR assay produced only a single primary band of 240 bp. When this assay was applied to the Chilean mussels and *M. galloprovincialis*, three primary bands of 200, 300 and 500 bp were produced (figure 4).

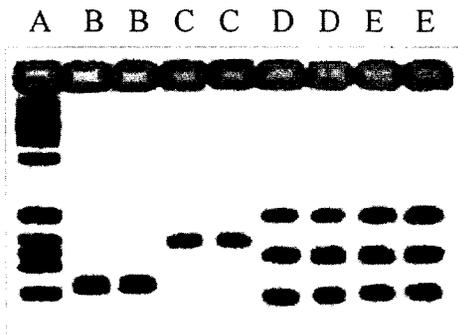


Figure 4. Scanned photonegative of an ethidium bromide stained 3 % agarose gel transilluminated with ultraviolet light showing the PCR (polymerase chain reaction) products generated using the *Glu-5* assay for *Mytilus trossulus* (lane B), *M. edulis* (lane C), *M. chilensis* (lane D) and *M. galloprovincialis* (lane E). Molecular weight marker (Gibco BRL 1 kb ladder) (lane A).

3.2.2. mt-DNA marker

The *EcoRI* restriction profiles of the 860-bp *COIII* segment amplified showed, in female *M. edulis* *M. galloprovincialis* and *M. chilensis*, a two band pattern (540 bp and 320 bp), while in males, a third uncut band at 860 bp was also present. The *M. trossulus* female showed a 860-bp band and the males showed two extra bands, one of 440 bp and the other of 320 bp (figure 5). The mussels were characterized by one or two mitotypes symbols (Fed, Ftr, Med and Mtr [28]) if they were to be homoplasmic or heteroplasmic respectively. All *M. edulis*, *M. chilensis*, *M. galloprovincialis* and *M. trossulus* females were found to be homoplasmic (Fed and Ftr). All *M. edulis*, *M. galloprovincialis* and *M. chilensis* males were heteroplasmic for F and *M. edulis* genome combination (Fed/Med). Sixty seven % of the *M. trossulus* males were heteroplasmic for Ftr/Mtr combination, the other 33 % showed a mitotype of only 860 bp band for *EcoRI*. The mitotypes for the Chilean mussels, *M. galloprovincialis* and *M. edulis* were similar for each of the sexes, while the *M. trossulus* female and male mitotypes are different from *M. edulis*.

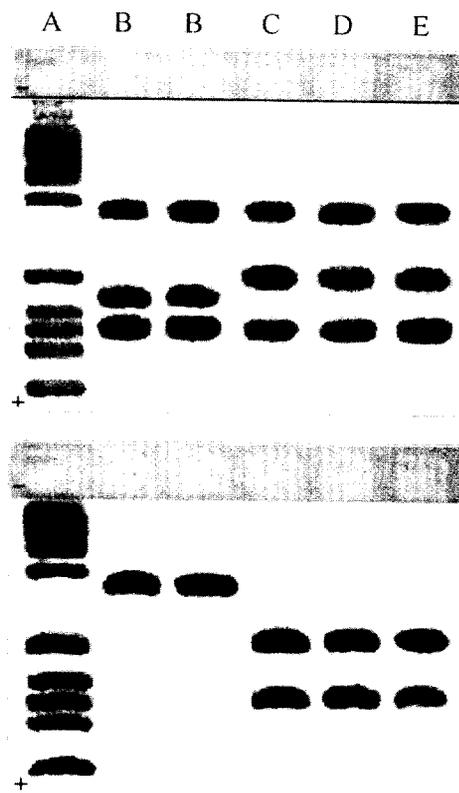


Figure 5. Scanned photonegative of an ethidium bromide stained 3 % agarose gels transilluminated with ultraviolet light showing the *EcoRI* profiles of the 860-bp *COIII* segment amplified from *Mytilus trossulus* (lane B), *M. edulis* (lane C), *M. chilensis* (lane D) and *M. galloprovincialis* (lane E) males (top gel) and females (bottom gel). Molecular weight marker (Gibco BRL 1 kb ladder) (lane A).

4. DISCUSSION

The three forms of *Mytilus* (*M. edulis*, *M. trossulus* and *M. chilensis*), differ in their shell morphology when measured by a multivariate analysis of several shell characteristics. The results presented here are similar to a previous study that also determined that *M. edulis* and *M. trossulus* are morphologically different [16]. However, the same study found that southern hemisphere *M. edulis* (formerly *M. chilensis* and *M. platensis*) were morphologically intermediate between northern *M. edulis* and *M. trossulus*. The higher discrimination found in the present study could be due, in part, to the use of the greater number of Chilean mussel shells examined ($n = 120$) instead of the 12 shells from Chile and 36 from Argentina and Kerguelen Islands used in the former study [16]. Because of the high phenotypic plasticity of the mussel shell, the latter being greatly influenced by the environment [24, 25], it would be necessary to carry out studies including reciprocal transplantations to evaluate the effect of the common environmental conditions. A recent study [8] found that *M. galloprovincialis* and *M. edulis* were morphologically distinct when allopatric populations were compared but were less distinct when individuals were sampled from populations in which both species coexist. In that particular case, aside from the possibility that common environmental conditions lead to greater similarity in shell morphologies in sympatric populations, hybridization and introgression could also play a role. The morphological distinctness among the three forms of *Mytilus* found in the present study is not due to differences in shell size of the populations sampled, because the size range of the individuals sampled for the study were restricted to adult size (47–73.3 mm) and any further differences in size were corrected by the log-transformed length standardization [15].

The study of the taxonomic status of the Chilean mussel should rely on an extensive suite of well-differentiated morphological traits and molecular genetic markers. The results obtained in the morphological study, based on a multivariate technique, was able to clearly distinguish among the three nominal mussel species. However, morphological studies, based exclusively on shell traits variation, may occasionally be used to identify mussel species on a local basis, when the mussels are sharing the same environment, but

these studies are less reliable over larger geographical scales [9, 10].

From previous studies and the results obtained here, using multivariate analysis of shell morphology and three PCR-based markers, it is clear that *M. edulis* and *M. trossulus* are two *Mytilus* forms that are genetically and morphologically different, and as discussed by other authors, this distinctness warrants taxonomic recognition at the species level [14, 16]. The morphologically different *M. edulis* and *M. chilensis*, however, were shown to share the same restriction patterns for the *ITS* nuclear PCR-marker and the *EcoRI* mtDNA marker. Despite differences in shell morphology, which can be related to differences in environmental conditions [16, 24], data presented here on the two DNA markers, as well as those from previous electrophoretic study [16], indicate that the Chilean mussel (*M. chilensis*) could be included tentatively in *M. edulis*. On the other hand, the other nuclear-DNA marker (*Glu-5*) produced different banding patterns in these two *Mytilus* forms. The Chilean mussel showed a banding pattern for the *Glu-5* marker that was similar to the pattern obtained when this marker was applied to a sample of *M. galloprovincialis* from New Zealand (figure 4). Another, recent study [18] described a similar banding pattern for the *Glu-5* marker in *M. galloprovincialis* from San Diego, CA, USA and Sete, France. In addition, an interesting observation [16] is that *Est-90*, the most common esterase allele in *M. galloprovincialis* and the rarest in *M. edulis* was the most common allele in the two mussel samples from Chile [16].

In a recent review of the taxonomic status of the Mediterranean mussel (*M. galloprovincialis*), Gardner [7] concluded, based on concordance principles, that the taxonomic status for this mussel is that of subspecies, using the trinomial form *M. edulis galloprovincialis*. The results obtained in the present study, which show evidence of genetic similarity for one nuclear-DNA and a mtDNA marker between *M. edulis*, *M. edulis galloprovincialis* and the Chilean mussel, suggest that the species status for *M. chilensis* seems inappropriate. On the other hand, because of the similar pattern found for the *Glu-5* marker and high frequencies of *Est-90* allele in both Chilean mussel and *M. edulis galloprovincialis* [16], it seems to be appropriate at this stage, until further studies show more evidence, that the taxonomic status of the Chilean mussel be as a subspecies, *M. edulis chilensis*, as proposed earlier in a report on the family Mytilidae [27].

Acknowledgments

I wish to acknowledge the support of research grants from Dr D.J. Innes and R.J. Thompson at Memorial University of Newfoundland. Also, I would like to thank Dr D.D. Heath for the helpful hints in setting up the PCR procedure for the *ITS* DNA marker, Dr O.R. Chaparro for help collecting and sending some of the samples from Chile and three anonymous reviewers for valuable suggestions. The author received a PhD fellowship from CIDA/MUN/Marine Science Scholarship.

REFERENCES

- [1] Aiken D., Bivalve culture in Chile, *World Aquacult.* 24 (1993) 7–19.
- [2] Bates J.A., Innes D.J., Genetic variation among populations of *Mytilus* spp. in eastern Newfoundland, *Mar. Biol.* 124 (1995) 417–424.
- [3] Beaumont A.R., Seed R., Garcia-Martinez P., Electrophoretic and morphometric criteria for the identification of the mussels *Mytilus edulis* and *M. galloprovincialis*, in: Rayland J.S., Tyler P.A. (Eds.), *Reproduction, genetics and distributions of marine organisms*, Olsen & Olsen, Denmark, 1989, pp. 251–258.
- [4] Beynon C.M., Skibinski D.O.F., The evolutionary relationships between three species of mussels (*Mytilus*) based on anonymous DNA polymorphisms, *J. Exp. Mar. Biol. Ecol.* 203 (1996) 1–10.
- [5] Côte-Real H.B.S.M., Holland P.W.H., Dixon D.R., Inheritance of a nuclear DNA polymorphism assayed in single bivalve larvae, *Mar. Biol.* 120 (1994) 415–420.
- [6] Coustau C., Renaud F., Delay B., Genetic characterization of the hybridization between *Mytilus edulis* and *Mytilus galloprovincialis* on the Atlantic coast of France, *Mar. Biol.* 111 (1991) 87–93.
- [7] Gardner J.P.A., *Mytilus galloprovincialis* (Lmk) (Bivalvia, Mollusca): the taxonomic status of the Mediterranean mussel, *Ophelia* 35 (1992) 219–243.
- [8] Gardner J.P.A., The *Mytilus edulis* species complex in southwest England: effects of hybridization and introgression upon interlocus associations and morphometric variation, *Mar. Biol.* 125 (1996) 385–399.
- [9] Gosling E.M., Systematics and geographic distribution of *Mytilus*, in: Gosling E. (Ed.), *The mussel Mytilus: ecology, physiology, genetics and culture*, Elsevier, Amsterdam, 1992, pp. 1–20.
- [10] Gosling E.M., Genetics of *Mytilus*, in: Gosling E. (Ed.), *The mussel Mytilus: ecology, physiology, genetics and culture*, Elsevier, Amsterdam, 1992, pp. 309–382.
- [11] Heath D.D., Rawson P.D., Hilbish T.J., PCR-based nuclear markers identify alien blue mussel (*Mytilus* spp.) genotypes on the west coast of Canada, *Can. J. Fish. Aquat. Sci.* 52 (1995) 2621–2627.
- [12] Inoue K., Waite J.H., Matsuoka M., Odo S., Harayama S., Interspecific variations in adhesive protein sequences of *Mytilus edulis*, *M. galloprovincialis*, and *M. trossulus*, *Biol. Bull.* 198 (1995) 370–375.
- [13] Inoue K., Odo S., Noda T., Nakao S., Takeyama S., Yamaha E., Yamazaki F., Harayama S., A possible hybrid zone in the *Mytilus edulis* complex in Japan revealed by PCR markers, *Mar. Biol.* 128 (1997) 91–95.
- [14] Koehn R.K., The genetics and taxonomy of species in the genus *Mytilus*, *Aquaculture* 94 (1991) 125–145.
- [15] Mallet A.L., Carver C.E., Comparative growth and survival patterns of *Mytilus trossulus* and *Mytilus edulis* in Atlantic Canada, *Can. J. Fish. Aquat. Sci.* 52 (1995) 1873–1880.
- [16] McDonald J.H., Seed R., Koehn R.K., Allozymes and morphometric characters of three species of *Mytilus* in northern and southern hemispheres, *Mar. Biol.* 111 (1991) 323–333.
- [17] Navarro J.M., Gutierrez A., Cultivo de moluscos en Chile, sinopsis sobre el estado actual del cultivo de mitilidos en Chile, in: Hernandez A. (Ed.), *Cultivo de moluscos en America Latina*, Bogotá, Colombia, 1991, pp. 189–210.
- [18] Rawson P.D., Joyner K.L., Meetze K., Hilbish T.J., Evidence for intragenic recombination within a novel genetic marker that distinguishes mussels in the *Mytilus edulis* species complex, *Heredity* 77 (1996) 599–607.
- [19] Saavedra C., Steward D.T., Stanwood R.R., Zouros E., Species-specific segregation of gender-associated mitochondrial DNA types in an area where two mussel species (*Mytilus edulis* and *Mytilus trossulus*) hybridize, *Genetics* 143 (1996) 1359–1367.
- [20] Sanjuan A., Zapata C., Alvarez G., *Mytilus galloprovincialis* and *M. edulis* on the coasts of the Iberian Peninsula, *Mar. Ecol. Prog. Ser.* 113 (1994) 131–146.
- [21] Sanjuan A., Comesaña A.S., De Carlos A., Macrogeographic differentiation by mtDNA restriction site analysis in the S.W. European *Mytilus galloprovincialis* Lmk, *J. Exp. Mar. Biol. Ecol.* 198 (1996) 89–100.
- [22] Sanjuan A., Zapata C., Alvarez G., Genetic differentiation in *Mytilus galloprovincialis* Lmk throughout the world, *Ophelia* 47 (1997) 13–31.
- [23] Sarver S.K., Foltz D.W., Genetic population structure of a species' complex of blue mussels (*Mytilus* spp.), *Mar. Biol.* 117 (1993) 105–112.
- [24] Seed R., Factors influencing shell shape in the mussel *Mytilus edulis*, *J. Mar. Biol. Assoc. UK* 48 (1968) 561–584.
- [25] Seed R., Absolute and allometric growth in the mussel *Mytilus edulis* L. (Mollusca: Bivalvia), *Proc. Malacol. Soc. Lond.* 40 (1973) 343–357.
- [26] Seed R., Systematics evolution and distribution of mussels belonging to the genus *Mytilus*: an overview, *Am. Malacol. Bull.* 9 (1992) 123–137.
- [27] Soot-Ryen T., A report on the family Mytilidae (Pelecypoda), *Allan Hancock Pacif. Exped.* 20 (1955) 1–175.
- [28] Steward D.T., Saavedra C., Stanwood R.R., Ball A.O., Zouros E., Male and female mitochondrial DNA lineages in the blue mussel (*Mytilus edulis*) species group, *Mol. Biol. Evol.* 12 (1995) 735–747.
- [29] Suchanek T.H., Geller J.B., Kreiser B.R., Mitton J.B., Zoogeographic distributions of the sibling species *Mytilus galloprovincialis* and *M. trossulus* (Bivalvia: Mytilidae) and their hybrids in the north Pacific, *Biol. Bull.* 193 (1997) 187–194.
- [30] Wilkinson L., SYSTAT. The system for statistics, Systat Inc., Evaston, Illinois, 1991, 676 p.