December, 2016

# Identification and Salinity Tolerance of the Western Hemisphere Mussel Mytella charruana (D'Orbigny, 1842) in the Philippines

Michael A Rice, *University of Rhode Island*
Paul D. Rawson, *University of Maine*
Ashley D. Salinas, *Bureau of Fisheries and Aquatic Resources, Philippines*
Westly R. Rosario, *National Integrated Fisheries Development and Training Center, Bureau of Fisheries and Aquatic Resources, Philippines*

*Journal of Shellfish Research*, Vol. 35, No. 4, 865–873, 2016.

# IDENTIFICATION AND SALINITY TOLERANCE OF THE WESTERN HEMISPHERE MUSSEL *MYTELLA CHARRUANA* (D'ORBIGNY, 1842) IN THE PHILIPPINES

**MICHAEL A. RICE,[1]* PAUL D. RAWSON,[2] ASHLEY D. SALINAS[3] AND WESTLY R. ROSARIO[3]**

[1]*Department of Fisheries, Animal and Veterinary Science, University of Rhode Island, 9 East Alumni Ave., Kingston, RI 02881;* [2]*School of Marine Sciences, 220 Murray Hall, University of Maine, Orono, ME 04469;* [3]*National Integrated Fisheries Technology and Development Center, Bureau of Fisheries and Aquatic Resources, Bliss Rd., Bonuan Binloc, Dagupan City 2400, Philippines*

***ABSTRACT*** Beginning in 2014, mussels have been found by shellfishers in parts of the Philippines that are morphologically distinct from native mytilids. These mussels, with a thick black periostracum, were first found in Manila Bay near an international shipping port, and later in 2014 appeared in western Tambac Bay (approximately 16.28° N, 119.9° E). The next year (July 2015), they were found near the village of Tucok (Dagupan City; 16.0272° N, 120.3147° E), and more recently (early 2016) they have been observed in Longos, San Fabian, Pangasinan (16.1887, 120.4043). About 50 mussels from Tucok were preserved in 95% ethanol and sent to the University of Maine for genetic evaluation. Sequencing of mitochondrial cytochrome oxidase I polymerase chain reaction products identified the nonnative mussels as *Mytella charruana*, the charru mussel, native to the tropical Caribbean and western Pacific coasts of South America. Further analysis indicates that populations on the Caribbean Coast of South America are the likely source of the Philippine charru mussels. Two salinity tolerance experiments were also conducted; in the first experiment, Philippine charru mussels were conditioned at salinity 5 (similar to the salinity of the collection site) for 7 days, then subjected to a salinity shock by directly transferring them to different salinity levels (10, 15, 20, 25, 30, and 35) for 12 days. In the second experiment, replicate groups of mussels were conditioned in 30 salinity, then for each replicate, salinity was gradually increased by five increments every 5 days until all mussels had died. In both the experiments, all mussels survived at salinities below 35. In the rapid salinity change trial, byssus formation was absent in mussels subjected to salinity 35 shock, and all mussels in this treatment died before the end of the experiment. In the acclimation trial, some mussels survived to salinity 60, but were inactive, and all died when salinity reached 65. These salinity shock and acclimation trials suggest that charru mussels may be best suited to Philippine waters during and after the monsoonal rainy seasons when salinities are routinely below 35, and may be spread among different estuaries via larval transport during the monsoonal rainy season. On the basis of these criteria, charru mussels may be a potential species for aquaculture complementing the culture of the native *Perna viridis*, a species with a higher preferred salinity that is traditionally cultured in the dry season.

***KEY WORDS:*** *Mytella charruana*, mtCO1, charru mussel, Philippines, exotic species, salinity tolerance

## INTRODUCTION

Beginning in early 2014, shellfishers in the region of Bacoor Bay (14.46° N, 120.89° E) near the Towns of Kawit and Binakayan in the province of Cavite in Manila Bay were finding unusual mussels that were different from the familiar native Philippine mytilids, *Perna viridis*, *Modiolus philippinarum* and *Modiolus modulaides* (=*Modiolus metcalfei*). These mussels attained moderately large sizes as adults, ranging from 5 to 7 cm in valve length, and had a thick black periostracum similar to that observed for mussels in the *Mytilus edulis* species complex found in more northern waters of Asia (Inoue et al. 1997). The northern area of Bacoor Bay is the location of the port facilities of Cavite City, which includes the primary naval base for the Philippines and docking for numerous international seagoing vessels. This suggests that ballast water discharges from vessels may have been a mechanism for the initial introduction of this exotic mussel to Philippine waters. Subsequent to the initial sightings in Bacoor Bay, these unusual mussels were observed 230 km to the north of Manila Bay in western Tambac Bay (16.2778° N, 119.9248° E) of Pangasinan Province by July 2014; transport to this new location was possibly by local shipping activities. In early 2015, they were found in the Calmay River Estuary (16.0263° N, 120.3136° E) near the village of Tucok (Dagupan City), with subsequent reports of them being found in late 2015 in Paombong, Bulacan Province in northern Manila Bay (14.7548° N, 120.7620° E), and in the estuary of Ternate, Cavite (14.2951° N, 120.7164° E) at the mouth of Manila Bay, and in January 2016 in Longos, San Fabian, Pangasinan (16.1887° N, 120.4043° E).

This study aimed to use a DNA-barcoding approach to identify this mussel, and reporting herein, it was determined that this new Philippine mussel is the charru mussel *Mytella charruana*. In addition to identifying this species, the purpose of this study is to provide information on the source populations and explore the environmental tolerances and invasion potential for Philippine *M. charruana*. On the basis of mussel growth and times of appearance, the period of greatest dispersion and recruitment for *M. charruana* in the Philippines appears to be in the months after the seasonal southwest monsoon as salinity in the estuaries gradually rises. Salinity is one of the most vital abiotic factors contributing to the temporal and spatial distribution and survival of marine bivalve species (Dame 1996); their distribution is profoundly affected by salinity as a result of their limited mobility (Castagna & Chanley 1973). Thus, salinity tolerance experiments were conducted to help determine whether this mesohaline/polyhaline, nonnative aquatic species could thrive once introduced to habitats in the Philippines characterized by seasonally varying salinity levels, and

*Corresponding author. E-mail: mrice@uri.edu
DOI: 10.2983/035.035.0415

whether an extended acclimation process would facilitate their survival. This information about salinity tolerance may allow prediction of areas in Philippine estuaries that may be colonized, and may also aid in the assessment of potential aquaculture sites for this species.

## MATERIALS AND METHODS

### *Genetic Studies*

Fifty of the exotic mussels collected from the Barangay Tucok site in Dagupan City on the Calmay River were preserved in 95% ethanol and sent to the University of Maine for genetic evaluation. Genomic DNA was isolated from a small (~20–30 mg) portion of the mantle tissue of 10 individual mussels using a QIAamp DNA Minikit, following the manufacturer's protocol (Qiagen Inc.). Quality and concentration of DNA were estimated by comparison with 200 ng of Hind III mass ladder (New England BioLabs) run in parallel with the 5 µl of the recovered DNA on a 1% agarose gel. A polymerase chain amplification reactions were used to generate a ~720-bp fragment of the mitochondrial cytochrome oxidase I (*mtCOI*) gene using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′; Folmer et al. 1994) from each DNA sample. Polymerase chain reactions (PCR) were performed in 25 µl volumes and contained 1 µl of DNA extract, 1.5 mM $MgCl_2$, 200 µM of each dNTP (Invitrogen), 0.5 µM each primer, 1 U of Taq polymerase (Invitrogen) in 1× PCR buffer supplied by the manufacturer. The reaction conditions included an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 20 s, 42°C for 30 s, and 72°C for 2 min with a final extension step of 72°C for 10 min. The resulting PCR products were spin column purified using a PureLink Quick PCR Purification Kit (Invitrogen) following the manufacturer's protocol. The purified PCR products were direct, cycle sequenced with the original PCR primers using a BigDye v3.1 Kit [Applied Biosystems (ABI)] and an ABI 3130XL Genetic Analyzer. The resulting chromatograms were visualized using the program FinchTV (Geospiza.com).

Upon obtaining 660 bp of unambiguous *mtCOI* sequence for one exotic Philippine mussel, Basic Local Alignment Search Tool searches to GenBank indicated that a portion of this initial sequence was 100% identical to bases 1–619 of *Mytella charruana mtCO1* haplotype A (accession no. EU917170) reported by Gillis et al. (2009) with a type location of Cartagena, Columbia. The next closest match in GenBank (excluding all *M. charruana* sequences), was to a sequence from *Modiolus brasiliensis* (syn: *Mytella guyanensis*; accession no. EU917193). The initial sequence (*M. charruana* Phil-1) was aligned with haplotypes A and H1 from two divergent *mtCOI* clades (accession nos. EU917170 and KP013759) that were reported for *M. charruana* by de Souza et al. (2015), haplotype EU917193 from *M. guyanensis*, and the native Philippine mussels *Perna viridis* (accession no. KM373589) and *Modiolus metcalfei* (syn: *Modiolus modulaides*; accession no. GQ480322). The sequences were then aligned using Clustal Omega (Sievers et al. 2011); from this alignment an unrooted neighbor-joining tree was developed to investigate the relationship between *M. charruana* and the other common Philippine mussels using MEGA6 (Tamura et al. 2013). A bootstrap analysis with 500 resamplings was used to estimate the significance attached to each node of the resulting phylogeny.

Two highly divergent *mtCOI* sequences and three chromatograms with numerous overlapping peaks were obtained. Marine mussels in the family Mytilidae have a complex pattern of doubly uniparental inheritance (Zouros et al. 1992, Rawson & Hilbish 1995, Zouros, 2000), in which there are separately transmitted female and male mitochondrial DNA lineages. While most individuals carry female mtDNA (*f-mtDNA*) in their somatic tissues, male mussels have a second lineage (*m-mtDNA*) that is retained from the sperm and amplified in the gonad, which develops in the mantle, and is passed on to their sons. Thus, it is not surprising to pick up overlapping sequences when genomic DNA is isolated from the mantle in mytilids and used in PCR reactions targeting mitochondrial genes. Basic Local Alignment Search Tool searches to GenBank with the ~650 bp of unambiguous *mtCOI* sequence from the two divergent sequences lacking overlapping peaks indicated these sequences had 93% identity to bases 1–542 of two *m-mtCOI* haplotypes from *Mytella charruana* reported by Alves et al. (2012). Two *m-mtCOI* sequences (*M. charruana* Phil-2 and *M. charruana* Phil-3) and initial *f-mtCOI* sequence were aligned with the *M. charruana* haplotypes given above, along with the two *m-mtCOI* haplotypes reported by Alves et al. (2012). As mentioned earlier, an unrooted neighbor-joining tree was estimated using MEGA6 (Tamura et al. 2013). A bootstrap analysis with 500 resamplings was used to estimate the significance attached to each node of the resulting phylogeny.

The sequences were aligned for both *m-* and *f-mtCOI* lineages with all of those available on GenBank for *Mytella charruana*. In this alignment, we could not confirm the accuracy of the *f-mtCOI* lineage-specific primers reported in the work of Gillis et al. (2009). Thus, the primers Mytel fCO1FOR (5′-TTTTCTAAAAAACGAAAGCTTGTAT-3′) and Mytel fCO1REV (5′-GGAACCGAAATAATTAACAGAACA-3′) were designed to specifically amplify female-lineage haplotypes. Although these primers amplify a PCR product that is only 462 bp in size, they distinguish *f-* and *m-mtCOI* sequences within *M. charruana*. These primers were used in PCR reactions following the protocol given above, with the exception that the annealing temperature was increased to 50°C. From these reactions, 397 bp of unambiguous *f-mtCOI* sequence were obtained from 10 *M. charruana* specimens from Barangay Tucok. The statistical parsimony approach of Clement et al. (2002) was used in the program Population Analysis with Reticulate Trees (http://popart.otago.ac.nz/index.shtml) to estimate a haplotype network for inferring the source population of our Philippine *M. charruana* samples.

### *Salinity Tolerance Studies*

For the first experiment on salinity tolerance of Philippine *Mytella charruana*, protocols similar to those of Yuan et al. (2010) were used. Samples of *M. charruana* were collected along the Bued River Estuary in Barangay Longos, San Fabian, Pangasinan, on January 12, 2016, at 11:00 AM. The time, weather condition, and environmental parameters of the site collection (e.g., water salinity, temperature, and dissolved oxygen) were recorded prior to sampling. Mussels were sampled from multiple locations along the river because each salinity trial (below) required 500 individuals, and one site did not have

sufficient mussels for the whole experiment. Collected mussels were transported to the BFAR-NIFTDC Laboratory located in Bonuan Binloc, Dagupan City, less than a kilometer away from the collection site. The shell length, width, and height of individual mussels were measured; small mussels (≤2.4 cm) were segregated from large mussels (≥2.5 cm). The small mussels had an average of 1.54 cm length, 0.63 cm width, 0.3 cm thickness, and 0.45 g wet weight, whereas the large mussels had an average of 3.1 cm length, 1.4 cm width, 0.8 cm thickness, and 2.64 g wet weight.

Mussels were conditioned in water with a salinity similar to that at the collection site (salinity 5) along with slight aeration for 7 days prior to the application of salinity shock. During conditioning, the mussels remained undisturbed except for the removal and replacement of dead mussels. Mussels in each aquarium were fed every other day by addition of 200 ml of *Isochrysis galbana* (T-ISO) from stock cultures of approximately 30 million cells/ml, for a final concentration of 300,000 cells/ml in each aquarium. The salinity and temperature of the water in each aquarium were monitored daily. If an individual mussel remained gaping and did not respond to physical stimulation, they were considered dead, removed and replaced with a live mussel; salinity shock experiments commenced only after no mussel deaths were observed for four consecutive days. There were six experimental treatments with respective salinity levels of 10, 15, 20, 25, 30, and 35 with three replicates for each salinity group. A control group was also included in the experiment; this group was kept at a salinity of 5 that matched the salinity at the collection site. Each plastic aquarium contained 20 l water accommodating 10 small and 10 large mussels with slight aeration. After 7 days of conditioning, individuals were randomly selected from the holding tanks and transferred to each of the experimental aquaria. Feeding protocols remained the same as during the acclimation period. The salinity shock trials ran for 12 days, with the water temperature at 25–26°C. No water changes were conducted, and salinity, temperature, and mortalities were monitored twice daily, in the morning (9:00 AM) and afternoon (3:00 PM).

In a second set of salinity tolerance trials, aimed at gauging ability of the mussels to acclimate to higher salinity regimes, *Mytella charruana* were collected from the Calmay River of Dagupan City. Small mussels (≤2.4 cm) were segregated from large mussels (≥2.5 cm). The biometric sampling conducted for small mussels yielded an average of 2.0 cm length, 1.0 cm width, and 0.54 cm thickness. Large mussels had an average of 4.3 cm length, 2.0 cm width, and 1.2 cm thickness. Mussels were transported to the laboratory in Bonuan Binloc and conditioned as previously described with water salinity similar to the salinity of collection site (salinity 30) for 7 days prior to salinity increment trials. The salinity increment trials consisted of a control group in which 20 mussels (10 each of the small and large) were held and fed at salinity 30 throughout the entire trial, and three replicate experimental groups in which salinity was increased by a factor of 5 when water was changed; a 50% water change was conducted every 5 days for all aquaria. The water temperature was held at 27–29°C, and all mussels were fed T-ISO every other day at a concentration of 300,000 cells/ml in each aquarium. The experiment lasted until no mussels were left alive in the experimental tanks. Survivorship data from both salinity trials were analyzed by *t* test to detect significant differences in mean salinity tolerances between larger and smaller mussels. In addition, one-way analyses of variance (ANOVA) were used to assess differences in survival among replicate salinity treatments using the data analysis package in MS-Excel, with significance reported at the $P < 0.05$ level.

Salinity profiling of surface waters at various stations in the Dagupan City Calmay River Estuary system was carried out using a refractometer on a monthly basis during 2015 to provide a field comparison of ambient salinities in areas of potential spread and colonization by *Mytella charruana*. Surface water was also sampled during 2015 in Sual Bay, an area in the open-water Lingayen Gulf to gauge coastal salinities during the dry and rainy southwest monsoon seasons.

## RESULTS

### *Identification of Mussels*

Genomic DNA was successfully isolated, and mtDNA sequences were obtained from preserved specimens of an exotic mussel species from the Barangay Tucok site on the Calmay River. Basic Local Alignment Search Tool searches to GenBank indicated that the *mtCOI* sequence (GenBank accession no. KX776480) initially obtained for one of these exotic mussels had highest affinity to female-lineage haplotypes from the charru mussel *Mytella charruana*. The Rawson laboratory at the University of Maine had not worked with *Mytella* previously, thus, there is little chance that this result is due to a prior contamination. This sequence had 100% identity with *M. charruana* "Haplotype A" reported by Gillis et al. (2009). de Souza et al. (2015) identified two divergent clades or haplogroups of f-mtCOI sequences among *M. charruana* sampled from South America and the southeastern United States. Neighbor-joining phylogenetic analysis was used to compare the initial f-mtCOI sequence to sequences from the two divergent *M. charruana* haplogroups reported by de Souza et al. (2015) and to sequences from other mytilids commonly found in the Philippines. Our results clearly indicate that the "exotic" mussels with shells with slightly purplish interior and a thick black periostracum are not only morphologically distinct but also genetically distinct from *Perna viridis*, the Asian green mussel, and the horse mussel *Modiolus modulaides* (Fig. 1). Furthermore, bootstrap analysis provides strong support for this sequence belonging to *Mytella* haplogroup B.

In the initial effort to isolate *mtCOI* sequences from Philippine mussels, two highly divergent sequences (*Mytella*

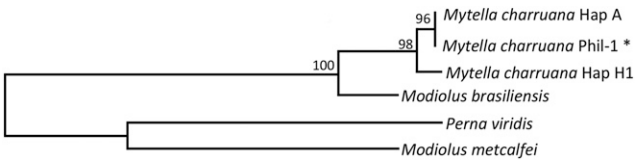


**Figure 1. Neighbor-joining phylogeny illustrating the genetic distance relationships among female-lineage mtCOI sequences from *Mytella charruana*, *Modiolus brasiliensis* (syn: *Mytella guyanensis*), *Perna viridis*, and *Modiolus metcalfei* (syn: *Modiolus modulaides*). Bootstrap support based on 500 iterations is shown adjacent to nodes with >80% support. For brevity, only haplotype A (Hap A) and H1 (Hap H1) are included from each of the haplogroups A and B, respectively, that were reported for f-mtCOI *M. charruana* sequences by de Souza et al. (2015). An asterisk marks the f-mtCOI sequence obtained from a Philippine mussel.**

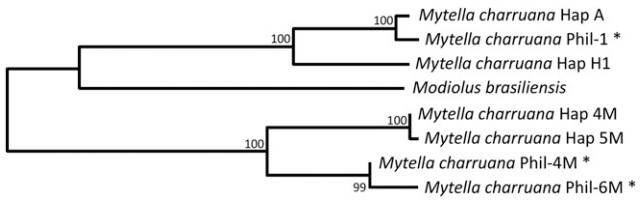


**Figure 2. Neighbor-joining phylogeny illustrating the genetic distance relationships among female- and male-lineage mtCOI sequences from *Mytella charruana*, along with a single f-mtCOI sequence from *Modiolus brasiliensis* (syn: *Mytella guyanensis*). Bootstrap support based on 500 iterations is shown adjacent to nodes with >80% support. As in Figure 1, here also only a single sequence from each of the two divergent *M. charruana* f-mtCOI haplogroups described by de Souza et al. (2015) was included, and sequences identified in this study are marked with asterisks.**

*charruana* Phil-4M and Phil-6M; GenBank accession nos. KX781339 and KX781340, respectively) were obtained that differ from the previous *f-mtCOI* sequences for Philippine *Mytella* by greater than 100 substitutions. These sequences are most closely related to haplogroup C of de Souza et al. (2015) that contains two *m-mtCOI* sequences (*M. charruana* 4M and 5M) first identified by Alves et al. (2012). These results confirm the presence of highly divergent male and female mtDNA lineages in *M. charruana*, a feature shared with other mytilids. A neighbor-joining phylogeny was constructed to examine the variation among representative sequences from the two *M. charruana f-mtCOI* haplogroups, one sequence from *Modiolus brasiliensis* (*Mytella guyanensis*) and two male lineage *mtCOI M. charruana* haplotypes (*M. charruana* 4M and 5M). This analysis confirms that the two male-lineage sequences obtained from Philippine *M. charruana* have closest affinity to the male-lineage haplotypes reported by Alves et al. (2012) for *M. charruana* they sampled from Bragança on the northern coast of Brazil (Fig. 2). Even so, bootstrap analysis suggests that the two Philippine *m-mtCOI* sequences are distinct from the Brazilian *m-mtCOI* haplotypes. Further, this phylogeny suggests that the male mtDNA lineage predates divergence between *M. charruana* and *M. guyanensis*. The doubly uniparental inheritance of mtDNA is however complex, and several reports have indicated the loss of male lineages and subsequent conversion of female haplotypes to male haplotypes in other mytilids (Zouros 2000). Given the current paucity of male sequences in *M. charruana* and the lack of male sequences for *M. guyanensis*, it is not feasible to date the origin of the male lineage in *Mytella*.

The *f-mtCOI* sequences were obtained from nine additional *Mytella charruana* from Barangay Tucok. Despite the shorter length of these sequences, haplotypes among the 10 total f-mtCOI sequences obtained from the Philippine charru mussels were distinguished. Statistical parsimony network analysis (Fig. 3) indicated that sequences clustered with *M. charruana*

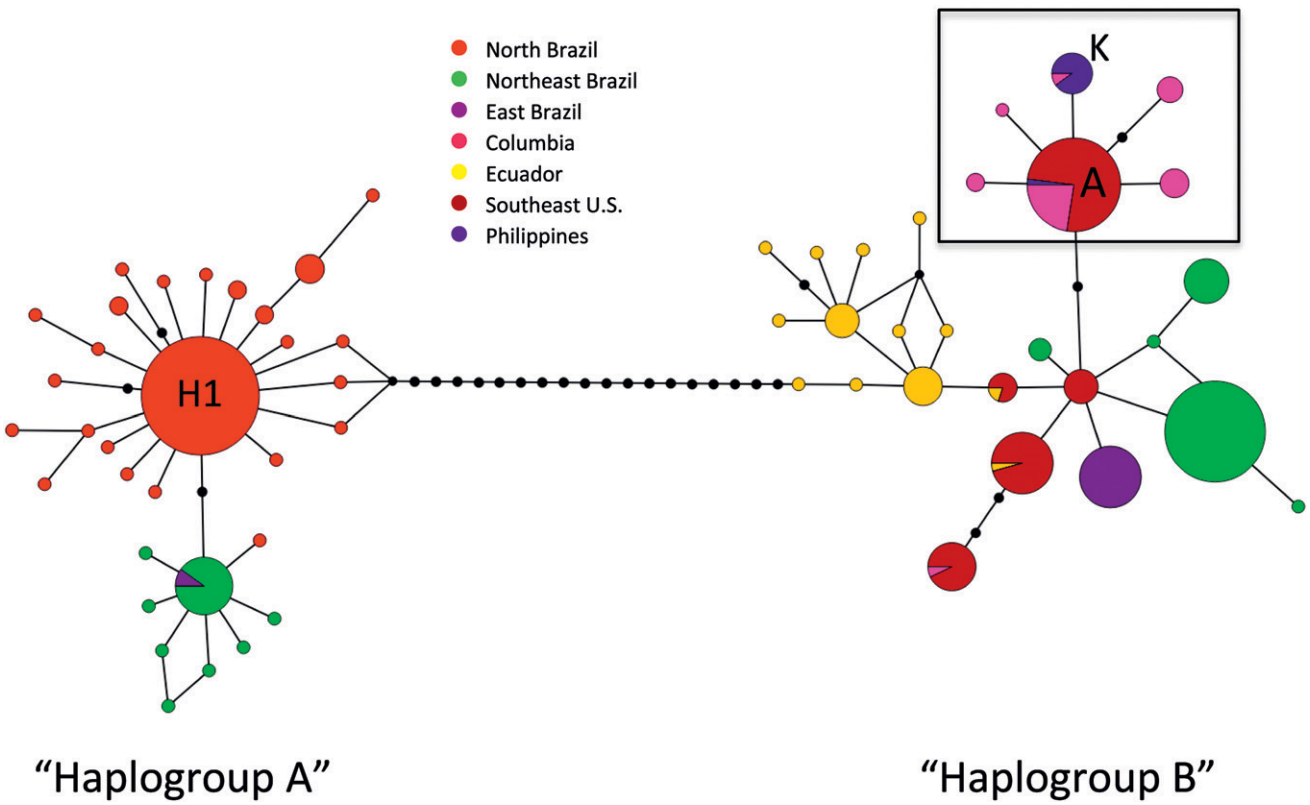


**Figure 3. Statistical parsimony network among f-mtCOI sequences from South American, southeastern United States and Philippine populations. This analysis is based on an alignment of 397 bp common to all sequences. Haplogroups A and B are indicated, as per de Souza et al. (2015). Missing or inferred haplotypes are indicated by black circles. The size of each circle indicates the overall frequency of each haplotype in the combined dataset while the color of each circle indicates the population in which each haplotype was observed. Haplotypes A and H1 are those used in the neighbor-joining phylogenies in Figures 1 and 2; the box indicates the position of all Philippine *Mytella charruana* sequences in the network.**

haplotypes A (accession no. EU917170) and K (accession no. EU917174) in haplogroup B with the majority ($n = 8$) being identical to haplotype K of Gillis et al. (2009). Both haplotypes have been observed among *M. charruana* from Cartegena, Colombia, whereas haplotype A has also been observed among charru mussels at several locations in southeastern United States (Gillis et al. 2009). This analysis thus suggests that populations on the Caribbean Coast of South America have served as the source population for the *M. charruana* introduced independently to the southeastern United States and the Philippines, with the likelihood of the mussels being carried to the Philippines via shipping traffic traversing the Panama Canal.

### *Salinity Tolerance Trials*

Throughout the 12-day salinity shock experiments, mortalities were observed only in the aquaria with salinity 35. Results of the *t* test between survivorships of smaller and larger mussels were not significant (>0.05), so smaller and larger mussel data were pooled for reporting purposes. Although there was variation among the replicate tanks, there was a steady decline in survival for the mussels directly transferred from 5 to 35 salinity, most of the mussels died after 4 days and all mussels had died by 11 days posttransfer (Fig. 4). In addition, no byssus formation was also observed for the mussels subjected to the salinity 35 shock. The mussels remained closed and unattached in the aquarium. In all other salinity treatments from the salinity 5 control up to 30, the mussels behaved normally including byssal attachment to the aquarium surface. The surviving mussels were held and fed for another 2 wk after the 12 days experimental period to determine whether there were any delayed behavioral changes or mortalities among mussels in the salinity 5 control and the treatment groups from salinities 10 to 30. No mortalities were observed in any of the treatments. From this, it was concluded that *Mytella charruana* can readily withstand a direct transfer into a wide range of salinity regimes (salinity 5–30), even without acclimation, but mussels transferred to salinities higher than 30 did not form byssal threads and eventually died after few days.

In the higher salinity acclimation trials (Fig. 5), all the mussels in the three replicate treatment groups died after being subjected to salinity 65 seawater. The results indicate that 100% of the mussels survived in both the salinity 55 treatment and the salinity 30 control aquaria. The experiment showed that *Mytella charruana* can tolerate a wide range of salinities, and that these mussels have the ability to acclimate to salinities above the nominal salinity 30 threshold if the salinity change is gradual. Mussels tolerated salinity 55 with 100% survival through gradual increase in salinity under controlled environmental conditions. As salinities began to exceed 60, however, even with acclimation, significantly greater mortalities ($P < 0.05$ ANOVA) occurred.

### *Measurement of Estuarine and Coastal Salinity Levels*

The salinity profiles at several stations on the Calmay River–Dagupan Estuary (Fig. 6) show peak salinities in the month of June at the hottest and driest part of the year just before the July

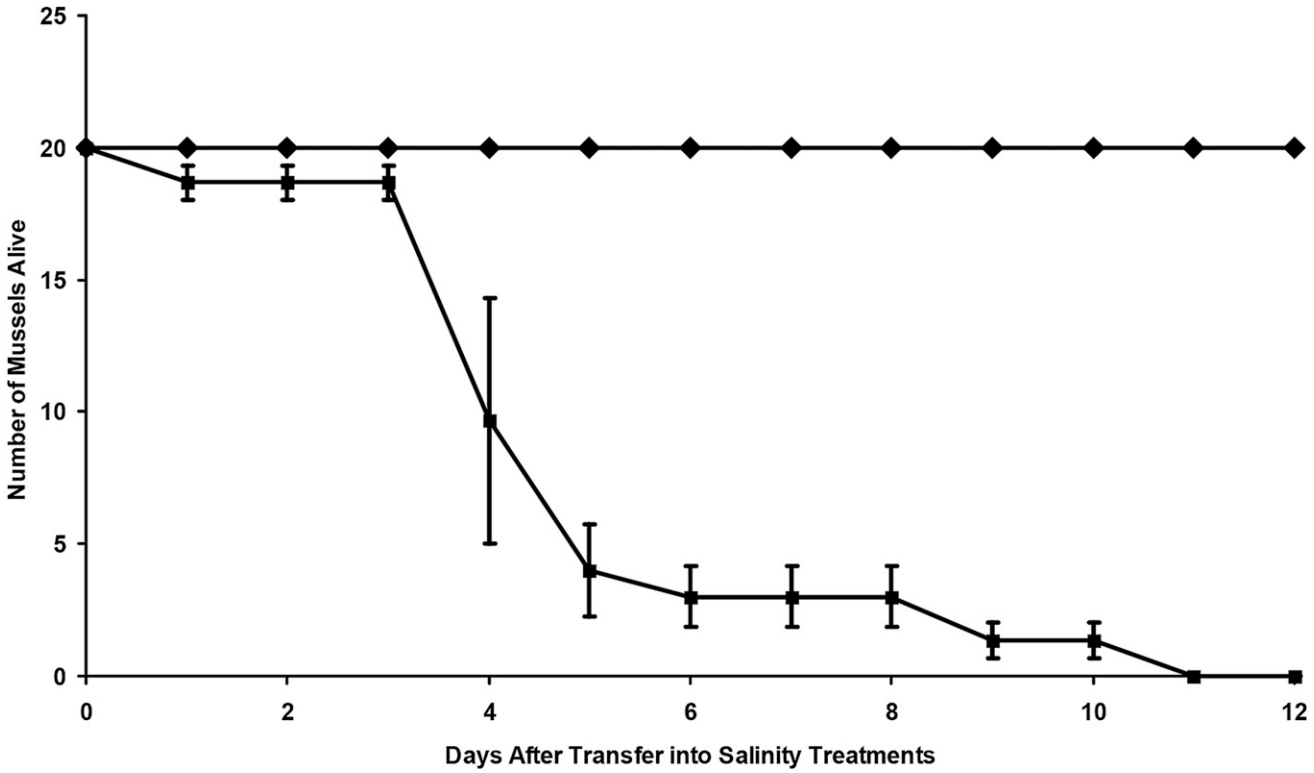


**Figure 4. The mortality of Philippine charru mussels exposed to various salinity regimes after collection from salinity 5 estuarine waters and a 7-day maintenance period in the laboratory at salinity 5. Survival data from the salinity 5 control and most test aquaria (salinity 10, 15, 20, 25, and 30) are indicated by diamond markers, whereas data from the salinity 35 test aquaria are indicated with square markers. Mortalities were noted only in aquaria with salinity 35 seawater. Most mussels died during day 4, and all mussels were dead by day 11. Error bars indicate standard error of the mean ($n = 3$).**

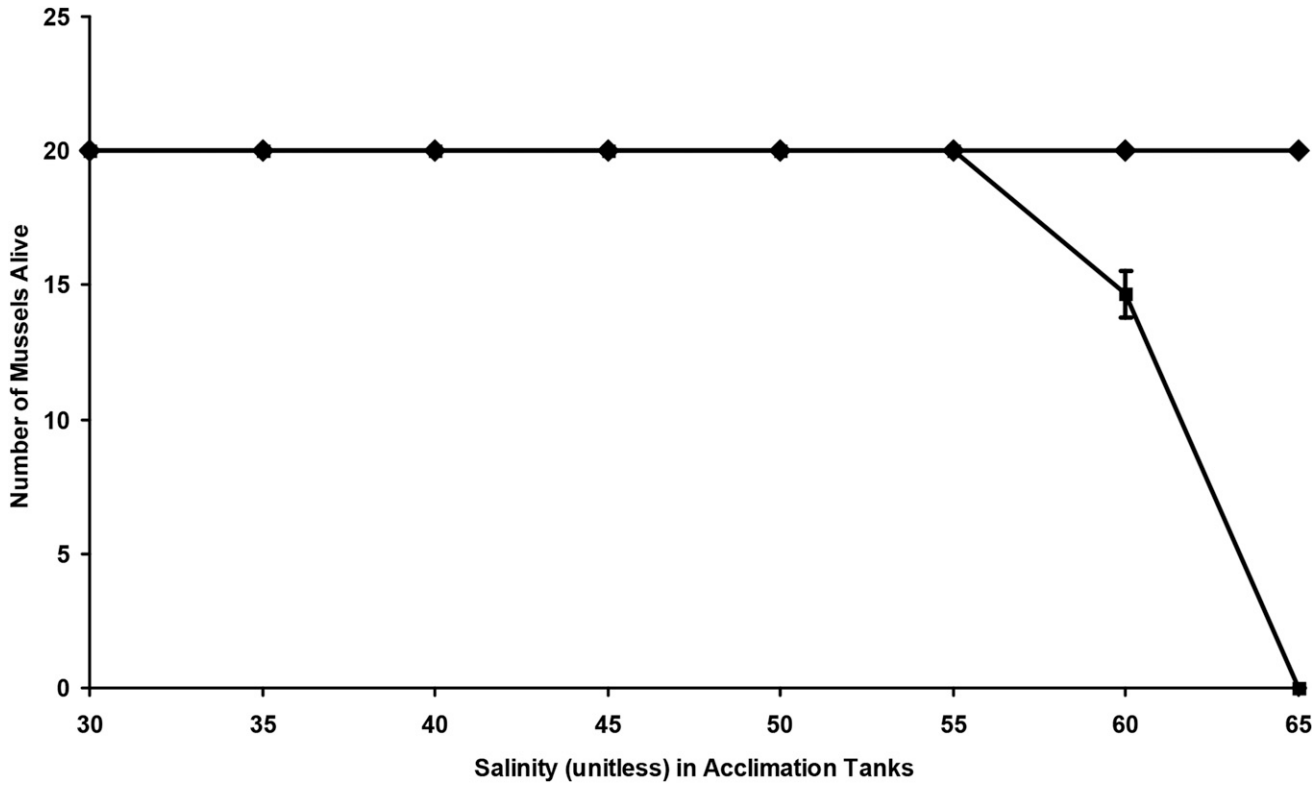


**Figure 5. The mortality response of charru mussel *Mytella charruana* acclimated to 30 psu salinity and subjected to a salinity 5 incremental increase every 5 days. Diamond markers represent survival of mussels in the salinity 30 control tank, and square markers represent response of mussels in the test tanks with the incremental salinity increases. Data from individual replicate experiments are shown with error bars indicating standard error of the mean ($n = 3$). Significant differences in means ($P < 0.05$ ANOVA) between the control and the treatment group by one-way ANOVA were only found once 60 and 65 psu salinities were reached.**

onset of the southwest monsoon rainy season. Surface water salinities may drop to zero during the rains, but gradually increase over several months as the rains cease in August or September and oceanic salinities become reestablished in the early months of the following year. Some locations upstream from the mouth of the river and the saline waters of the Lingayen Gulf remain below salinity 30 even during the height of the hot dry Philippine summer. These are likely areas for year- around survival of charru mussels. One of the initial charru mussel colonization sites in the Calmay River, Barangay Tucok, is one site that does not reach salinities above 35, and the charru mussels have remained alive there year-around so far.

Salinity fluctuation of the coastal waters of the Lingayen Gulf was investigated by monitoring salinity at six stations in nearby Sual Bay during 2015 (Fig. 7). Sual Bay is a highly flushed embayment along the coast of Pangasinan with relatively modest freshwater input even during the height of the monsoon season because of no large river nearby. During the postmonsoon period, when the rains do stop, salinity remains below salinity 30 for a couple of months, so this may be an opportune time when charru mussel larvae could be dispersed to adjacent estuaries if carried by longshore currents.

## DISCUSSION

The charru mussel *Mytella charruana* is an epifaunal tropical and subtropical mussel colonizing rocky substrates in estuarine areas primarily along the Atlantic and Caribbean coasts of South America (Scarabino et al. 1975). Charru mussels have great dispersal ability and appear to readily colonize a variety of habitats; traits that no doubt have facilitated this mussel becoming an important invasive species in several regions of the world (Gillis et al. 2009).

Large numbers of charru mussels first appeared in the United States in 1986 in the seawater intake pipe of a powerplant in Jacksonville, FL (Lee 1987). These mussels died out in early 1987 and were considered extirpated from the state at that time. Charru mussels were, however, reintroduced into Florida waters via the Mosquito Lagoon in the Indian River Lagoon system in 2004 (Boudreaux & Walters 2006), and they have spread considerably along the Atlantic Coast from South Carolina to Central Florida since this secondary introduction (Spinuzzi et al. 2013). In their analysis of female-lineage mtCOI sequence variation, Gillis et al. (2009) concluded that charru mussels in the southeastern United States originated from source populations on the Caribbean Coast of South America. Our analysis of 10 female-lineage sequences suggests a similar origin for Philippine charru mussels. The 10 female-lineage sequences obtained in this study cluster with haplotypes A and K reported by Gillis et al. (2009) in a statistical parsimony network. Haplotypes A and K have been observed among charru mussels from Cartagena, Colombia; the former is also common among nonnative charru mussels in the

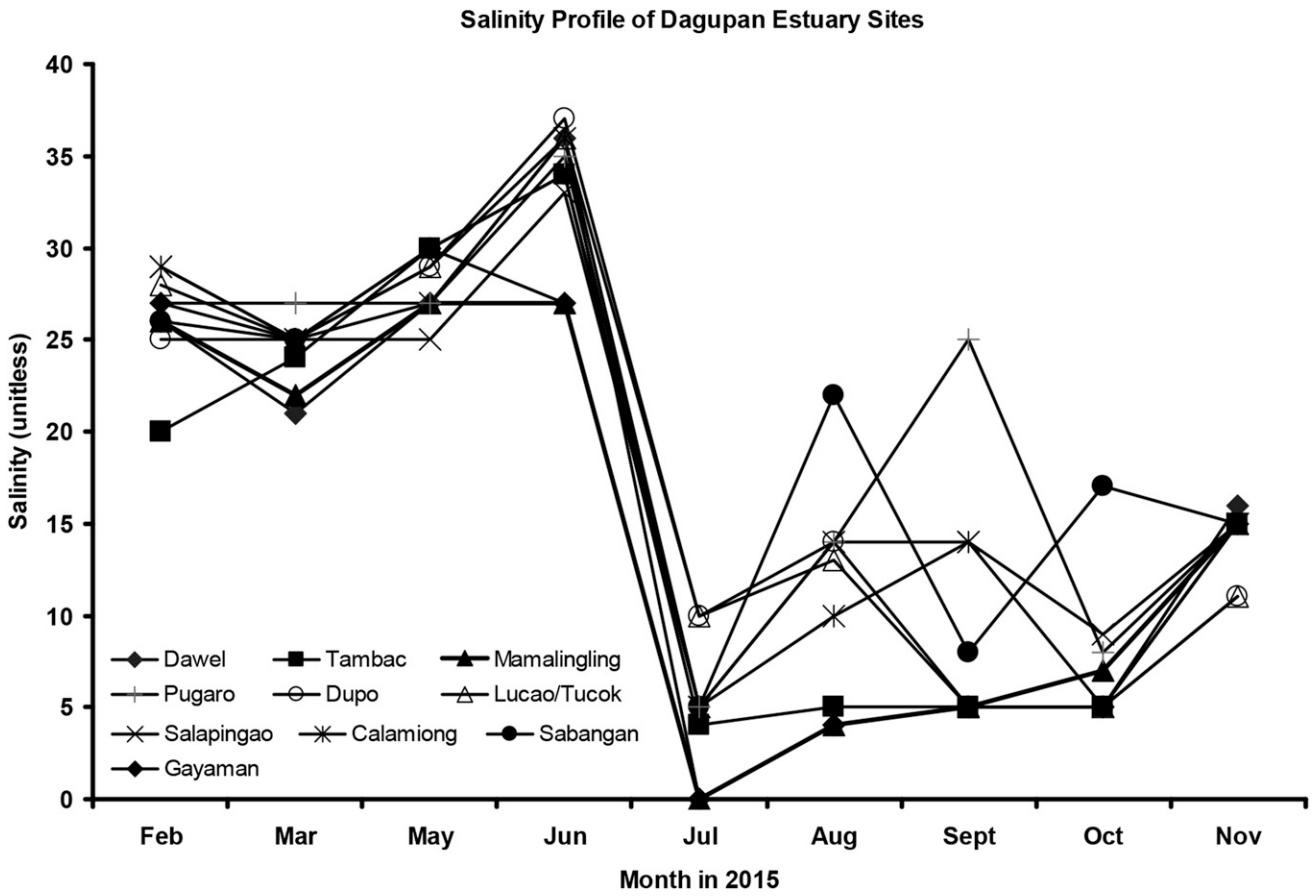


**Figure 6. Surface salinity profile (unitless) in several locations in the Dagupan City Estuary–Calmay River from monthly measurements taken between February and November 2015. Highest salinities above 30 were found at locations nearest the mouth of the estuary exchanging with the waters of the Lingayen Gulf. Areas for highest likelihood of year-around survival of charru mussels were at locations further up the estuary that remained below the salinity 30 threshold year-around.**

southeastern United States. We have also observed that divergent male-lineage mtDNA is common, at least among the mussels sampled from Barangay Tucok. The two unambiguous m-mtCOI sequences obtained in this study were substantially divergent from the only other *Mytella charruana* male-lineage sequences on GenBank. Further analysis is limited by the paucity of male-lineage sequences for *Mytella* mussels. The results of this study, however, indicate that it is unlikely that Philippine mussels originated from northern Brazil populations of *M. charruana*, consistent with the results obtained with the female-lineage sequences. To our knowledge, this and preliminary reports (Rawson et al. 2016, Rice et al. 2016) are the first reports of an instance of a trans-Pacific introduction of this neotropical mussel species into an Asian location.

Charru mussels can reach 65 mm in shell length; the shells of this species are frequently characterized as having a dark brown to black external color with semicircular rings and an iridescent purple interior. Close inspection of the shell reveals a wavy dark (brown, purple, and dark green) and light (cream) patterns in the shell (Spinuzzi et al. 2013). The holotype specimen in the Smithsonian Institution United States National Museum identified under the synonym of *Modiolus arciformis* (Dall, 1909) (USNM Holotype 207,756) is light in color with a cream-colored exterior. The shell color of the Philippine specimens inspected in this study tends toward the darker shades. Most specimens collected in the Philippines so far exhibit the iridescent purple interior along with a thick black periostracum that may occasionally tend toward dark brown in smaller specimens.

After the 2004 reintroduction of *Mytella charruana* to Florida, they spread rapidly and became firmly established in many estuaries along the southeast Atlantic Coast. In the Mosquito Lagoon area, they exhibit peak spawning during August, which corresponds with the period of peak in precipitation for the area (Gilg et al. 2010). If the reproductive pattern of *M. charruana* follows suit in the Philippines with spawning period maximum during the southwest monsoonal rainy season, this may also be the period of most likelihood of spread of the mussels from estuary to estuary along the coast. Charru mussels collected from the Bued River-Longos, San Fabian Estuary, for the salinity shock trials are separated from the mouth of the Calmay River–Dagupan Estuary by a distance of 8 km by the waters of the Lingayen Gulf that are usually greater than 30 salinity for most of the year. Salinity dips in the Lingayen Gulf below the salinity 30 threshold during the rainy season (Fig. 7) however may provide ideal conditions for longshore dispersal of mussels from estuary to estuary. Further studies of

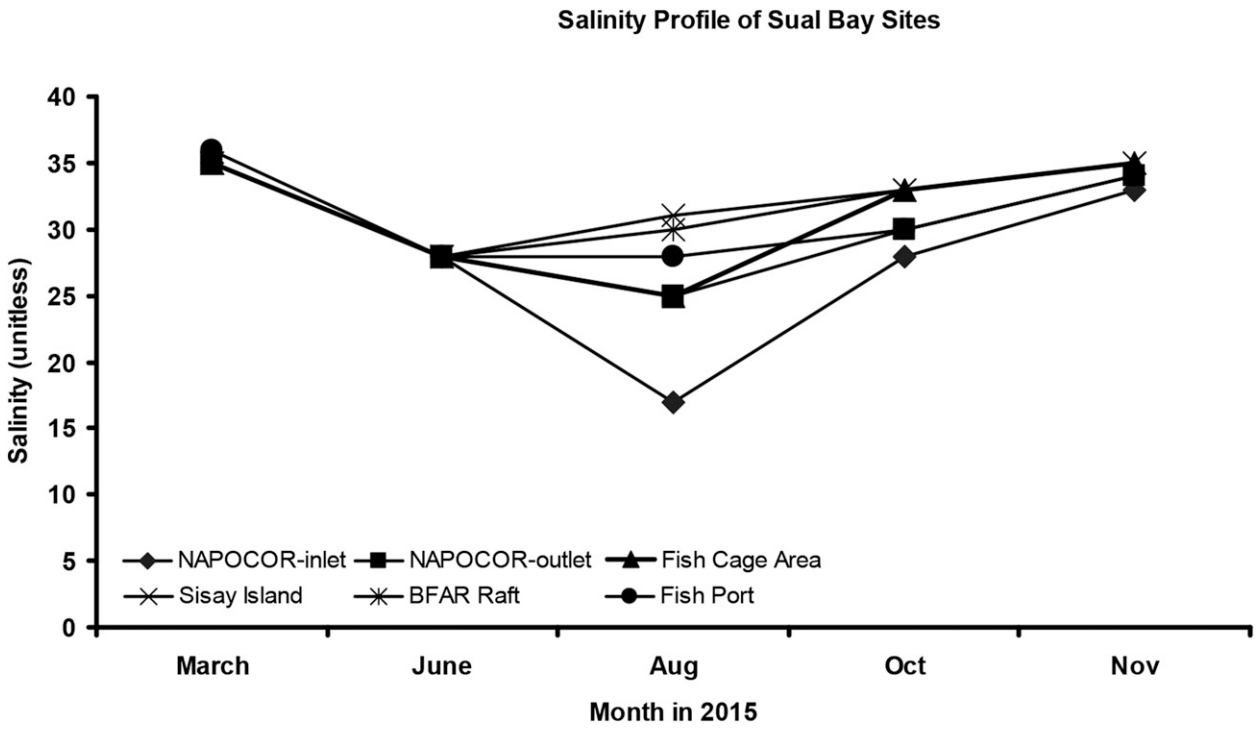


**Figure 7. Surface salinity profile (unitless) at several locations in Sual Bay, an embayment of the Lingayen Gulf at about 16.1° N and 120.1° E taken between March and November 2015. The salinity profile of the Bay was lowest in August after the onset of the July onset of monsoonal rains, but exceeded salinity 30 by November. When coastal salinity drops below the nominal salinity 30 mortality threshold for charru mussels, spread of larval stages from estuary to estuary may potentially occur.**

spawning times and salinity tolerances of the pelagic larvae of the Philippine charru mussels should shed further light on this speculative dispersion pathway.

The charru mussel *Mytella charruana* is considered a mesohaline and polyhaline species that is found from marine environments with salinities higher than 25 to estuarine environments with salinities as low as 5. A population of mussels at the Longos site was found that appears to be able to survive salinities as low as 2 for several weeks (A. D. Salinas, unpublished data). Salinity tolerance data, estimated for 36 bivalve species by Castagna and Chanley (1973), suggest that acclimation to small incremental changes in salinity may allow species to broaden their tolerance threshold. The salinity tolerance results show that even without small incremental changes in salinity, charru mussels have a very broad tolerance for changing salinity. Yuan et al. (2010) explored survival threshold of charru mussels by experimenting on the salinity tolerance of mussels from the Florida population. In their study, large *M. charruana* (20- to 54-mm shell length) survived best in salinities from 2 to 23, with 100% mortality at 0 and 45 salinities. Larger mussels were able to tolerate up to salinity 40 with gradual salinity adjustment. Smaller *M. charruana* (3–19 mm) survived in a wider range of salinities (2–40 ppt) and could acclimatize to more extreme levels when salinity was adjusted gradually. Their observations indicated that charru mussels have the potential to invade a wide variety of habitats particularly when they have an opportunity to acclimate to gradual changes in salinity. Acclimation experiments in this study on the Philippine charru mussels provided very similar results; however, we did not detect statistically significant size-based differences in salinity tolerances. Both studies indicate that charru mussels have the capacity to invade a wide variety of saline environments with significant freshwater or marine input, possibly surviving wide salinity swings in Philippine estuaries associated with the rapid onset of abundant monsoonal rains. Their ability to gradually adapt to salinities higher than 55 suggests that under some circumstances, the mussels may have potential for spread throughout the Philippine archipelago.

Results indicating that gradual acclimation over a 5-day period allowed charru mussels to survive exposure to high salinities are consistent with previous studies with other mytilids. These studies have shown that mytilids adjust to high salinity by activation of metabolic pathways, such as the induction of peptidases to depolymerize proteins and activation of enzymes catalyzing the alanine dehydrogenase reaction that utilizes ammonia and pyruvic acid in the formation of free alanine, to increase the concentration of osmotically active intracellular free amino acids in their tissues (e.g., Baginski & Pierce 1977, 1978, Berger & Kharazova 1997, Livingstone et al. 1979). Although the metabolic basis of salinity acclimation in *Mytella* remains unstudied, it is predicted that similar pathways are likely important for the charru mussel.

A previous report (Rice et al. 2016) discussed Philippine mussel farmers successfully adapting some of their mussel culture gear primarily designed for the culture of green mussels *Perna viridis* for the culture of *Mytella charruana* during and in the months after the rainy season. They

reported that green mussels die off during the onset of the rainy season. Indeed, Nair and Murugan (1991) have shown that *P. viridis* have salinity tolerance thresholds of 19 and 44. With data of this study and of others showing that *M. charruana* are viable at much lower salinities, there is the possibility that mussel farmers in some sites will be able to expand mussel production into a two-season enterprise with alternation of the two species between the dry and wet growing seasons.

**ACKNOWLEDGMENTS**

We gratefully acknowledge the technical assistance of Chastity Bowman of the University of Maine for conducting some of the genetic analyses of the charru mussels. Partial funding for this project has been through the University of Maine Agricultural Experiment Station, The Philippine Bureau of Fisheries and Aquatic Resources, and the University of Rhode Island Cooperative Extension.

**LITERATURE CITED**

Alves, F. A., C. R. Beasley, W. R. Hoeh, R. M. Rocha, L. R. L. Simone & C. H. Tagliaro. 2012. Detection of mitochondrial DNA heteroplasmy suggests a doubly uniparental inheritance pattern in the mussel, *Mytella charruana*. *Rev. Bras. Biocienc.* 10:176–185.

Baginski, R. M. & S. K. Pierce. 1977. The time course of intracellular free amino acid accumulation in tissues of *Modiolus demissus* during high salinity adaptations. *Comp. Biochem. Physiol. A* 57:407–412.

Baginski, R. M. & S. K. Pierce. 1978. A comparison of amino acid accumulation during high salinity adaptation with anaerobic metabolism in the ribbed mussel, *Modiolus demissus demissus*. *J. Exp. Zool.* 203:419–428.

Berger, V. J. & A. D. Kharazova. 1997. Mechanisms of salinity adaptations in marine molluscs. In: Naumov, A. D., H. Hummel, A. A. Sukhotin & J. S. Ryland, editors. Interactions and adaptation strategies of marine organisms. Developments in hydrobiology. Berlin, Germany: Springer Verlag. pp. 115–126.

Boudreaux, M. L. & L. J. Walters. 2006. *Mytella charruana* (Bivalvia: Mytilidae): a new, invasive bivalve in Mosquito Lagoon, Florida. *Nautilus* 120:34–36.

Castagna, M. & P. Chanley. 1973. Salinity tolerance of some marine bivalves from inshore and estuarine environments in Virginia waters on the western mid-Atlantic coast. *Malacologia* 12:47–96.

Clement, M., Q. Snell, P. Walke, D. Posada & K. Crandall. 2002. TCS: estimating gene genealogies. Proceedings of the 16th International and Parallel Distributed Processing Symposium, IEEE Computer Society, Washington, DC.

Dame, R. 1996. Ecology of marine bivalves: an ecosystem approach. CRC Marine Science Series. Boca Raton, FL: CRC Press. 272 pp.

de Souza, T. O., F. A. dos Santos-Alves, C. R. Beasley, L. R. Lopes de Simone, N. S. Marques-Silva, G. C. Santos-Neto & C. H. Tagliaro. 2015. Population structure and identification of two matrilinear and one patrilinear mitochondrial lineages in the mussel *Mytella charruana*. *Estuar. Coast. Shelf Sci.* 156:165–174.

Folmer, O., M. Black, W. Hoeh, R. Lutz & R. Vrijenhoek. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 3:294–299.

Gilg, M. R., E. A. Hoffman, K. R. Schneider, J. Ryabinov, C. El-Khoury & L. J. Waters. 2010. Recruitment preferences of non-native mussels: interaction between marine invasions and land-use changes. *J. Molluscan Stud.* 76:333–339.

Gillis, N. K., L. J. Walters, F. C. Fernandes & E. A. Hoffman. 2009. Higher genetic diversity in introduced than in native populations of the mussel *Mytella charruana*: evidence of population admixture at introduction sites. *Divers. Distrib.* 15:784–795.

Inoue, K., S. Odo, T. Noda, S. Nakao, S. Takeyama, E. Yamaha, F. Yamazaki & S. Harayama. 1997. A possible hybrid zone in the *Mytilus edulis* complex in Japan revealed by PCR. *Mar. Biol.* 128:91–95.

Lee, H. 1987. Immigrant mussel settles in northside generator. The Shell-O-Gram. Jacksonville, FL: Jacksonville Shell Club.

Livingstone, D. R., J. Widdows & P. Fieth. 1979. Aspects of nitrogen metabolism of the common mussel *Mytilus edulis*: adaptation to abrupt and fluctuating changes in salinity. *Mar. Biol.* 53:41–55.

Nair, K. V. K. & P. Murugan. 1991. Biofouling by *Perna viridis* in a deep submarine tunnel system at Kalpakkam. *J. Mar. Biol. Assoc. India* 33:366–373.

Rawson, P. D. & T. J. Hilbish. 1995. Evolutionary relationships among the male and female mitochondrial DNA lineages in the *Mytilus edulis* species complex. *Mol. Biol. Evol.* 12:893–901.

Rawson, P. D., C. Bowman, M. A. Rice & W. R. Rosario. 2016. Genetic evidence of recent introduction of the charru mussel, *Mytella charruana*, to the Philipine Islands. 45th Annual Benthic Ecology Meeting, sponsored by Bowdoin College, March 16–19, 2016, Portland, ME (poster).

Rice, M. A., P. D. Rawson & W. R. Rosario. 2016. Notes on the identification and distribution of the exotic western hemisphere mussel, *Mytella charruana* D'orbigny 1846, in the estuaries of Pangasinan, Philippines. In: Blogoslawski, W. J. & L. Milke, editors. 36th Milford Aquaculture Seminar. U.S. Department of Commerce, Northeast Fisheries Science Center Ref Document 16-06. 52 pp.

Scarabino, V., S. Maytía & M. Caches. 1975. Carta binómica litoral del departamento de Montevideo. I. Niveles superiores del sistema litoral. *Comun. Soc. Malacol. Urug.* 4:117–129.

Sievers, F., A. Wilm, D. Dineen, T. J. Gibson, K. Karplus, L. Weizhong, R. Lopez, H. McWilliam, M. Remmert, J. Söding, J. D. Thompson & D. G. Higgins. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol. Syst. Biol.* 7:539.

Spinuzzi, S., K. R. Schneider, L. J. Walters, W. S. Yuan & E. A. Hoffman. 2013. Tracking the distribution of non-native marine invertebrates (*Mytella charruana*, *Perna viridis* and *Megabalanus coccopoma*) along the south-eastern USA. *Marine Biodiversity Rec.* 6:1–13.

Tamura, K., G. Stecher, D. Peterson, A. Filipski & S. Kumar. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30:2725–2729.

Yuan, W., L. Walters, K. Schneider & E. Hoffman. 2010. Exploring the survival threshold: a study of salinity tolerance of the nonnative mussel *Mytella charruana*. *J. Shellfish Res.* 29:415–422.

Zouros, E., K. R. Freeman, A. O. Ball & G. H. Pogson. 1992. Direct evidence for extensive paternal mitochondrial DNA inheritance in the marine mussel *Mytilus*. *Nature* 359:412–414.

Zouros, E. 2000. The exceptional mitochondrial DNA system of the mussel family Mytilidae. *Genes Genet. Syst.* 75:313–318.