Determining the population distribution of invasive mussels *Mytilus galloprovincialis*, *Mytilus edulis*, native mussel *Mytilus trossulus* and their hybrids in Vancouver ecosystems and markets.

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Abstract.

The purpose of our study was to determine the population distribution of invasive Mediterranean blue mussel *Mytilus galloprovincialis,* invasive Atlantic *Mytilus edulis*, and native North-Pacific *Mytilus trossulus*. The introduction of invasive species can displace the native mussel species and alter the biota of the ecosystem. DNA isolation, polymerase chain reaction (PCR) and electrophoresis gel techniques were performed on three different populations to determine the species types. Twelve replicates were sampled from each local population: the Jericho Beach Pier, the Maritime Market Pier in Granville Island and the Lobster Man seafood market in Granville Island. Results reveal that both invasive and native species are present in the Jericho Beach Pier, with five individuals confirmed to be *M.edulis* (invasive) and six individuals confirmed to be *M.trossulus* (native). Additionally, there were six individuals confirmed to be *M.edulis* in the Maritime Market Pier, suggesting that only the invasive species is present. In the Lobster Man seafood market, there were two individuals confirmed to be *M.edulis*, three individuals confirmed to be *M.galloprovincialis* (invasive) and six individuals confirmed to be *M.trossulus*, which suggests that some of the samples were mislabeled. No hybrids were found in any of the populations. Our findings show a potential population distribution of the *Mytilus* spp. in Vancouver.

Introduction.

B.C.'s coastal waters contain a mix of mussel species. Two native species, *Mytilus trossulus* (bay mussel) and *Mytilus californianus* (California mussel) have had to adapt to the introduction of non-native species *Mytilus edulis* (blue mussel) and *Mytilus galloprovincialis* (Mediterranean mussel) in the 1980s. These two non-native species were introduced for reasons of aquaculture and commercial viability (Helen *et al.* 2017).

Our objective is to use DNA isolation, PCR and electrophoresis to determine the species population distribution of 3 mussel species in Vancouver's waters and markets. Differentiation of species by morphology is difficult, as native *M. trossulus,* and invaders *M. edulis* and *M. galloprovincialis* are similar enough that they are collectively referred to as the 'Mytilus complex', and readily mix, often creating hybrid species. Therefore, DNA banding techniques are the preferred methods for identifying population diversity (Wimberger *et al*., n.d.).

We used two primers (Me15 and Me16) to test for three of the four aforementioned species, *M. californianus* being the exception. Our expected survey result is that *M. trossulus, M. edulis* and *M. galloprovincialis* will all be present in local waters, but that *M. edulis* will be the most abundant (Gurney-Smith *et al*., 2017). We also purchased a sample of 12 mussels labeled Salt Spring Island (Gallo variety) from Lobster Man in Granville Island to see if they were sold as advertised. In our market sample, we are expecting all *M. galloprovincialis* due to the label.

Since mussels are filter-feeders, we must consider the effects that invasive species will have on primary food sources, such as phytoplankton. For example, the Great Lakes invasion by the Quagga mussel, which has increased survivability like the Gallo, allowed it to colonize deeper areas of the water column. The resulting shift in biomass to the lake bottom from filter feeding primary food sources caused a decline in other species such as *Diporeia*, a native shrimp-like organism that supported local fish populations (GLEAM, n.d.). In Lake Michigan specifically, invasive mussels also caused a decrease in the Chinook salmon population by depleting nutrient sources (Fletcher, 2017; De Stasio *et al.,* 2014).

M. galloprovincialis is one of the top 100 most invasive species, displacing native species in South Africa and many other regions worldwide. It shows an increased survivability over other species of mussel and therefore could act as a nutrient sink in ecosystems adapted to less robust species (GISD, 2017; Gardner *et al*., 2016). Invasive species are considered the second largest threat to an at-risk species next to habitat destruction (Rankin *et al*., 2004). Therefore, monitoring the effects of an invasive species is important, especially when a keystone species such as salmon are the at-risk species, as is the case in British Columbia (Price et al. 2017).

Methods.

For mussel collection, we used convenience sampling to obtain mussels from each sample site. A handful of mussels were pulled from two to three points along the side of both Jericho Pier (49.276845, -123.201510) and Maritime Pier (49.271202, -123.137234), totalling around 30 individuals from each site. For the Lobster Man sample, we picked 12 mussels from the tray of mussels available. We then randomly selected 12 mussels from within each population. Using a caliper, we measured the length (l), width (w) and height (h), then calculated the average diameter of each mussel (($l * w * h$)/3).

In a sterile environment, we obtained tissue for DNA isolation by using scissors to excise an approximately 3mm by 3mm piece of the mussel mantle and mantle edge. Then we rinsed each sample of tissue and placed them into separate 1.5mL eppendorf tubes, and mashed each one with a new, sterile toothpick. Next, we added 300 µL of "Cell Lysis Solution with Proteinase K" to each tube. Each sample was incubated at 65°C for 15 minutes, vortexing every 5 minutes until the solution turned cloudy. After that, we placed

samples on the ice bucket for 5 minutes, then added 150 µL of "Protein Precipitate Reagent" to each tube and vortexed for 10 seconds. We then used the centrifuge machine to centrifuge each tube at 16.1 rcf for 10 minutes. When the centrifuge finished, we transferred the supernatant to new 1.5mL Eppendorf tubes, avoiding the layer of fat and the protein pellet while pipetting. Then, we added 500 µL of ice cold isopropanol to the supernatant and inverted them 30 - 40 times. Again, we centrifuged the samples at 16.1 rcf for 10 minutes. Afterwards, the isopropanol was poured off without disturbing the pellet. We next did two rounds of adding 500 µL of ethanol to the pellet and pouring off the ethanol. Finally, we left the caps open at room temperature overnight to evaporate any remaining ethanol. The next day, we added 30 µL of TE Buffer to each dry DNA pellet and pipetted the sample up and down to resuspend the DNA.

Basic Recipe for Master Mix

COMPONENT	AMOUNT
a. Sterile distilled water	11.5 µL
b. 50% glycerol	5.0 µL
c. 10X PCR buffer	2.5 µL
d. 25 mM MgCl2	1.0 µL
e. 5' Primer 10uM (Me15)	1.0 µL
f. 3' Primer 10uM (Me16)	1.0 µL
g. 10 mM dNTPs	0.5 µL
h. Taq polymerase	0.5 µL

Figure 1. Recipe for 23 **µ**L of Master Mix (one sample).

To perform PCR, we made a Master Mix based on the recipe shown in Figure 1 and pipetted it up and down to mix thoroughly. Then we combined 23 µL of the Master Mix and 2 µL of DNA from each sample in separate PCR tubes. For our negative control, we used 2 µL of distilled water in place of DNA. To start PCR, we placed the tubes in a

thermal cycler and waited for the PCR cycle to complete overnight. The procedure of thermal cycler is shown in Figure 2.

PCR Cycle

TEMPERATURE	TIME
(1). 95°C	2 min
(2). 95°C for 30 sec —> 54°C for 40 sec —> 72°C for 5 min	x 35
(3). 4°C	overnight
(4). Store in freezer	

Figure 2. Procedure of thermal cycler.

To prepare for electrophoresis, the next day we thawed our samples and then placed them back on ice. We next added 2.7µL of 10X loading dye into each PCR tube and pipetted up and down few times to mix. Then we loaded 15µL of the PCR tube contents into each well of a 1% agarose gel submerged in 100 mL TAE buffer. We placed the negative control, PCR samples of 36 mussels and the ladder in an order shown in Figure 3. Finally, we ran the gel at 80 Volts for 10 minutes before increasing to 100 Volts for 120 minutes.

Left Right

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Nega-tive control	L1	L2	L3	L4	L5	L6	L7	L8	Ladder	L9	L10	L11	L12	M1	M2	M3	M4	M5	M6

Left Right

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Ladder	M7	M8	M9	M10	M11	M12	J1	J2	Ladder	J3	J4	J5	J6	J7	J8	J9	J10	J11	J12

Figure 3. Electrophoresis gel order. Samples beginning from L1-L12 are species from Lobsterman Seafood market, M1-M12 represents samples from Maritime Pier, and J1-J12 samples are from Jericho Pier.

For analysis, we examined the gel to determine the number of bands present for each base pair to conclude the number of invasive, native and hybrid species. As a side analysis, we compared the average diameter of each mussel species within each population, and between populations. Lastly, we conducted a BLAST search (blast.ncbi.nlm.nih.gov/Blast.cgi) the 5′ Me15 and 3′ Me16 primers used during PCR.

Results.

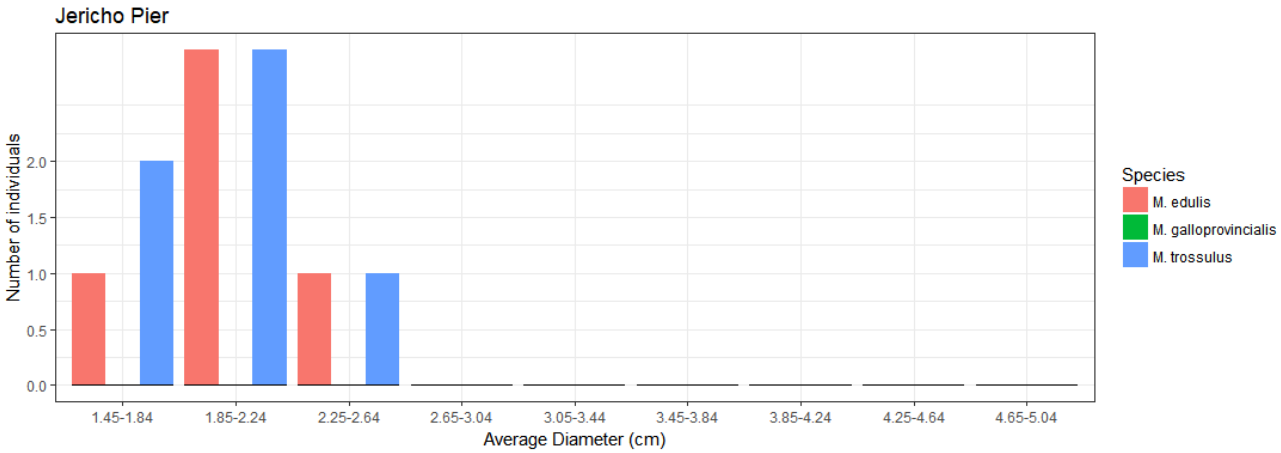

Figure 4. Histogram of average diameter of mussel individuals from Jericho Pier. Average diameter was calculated from the mean of the mussel length, width, and height. Groups of mussels were gathered along the side of the pier located at Jericho Beach in Vancouver, B.C. on November 1, 2017. 12 replicates were gathered and 11 replicates resulted in bands on the electrophoresis gel and is included in this figure.

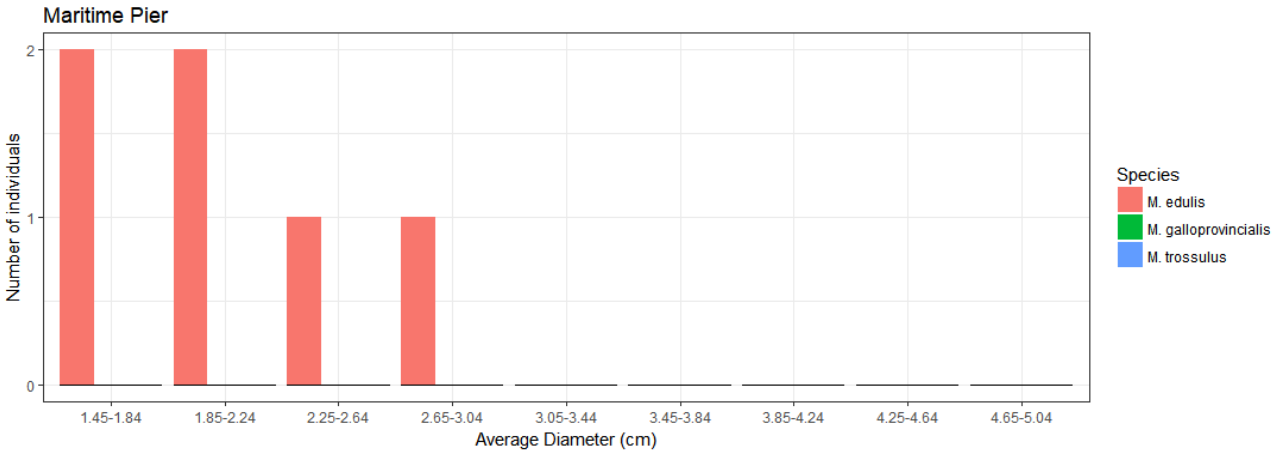

Figure 5. Histogram of average diameter of mussel individuals from Maritime Pier. Average diameter was calculated from the mean of the mussel length, width, and height. Groups of mussels were gathered along the side of the pier located at Granville Island in Vancouver, B.C. on November 1, 2017. 12 replicates were gathered and 6 replicates resulted in bands on the electrophoresis gel and is included in this figure.

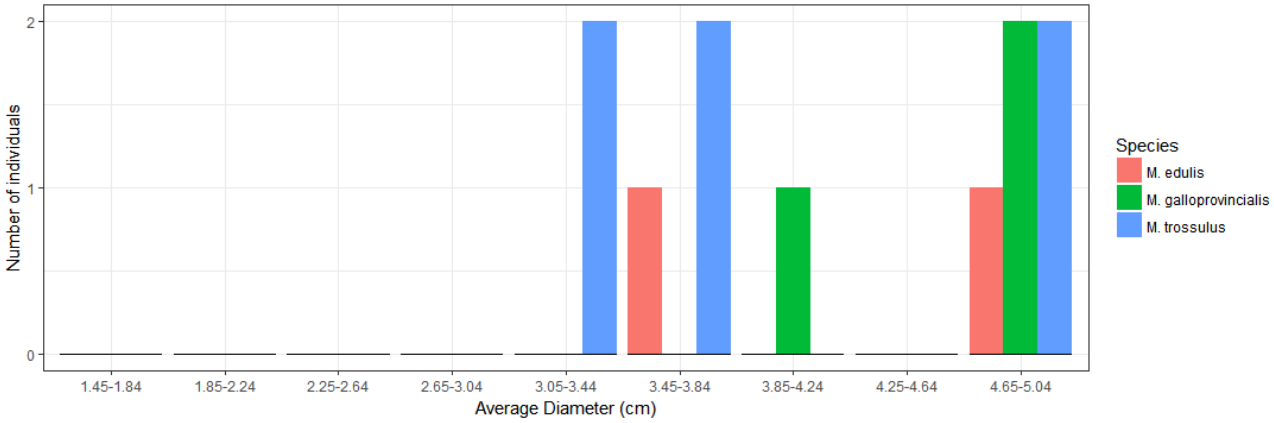

Figure 6. Histogram of average diameter of mussel individuals from Lobsterman. Average diameter was calculated from the mean of the mussel length, width, and height. 12 replicates were bought from the Lobsterman seafood store in a section labeled “Gallo Mussels”. 11 replicates resulted in bands on the electrophoresis gel and are illustrated on this figure. The store is located in located at Granville Island in Vancouver, B.C. Data was gathered on November 1, 2017.

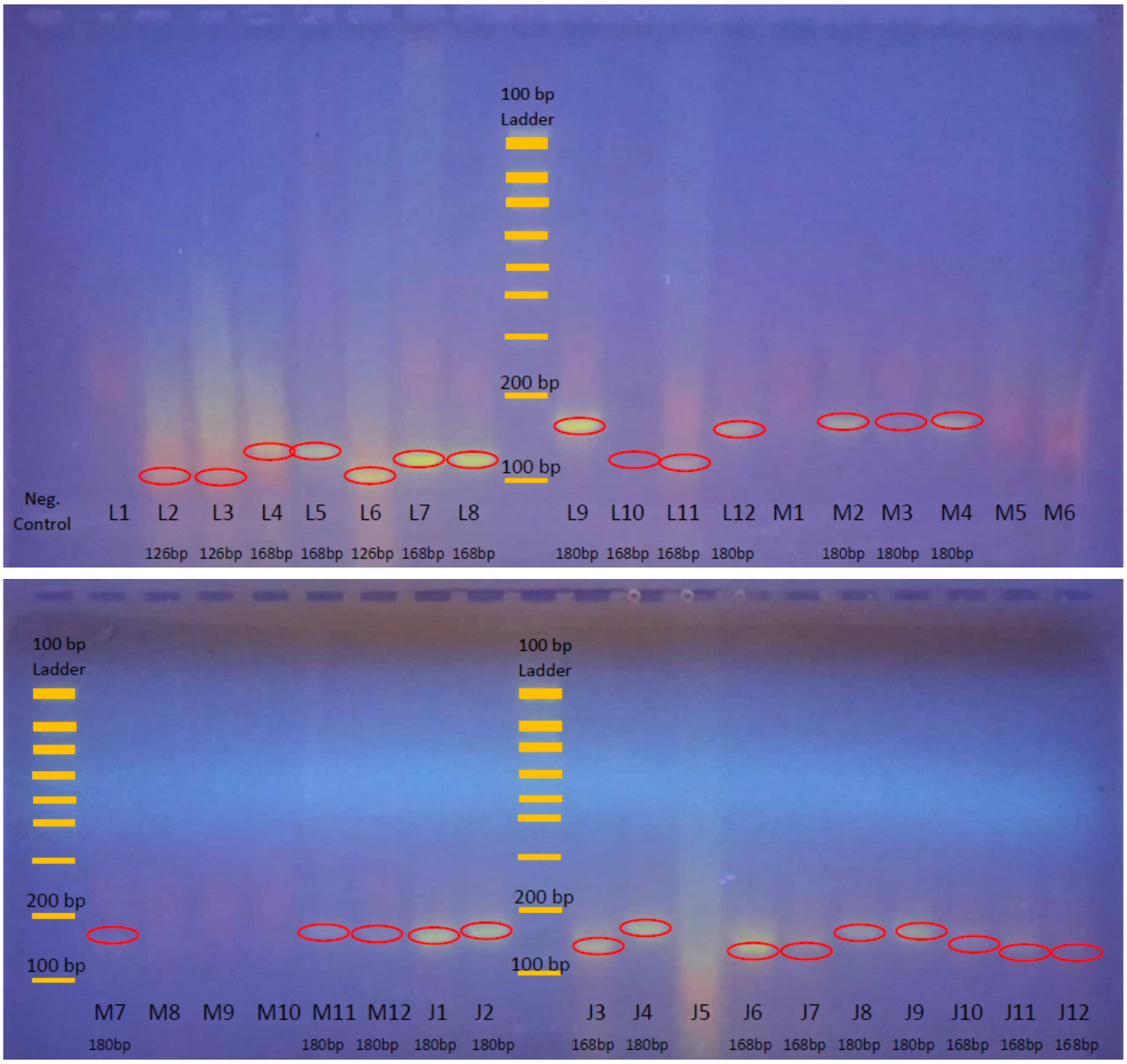

Figure 7. Gel electrophoresis run on all 36 mussel individuals. Bands of each individual are circled in red. Bands of the DNA ladder are represented in yellow. Banding at 126 bp indicates the replicate is of the *M. galloprovincialis* species, 168 bp indicates *M. trossulus* and 180 bp indicates the replicate is *M. edulis*. Approximate band size of each mussel individual were determined by comparing to the ladder. The negative control used was distilled water. Data was gathered in Vancouver, B.C. on November 1, 2017. Gel electrophoresis run completed on November 9, 2017.

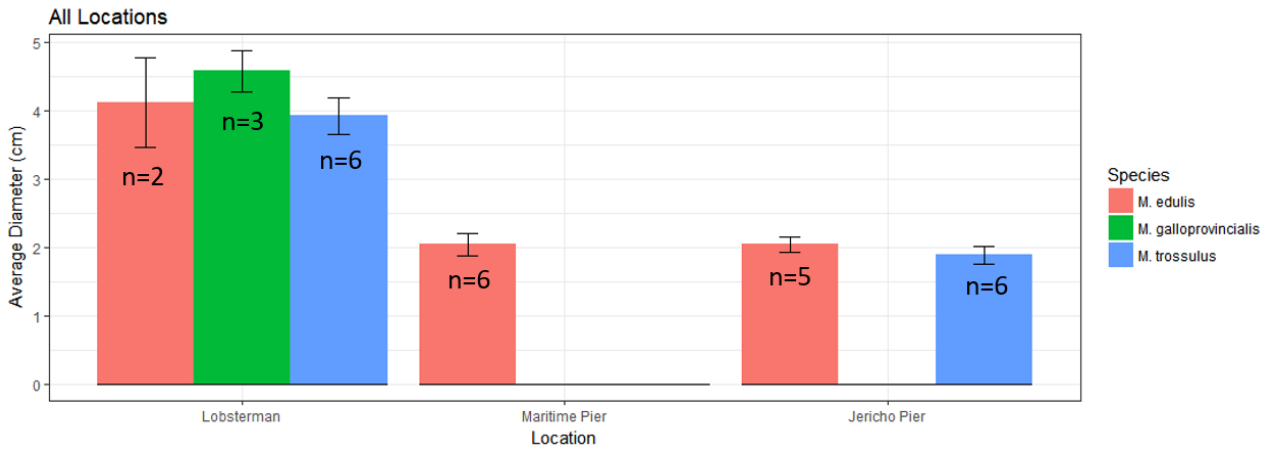

Figure 8. Average diameter of all mussel individuals according to location. Location includes the Lobsterman seafood store, Maritime Pier and Jericho Pier. Average diameter of each individual was calculated from the mean of the mussel length, width, and height. Average diameter overall was calculated from the mean of all mussels of the same species from one location. Error bars represent 95% confidence intervals. 12 replicates were gathered from each site, resulting in 36 replicates. 28 replicates showed banding on the electrophoresis gel are included in this figure, and number of individuals (n) noted. Data was gathered in Vancouver, B.C. on November 1, 2017.

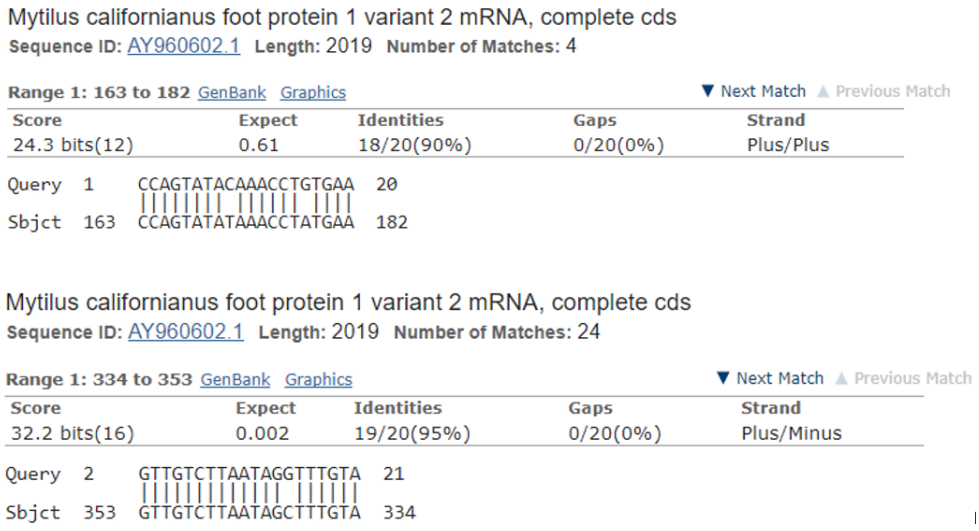

Figure 9: BLAST search results of *M. californianus*. The primer Me15 (22 base pairs) resulted in four base pair mismatches. The Me16 primer was 24 bp long, indicating a five base pair mismatch.

To illustrate the population distributions and mussel average diameter, Figures 4, 5 and 6 plot the number of each species found per range of size (average diameter) for our

three populations: Jericho Beach Pier, Maritime Market Pier and the Lobster Man seafood market, respectively.

The population distribution of the twelve replicates in each sample was determined by DNA banding patterns. Figure 7 is an image of the gel electrophoresis run, showing the circled bands of successful runs. From this image, it is possible to locate the size of the DNA fragment in relation to the ladder. Banding at 180 bp indicates the replicate is of the invasive *M. edulis* species, 168 bp bands shows up for *M. trossulus* and 126 bp bands indicate the replicate is *M. galloprovincialis* (Wimberger *et al.*, n.d.; Inoue *et al.*, 1995). In the results, it is observable that there are two individuals of the *M. edulis*, six individuals of the *M. trossulus* species and three of the marketed *M. galloprovincialis* invasive individuals at the Lobsterman market, as seen in Figure 8. At Jericho Pier, results indicate that there were five invasive *M. edulis* individuals and six *M. trossulus* individuals (Figure 8). At Maritime Pier, there were six individuals of the *M. edulis* species (Figure 8). No replicates were hybrid or of the *M. californianus* species.

Discussion.

After investigating whether the invasive *M.galloprovincialis* or *M.edulis* species has displaced the native *M.trossulus* species in our three populations, we were able to draw certain conclusions about the population distribution. First, we were not able to confirm the presence of any hybrid species in any of the populations. From Figure 8, it appears that both invasive *M.edulis* and native *M.trossulus* are present at the Jericho Beach Pier, which suggests that there is a potential for hybrids and that *M.edulis* could be displacing the *M.trossulus* species. Figure 7 also reveals the presence of only *M.edulis* in all of the

successful DNA sample runs of the Maritime Market Pier, suggesting that the *M.edulis* has displaced *M.trossulus*. We expected the results for the Lobster Man seafood market's samples labeled "Salt Spring Island (*Gallo* variety)" in Figure 7 to be all *M.galloprovincialis,* but only three of the samples actually revealed to be *M.galloprovincialis.* Wimberger *et al*. (n.d.) mentioned that *M.galloprovincialis* species can be relative larger than other species, but it is not always easy to differentiate by size alone. Since all three species appeared in the Lobster Man seafood market, this suggests that these mussel species could be indistinguishable and easily misidentified by size, specifically in the seafood market industry.

As a side analysis, we investigated whether or not there is a difference in the relative sizes between the species. We calculated a 95% confidence interval and plotted it, as seen in Figure 8 to determine if the mean size (average diameter) is correlated to the species in each location and in comparison to the other locations. We calculated the mean average diameter and used it as our mean size. Figure 8 shows that the error bars for each species in each population overlap, allowing us to conclude that for each population there is not a significant difference in the mean size between the species. However, comparing the two field sites to the seafood market population, Figure 8 shows non-overlapping error bars, allowing us to conclude that there is a significant difference in the mean sizes. This is expected because aquaculture is used as a means of increasing wild species to a marketable size (Naylor *et al*., 2000) and so the farmed seafood market mussels are likely to be much larger than the field samples. From size alone, we may not be able to determine the species type in each population of the field sites but we could potentially use average size to differentiate between the farmed and field samples.

There were potential issues in our sampling method. Our practice of convenience sampling may have caused sampling bias and therefore the samples may not be a good representation of the entire population. We were limited to convenience sampling because we did not have access to do random sampling of an entire body of water or region. In addition, within our samples, our sampling method may not have included fully grown mussel samples. Most of the field site samples were rather small and were likely to be juvenile. Samples from the market were rather large in comparison and likely have reached adult development.

In our experiment, six of the replicates in the Maritime Market Pier, one from the Jericho Beach Pier, and one from The Lobsterman market did not successfully display bands on our gel. One potential source of error could have been our practice of DNA isolation in a non-sterile environment. Another suggestion for our empty lanes is that it could possibly be the *M.californianus* mussel that our primers did not test for. The BLAST results and calculations (Figure 9) show that we cannot test for *M. californianus*, as the closest DNA sequence match includes four base pair mismatches in Me15 and five mismatches in Me16, which results in failure of the primers binding and cleaving off the DNA segment, therefore leading to no banding result. Although it is assumed that *M. californianus* would not be in our samples because we gathered our field samples at piers (Jericho Pier and Maritime Pier), and this species prefers open rocky intertidal zones (Schmidt, 1999).

To test for *M.californianus*, another primer is required. Another study conducted using the Myti-F/R primer successfully produced bands at 190 bp for the *M. californianus* during electrophoresis (Fernández-Tajes *et al.*, 2011).

For future experiments, it would be a better representation of population diversity to do random sampling, have more replicates, and to gather consistent data on fully grown mussels. In addition, our study may have benefitted from better practices of DNA isolation, to prevent contamination and possibly avoid losing as many lanes of data in our results. DNA spectrophotometry could also be conducted to test for RNA or protein contamination, and provide the actual yield of DNA being run.

Due to the aforementioned limitations of our study, our results may not be indicative of the actual mussel population in Vancouver. However, it does present a potential population distribution, and gives a bit of insight into the relative presence of native and invasive species.

Conclusion.

We were not able to conclude that any *M. galloprovincialis* or its hybrids are present at the Jericho Beach pier or the Maritime Market pier. The *M. edulis* was the only species present at the Maritime Market pier, and a mix of *M. edulis* and *M. trossulus* were present at the Jericho beach pier. No hybrids were identified at either location.

At the Lobster Man, despite being advertised as “Gallos”, the sample returned our most diverse sample of six *M.trossulus,* three *M. galloprovincialis* and two *M. edulis.* No hybrids were identified in this sample either.

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Appendix.

Materials needed:

- 37 x 23 µL of Master Mix
- 37 x 500 µL of ice cold isopropanol
- 37 x 300 µL of Cell Lysis Solution with Proteinase K
- 37 x 150 µL of Protein Precipitate Reagent
- 37 x 500 µL of ethanol
- 37 x 30 µL of TE Buffer
- 37 x 2 µL of DNA
- 37 x 2.7µL of 10X loading dye
- calipers
- 37 x toothpicks
- sterile scissors
- ice bucket
- centrifuge
- 37 x PCR tubes
- 3 x PCR racks
- 74 x eppendorf tubes
- 4 x eppendorf tube racks
- thermal cycler

Table 1. Materials needed for this project.

Lobsterman – Gallo Mussels					
Replicate	Length (cm)	Width (cm)	Height (cm)	Average (cm)	Notes
1	7.8	4.0	3.1	5.0	Light Beige DNA Pellet
2	8.5	4.0	2.6	5.0	Light Beige DNA Pellet
3	7.9	3.5	2.7	4.7	White DNA Pellet
4	5.8	2.8	2.1	3.6	Light Beige DNA Pellet
5	5.2	2.7	1.8	3.2	White DNA Pellet
6	6.5	3.2	2.3	4.0	Light Beige DNA Pellet
7	5.5	2.9	1.9	3.4	White DNA Pellet
8	6.0	3.3	2.1	3.8	Light Beige DNA Pellet
9	7.6	3.8	2.9	4.8	Light Beige DNA Pellet
10	7.6	4.1	2.8	4.8	Light beige DNA pellet; pellet after step#7 is not in complete pellet form/scattered
11	7.8	4.1	2.1	4.7	White DNA Pellet
12	5.8	2.6	2.0	3.5	White DNA Pellet

Maritime Pier – Granville Island					
Replicate	Length (cm)	Width (cm)	Height (cm)	Average cm)	Notes
1	4.0	2.1	1.5	2.5	White DNA Pellet
2	3.8	1.8	1.5	2.4	Beige DNA Pellet
3	3.4	1.6	1.1	2.0	Beige DNA Pellet
4	4.4	2.2	1.4	2.7	Light Beige DNA Pellet
5	3.6	1.6	1.2	2.1	Light Beige DNA Pellet
6	3.1	1.6	1.2	2.0	Beige DNA Pellet
7	3.2	1.5	0.9	1.9	Beige DNA Pellet
8	3.0	1.6	1.0	1.9	Light Beige DNA Pellet
9	5.1	2.2	1.7	3.0	Light Beige DNA Pellet
10	3.6	1.8	1.3	2.2	Light Beige DNA Pellet
11	2.8	1.4	1.0	1.7	Light Beige DNA Pellet
12	2.1	1.6	1.1	1.6	White DNA Pellet

Jericho Pier					
Replicate	Length (cm)	Width (cm)	Height (cm)	Average cm)	Notes
1	3.5	1.6	1.2	2.1	Light Beige
2	3.6	1.6	1.3	2.2	Light Beige
3	3.6	1.8	1.4	2.3	Beige
4	2.7	1.3	0.9	1.6	Beige
5	2.9	1.4	1.0	1.8	Beige
6	3.4	1.8	1.3	2.2	Beige
7	3.1	1.6	1.1	1.9	Beige
8	3.8	1.8	1.4	2.3	Light Beige
9	3.4	1.4	1.2	2.0	White
10	2.6	1.4	0.8	1.6	Beige
11	2.6	1.0	0.8	1.5	Light Beige
12	3.1	1.6	1.1	1.9	Beige

Table 2. Dimensions of all individuals and additional notes on the colour of the DNA pellet after DNA isolation.