UFF – UNIVERSIDADE FEDERAL FLUMINENSE

SÁVIO HENRIQUE CALAZANS CAMPOS

Investiganting the origins of invasive *Perna* and *Mytella* mussels: where do these Mytilidae come from?

Niterói
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Tese de doutorado submetida ao Programa de Pós-Graduação Dinâmica dos Oceanos e da Terra – DOT da Universidade Federal Fluminense - UFF, como parte dos requisitos para a obtenção do grau de Doutor.

Orientadores:
Dr. Carlos E. L. Ferreira – UFF, Brasil
Dr. Eric A. Hoffman – UCF, USA
Dr. Flavio da C. Fernandes – IEAPM, Brasil

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Niterói
2017

Dedico esta tese às pessoas que acreditaram em mim, a minha família, aos meus amigos e aos que possuem a sede do saber.

com amor e admiração eu dedico,
meu suor, meu cansaço meu afinco,
a elaboração deste trabalho típico,
com prazer ou não; fui sem medo,
a escolha sempre ao alcance das mãos,
cabe segurar ou não, o que queres perto do coração,
o trabalho não será em vão, se feito com emoção,
nas mãos a dor do trabalho sem descanso,
nos olhos a certeza alegre da conquista do sonho,
falhas, frustrações e o tempo, vincaram meu rosto,
na boca o desejo de matar a minha sede do saber,
junto a realidade, mostram-me uma nova dimensão,
e agora sinto o cheiro de uma nova direção,
buscar algo, dá sentido às suas atitudes,
te motiva enfrentar as dificuldades, seguir em frente,
só existirá melhor momento no futuro,
se para você, o melhor momento for o agora.
tentar, não será suficiente para conquistar,
manter o foco e perseverar,
sendo que o mais importante será,
não desistir e sonhar.

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Resumo

Os mexilhões da família Mytilidae são historicamente reconhecidos como importantes fontes de proteína para as comunidades costeiras, espécies-chave ecológicas e bioinvasores conhecidos. Usando padrões filogeográficos das populações é possível compreender a diversidade genética e o grau de conectividade entre as populações localizadas ao longo da distribuição de uma espécie. Da mesma forma, as características genéticas fornecem informações sobre como barreiras biogeográficas e o fluxo de genes influenciam o estabelecimento de espécies em novos ambientes. Usando duas espécies da família Mytilidae, *Mytella charruana* e *Perna perna*, como modelos, procurei avaliar os padrões genéticos entre populações nativas e introduzidas de ambas as espécies para contrastar as semelhanças e diferenças de sua relação filogenética e história evolutiva. No capítulo 1, investiguei qual a população nativa seria a origem mais provável da população invasora no sudeste da costa leste dos Estados Unidos e testei possíveis mudanças temporais na composição genética ao longo do tempo. No capítulo 2, procurei determinar se as populações brasileiras de *P. perna* apresentam características genéticas esperadas para uma população recentemente descendente da África. No capítulo 1, Eu pude determinar que a fonte da população de *Mytella charruana* dos Estados Unidos se originou provavelmente do Panamá, na costa do oceano Pacífico, e que virtualmente não houve nenhuma modificação na composição genética nas populações invasoras ao longo do tempo. No capítulo 2, pude identificar que as regiões estudadas de *P. perna* estão igualmente diferenciadas geneticamente entre si, e que a população Brasileira de *P. perna* apresentou características esperadas para uma população nativa estabelecida e isolada á muito tempo. Em geral, os resultados mostram como as análises genéticas podem ser usadas para comparar populações nativas e introduzidas tal como para entender melhor a história evolutiva das bioinvasões marinhas.

Palavras-chave: Mytilidae; *Mytella charruana*; *Perna perna*; Charru Mussel; Sururu; Brown mussel; Mexilhão; Bioinvasão; Genética de populações; DNA; Citocromo oxidase I - COI; Microssatéletes; Origem; Espécie criptogênica.

Abstract

Mussels from the Mytildae family are historically recognized as important source of protein for coastal communities, ecological keystone species and known as bioinvaders. Using phylogeographic patterns of populations, it is possible to understand the genetic diversity within-populations and the degree of connectivity among populations located throughout a species distribution. In the same way, genetic analysis provides information on how biogeographic barriers and gene flow influence species establishment in new environments. Using two species from the Mytilidae family, *Mytella charruana* and *Perna perna,* as a model, I sought to assess the genetic patterns between native and introduced populations of both species to contrast the similarities and differences of their phylogenetic relationship and evolution history. In Chapter 1, I investigated which native population would be the most likely source of the invasive populations and tested for possible temporal change in genetic composition over time. In Chapter 2, I sought to determine whether Brazilian populations of *P. perna* present genetic characteristics expected for a recently descended population from Africa. I was able to determine that the source of United States populations of *Mytella charruana* most likely originated from the Pacific coast of Panama and that there was virtually no modification in the gene composition in the invasive populations over time. I was also able to identify that all regions of *P. perna* were approximately equally genetically differentiated from each other and the data were entirely consistent with Brazilian populations of *P. perna* presenting characteristics expected for a native population being established and isolated for a long time. Overall, these studies show how genetic data can be used, in conjunction with native and introduced populations, to better understand the evolutionary history of marine bioinvasions.

General Introduction

Biological invasion or bioinvasion, is defined when a species outside its natural distribution, carried by human activity, either deliberate or accidental, threatens the biological diversity in a new geographic area, causing damage to the environment and the economy, increasing health risks, and/or creating a social impact (Elton, 1958; Mooney & Drake, 1989; Holand, 2000; UNCED, 1992; Primack, 2004; Tsiamis *et al.,* 2016). Other terms used to describe bioinvasions include: introduced, alien, exotic, non-indigenous, or non-native species. On the other hand, a species is considered *native* when it occurs in its region of origin (IUCN, 2000) and *cryptogenic* when there is an absence of clear evidence of the biogeographic origin (Carlton, 1996).

Ever since humans started moving throughout the world, they have been moving species and introducing them on a global scale and with the advancement of modern technology, the transport of non-native species has increased by orders of magnitude with consequent changes in ecosystems around the world (Ruiz *et al.*, 1997; Mack *et al.*, 2000; Kolar & Lodge, 2001; Lockwood *et al.*, 2007). Although some studies have questioned if invasive species really cause extinctions of native species (Gurevitch & Padilla 2004; Briggs, 2007), it is well established that invasive species are one of the greatest threats to marine biodiversity due to their capacity to unbalance the ecological equilibrium (Sala *et al.*, 2000; Molnar *et al.*, 2008; McGeoch *et al.,* 2010).

Introduced species can be classified by its condition and or competitive capacity within the environment in which it is found. When an exotic species is identified only in isolated environments (e.g. aquaria, tanks, ships) the species is considered *CONTAINED*. When the exotic species is isolated in the natural environment but without an increase of abundance or dispersal, the exotic species is generally considered as *DETECTED*. When the exotic species is characterized by increasing population number but without presenting ecological and/or apparent socioeconomic impediments this exotic species is referred to as *ESTABLISHED*. However when a species is established in a non-native habitat and its life cicle generates ecological and socioeconomic impacts,

this exotic species is referred to as *INVASIVE*. Therefore, an "alien invasive species" means that the
112 non-native species has become established in novel ecosystems or habitat, become an agent of change, and threatens native biological diversity or socioeconomic human activity. The most
114 important aspect of these concepts is that these processes are variable and dynamic over time on the spatial distribution (IUCN, 2000).

116 To be able to understand biological invasions is important to characterize the history of the species and to know the origin of the vectors, sources responsible for these introductions, and to
118 develop effective prevention and management strategies (Kolar & Lodge 2002; Fofonoff *et al.* 2003; Lodge *et al*. 2006; Rollins *et al.* 2006). Systematic, genetic, and geographical distribution
120 have shown to be important characteristics for discover the ancestry and origin of organisms (Avise, 2000) and many studies have investigated these characteristics to discover and understand
122 the origin of bioinvasions on land (Davies, *et al.* 1999), and in the sea (Holland, 2000; Bernardi *et al* 2010).

124 In marine ecosystems, the globalization and food demand growth has transformed shipping and aquaculture into the major vectors for species dispersal between ecosystems (Carlton, 1985
126 Gollasch *et al*., 2000; Kolar & Lodge, 2002; Hulme, 2009, Azevedo-Santos *et al.* 2011). However, the pathways for species dispersal remain poorly understood on a global scale (Mack *et al.*, 2000;
128 Hulme, 2009). Dispersal is often mediated through shipping traffic, which increases propagule pressure, making it feasible for species to transpose barriers and establish gene flow among isolated
130 populations, which would not naturally occur (Smith *et al.* 1999; Seebens *et al.* 2013). Studies have shown that the likelihood of new introductions depends on shipping intensity, ecological, and
132 environmental factors (Keller *et al.*, 2010). Associated with shipping activity, ballast water and hull fouling are the primary mechanisms for introduction in the marine realm (Norse, 1993; Seebens *et*
134 *al*. 2013). Also, aquaculture has been shown to lead the release of organisms into new areas and is an additional vector of exotic species introduction (Gollasch 2007; Azevedo-Santos *et al.* 2011).

In natural populations, genetic composition changes across generations under the influence of micro-evolutionary processes (e.g. migration, genetic drift, natural selection, and mutation). Typically, migration can bring propagules to a new territory causing a change in allele frequencies in that region. Selection can then act on those propagules such that those that are adapted in the new environment may flourish (Mayr, 1963; Lee 2002; Prentis, 2008). Genetic drift is the change in the frequency of a gene variant (allele) in a population due to random sampling of organisms and is likely to impact allele frequencies in small, recently established populations. Alternately, mutation is a change in DNA which produce new alleles, the ultimate source of all genetic variation, but acts too slow to justify genetic differentiation among populations connected by migration or impacted by genetic drift or selection. The genetic reconstruction of invasion history has been used in many studies designed to understand the causes and consequences of invasiveness (Cristescu, 2015). These molecular data can be used to infer patterns of population relationship as in phylogenetic and phylogeographic studies (Holland, 2001). These types of studies help to understand the degree of connectivity that exists along species distributions (Mayr, 1963; Hartl & Clark, 1997). In the same way, genetic data provide information on how biogeographic barriers and gene flow influence species establishment in a new environment. These data can be used to answer evolutionary questions, to identify geographic sources of invasive populations and invasion pathways, thus aiding in the management of invasive species (Holland, 2000).

The model organisms used in this thesis belong to the family Mytilidae, which is comprised of species that are economically, socially and ecologically important. The ecological importance of Mytilidae is generally related to their key role as ecological engineers, while holding a central position on the tidal habitat in a wide range of substrates and systems such as mud, open rocky shores, estuaries and mangroves (Gosling, 2003; Hicks, 2001; Siddall, 1980). The Mytilidae (order Mytiloida) is a family composed of some 52 genera mainly marine bivalves, with the exception of *Limnoperna* that inhabits brackish or freshwater environments (Bouchet & Rocroi 2010; Hayward,

1990). In fact, these organisms are known by their socioeconomic importance, being cultivated and collected in sea beds as food resource in many countries (Vakily, 1989; Marenzi & Branco, 2006). They are historically recognized as important resource for humans (Vermeij, 2012), having been a resource which helped human species to escape from extinction in the last glaciation (Griffiths & Branch, 1997, Marean 2011, Roberts 2011). One example of a well-studied group from the Mytilidae family is species from the genus *Mytilus*. Apart from being considered an important marine resource, *Mytilus* spp. are also known for their history of taxonomic uncertainty and hybridization across established species boundaries (Siddall, 1980; Vakily, 1989; Bouchet & Rocroi, 2010; Hayward, 1990). The Mytilidae family have also been well studied with regard to the attributes that make them efficient invaders (rapid sexual maturity, aggregation, fast growth, resilience, plasticity, resistance and association with human activities) (Zardi *et al.,* 2005; Doğan *et al.,* 2007; Taylor *et al.,* 2013; Darrigran *et al.,*2000).

The species that are the focus of this thesis present an interesting contrast due to the different evolutionary history as well as different impacts of contemporary human-influenced dispersal. *Mytella charruana* is a species that is native to the Brazilian coast and was discovered as invasive along the Southeastern United States coast in 1986 (Lee, 1987). The current hypothesis for the founding of *M. charruana* along the US coast is the transport via oil tankers from Venezuela. In contrast, *Perna perna* is native throughout the coast of Africa and the Mediterranean, but is cryptogenic along the Brazilian coast. Studies proposing that *P. perna* is not native to Brazil had suggested that this species was transported as biofouling in ships during the human slave trade (Souza *et al.*, 2003). Overall, this thesis shows how we can use these species to better understand how the human global activities can influence on the evolutionary history and contemporary distribution of invasive species.

General Objective

This study aimed to evaluate the relationship and connectivity between natural and introduced populations, using molecular analyses to help understand patterns of invasions by mussels. Both species studied here were evaluated using genetic markers to address general questions of genetic diversity and differentiation among populations from the native and invaded area as well as the use of genetic diversity to identify the source of populations. In chapter one, I used the mtDNA COI gene to identify the most likely source for the introduced *Mytella charruana* populations in Southeastern United States and to determine whether genetic composition of the invaded population had changed over time, influenced by a cold weather event that caused a demographic decline in the invaded range. In chapter two, I used microsatellite markers to address the primary question of whether Brazilian populations of *P. perna* present genetic characteristics expected for a population that had recently (i.e. in the past 500 years) descended from Africa.

Chapter 1

Genetic structure provides insights into the geographic origins and temporal change in the invasive charru mussel (Sururu) in the southeast United States

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Abstract

242 In 2004, *Mytella charruana* (d'Orbigny, 1842) (Mollusca: Bivalvia: Mytilidae) became established along the coast of the southeastern United States (SE-US). Using mitochondrial DNA
244 sequencing (cytochrome c oxidase subunit I), we compared genetic variation throughout its native range in South America to its invasive range in the SE-US. Samples from the SE-US were
246 collected in 2006 and 2010 enabling a temporal comparison to evaluate possible genetic changes of the invasive population. We addressed two questions. First, what are the potential source
248 populations (or geographic regions) for the SE-US invasion? Second, how has genetic diversity changed between the two sampling periods within the SE-US? We identified a total of 72
250 haplotypes, 64 of which were isolated to geographic sites and only 8 were shared among sites. The highly structured native range provides insight into the origin of invasive populations where
252 our results suggest that the introduced SE-US population originated from multiple source populations with the Panama region as the primary source. Additionally, our results indicate that
254 genetic composition of the non-native populations was unchanged between the two sampling periods. *Mytella charruana* exhibit a significant pattern of genetic structure among natural
256 populations, owing to biogeographic barriers that limit natural dispersal, and an ability to persist in novel habitats, owing to a suite of life-history characters that favor survival under variable
258 conditions. Overall, this study explains why *M. charruana* may become an increasing threat to locations founded by anthropogenic transportation.

260

Introduction

Human activities pose a significant threat to biodiversity and natural ecosystems due to the potential introduction of non-native species [1, 2]. Although the majority of introductions are unsuccessful, when a species becomes established in a novel environment, the consequences can be catastrophic [3] Once a species arrives in a new environment it is likely that other species will follow (i.e. invasional meltdown [4]), further altering the native habitat, either by linked (i.e. pests and diseases [5, 6]) or facilitated association [7, 8]. Given that biological invasions are changing natural systems on a large scale, it is important that we understand general characteristics of species introductions and mechanisms of species invasions [9, 10]. Investigating ecological and genetic questions allow us to illuminate how evolutionary characteristics of invading species favor invasion success and help to understand a species' potential of invasion [11].

In particular, genetic characterization of invasive populations has proven to be important for several reasons, including species identification [12, 13], distinguishing population of origin [14], and characterizing levels of diversity in the invaded range [15]. A number of studies have shown that non-native populations tend to have greater genetic variation when compared to native populations which may increase the likelihood of establishment [15-17]. Moreover, the high levels of genetic diversity found in invasive populations are usually ascribed to multiple population founders coalescing in the invaded range [15, 17 - 27].

Despite the detrimental conditions that may follow an invasion, initial colonizers do not always pose a threat when they first settle in an area due to low density and the likelihood that the invasion will fail to flourish [11]. Indeed, during the establishment phase non-native species typically experience a lag-time before they become invasive [28]. During this phase, the introduced population may evolve as an adaptive mechanism to survive in the new environment [29 - 31]. However, few studies have investigated genetic change over time within the invaded range [29, 32, 33].

Studies seldom have the opportunity to characterize an invasion from its very beginning because of low densities and lack of ecological and economic impact of recent invaders. One exception to this is the invasive charru mussel *Mytella charruana* (d'Orbigny, 1842), a 3-5cm mussel that lives in muddy areas of bays and estuaries forming dense aggregations or covering mangroves roots and other hard substrates [3434]. *Mytella charruana* exhibit broad salinity and temperature tolerances, surviving in salinities ranging from 2 – 40 ppt [35] and temperatures between 6° – 31° C [36].This mussel is native to Central and South America, ranging on the Pacific coast from Guaymas Sonoro, Mexico to Southern Ecuador, and along the Atlantic coast from Colombia to Argentina [37 - 41]. In 1986, *M. charruana* was first discovered in Jacksonville, Florida, USA, attached to a seawater pipe of a power plant [42]. This species was presumably introduced to Florida by ship ballast water or hull fouling from South America [24, 43]. In 1987, after a cold winter, *M. charruana* was considered extirpated from the Florida coast. However, in 2004 several individuals were discovered in the Mosquito Lagoon located 212 km south of the original discovery site [44]. Since 2004, *M. charruana* has been found along the southeastern coast of the United States from Florida to South Carolina [45] and several studies have investigated ecological aspects of invasion in that area [45 - 48]. Moreover, a recent study verified negative impacts of *M. charruana* on native eastern oysters (*C. virginica*), confirming the invasiveness of the species [35, 49].

Molecular genetic studies have found that native populations of *M. charruana* along the Brazilian coast were genetically diverse, more structured than other related species (i.e. *M. guyanensis* [50]) and resistant to extreme environmental conditions [35, 51]. Samples from the Southeastern United States (i.e. Georgia and Florida – referred to as SE-US from now on) were found to be genetically similar to each other and exhibited higher levels of genetic diversity than and differentiated from native populations [24, 52]. Additionally, studies within the native range have identified an uncommon mitochondrial (mtDNA) evolutionary history of *M. charruana,* with

312 three different mtDNA lineages recognized for this species [52]. These three lineages include two maternal lineages and one paternal lineage (i.e. double uniparental inheritance – DUI [53]). 314 Previous studies that have investigated these lineages have been geographically constrained to the east coast of Brazil limiting our understanding of how they exist throughout the species range.

316 In this study, we analyzed the mtDNA cytochrome c oxidase subunit I (COI) gene of *M. charruana* from 12 populations in 7 countries to investigate the relationship between native and 318 nonnative populations of this invasive species. We used these data to address two primary questions: First, what were the possible source populations for the introduced populations? 320 Second, did mtDNA haplotype composition change over time within the invaded region? Considering that a previous study [24] suggested that *M. charruana* in the SE-US was of mixed 322 origin with likely sources from the southern Caribbean, we predicted that source populations would originate from the northern region of South America. Moreover, we used these data to 324 investigate why invaded populations do not show marked founder effects (e.g. low genetic variability compared to source population areas) expected with typical new population founding 326 [24, 54]. With regard to our second question, the time-scale investigated here is too short for us to expect to see mutation or selection driven changes in mtDNA markers that might occur over time- 328 periods for quickly evolving loci. Rather, we aimed to see whether new propagules influenced genetic composition owing to the founding of new mussels. We predicted that we would see a 330 change in the genetic composition over time due to fluctuations of population densities in the introduced area. In particular, Spinuzzi [45] found that a sharp die-off of *M. charruana* after 332 2009/2010 extreme cold events throughout the invaded range. Finally, we discuss how spatial and temporal genetic structuring of populations can be used to better understand the initial phases of 334 marine invasions.

336 ## Materials and methods

Sample collection

For this study, we employed mtDNA haplotypes from six newly sequenced geographic regions from the native range [Panama (PANA), Trinidad & Tobago (TRIN), Northeast Brazil (MACO), Southeast Brazil (VITO), South Brazil (PARA) and Uruguay (MONT) as well as previously published data representing the invaded region (SE-US [24]) with three native regions Ecuador (ECUA), Colombia (COLO [24]) and Northern Brazil (BRAG [53]) to evaluate the genetic relationships within the native region and between native and introduced populations of *M. charruana* (Fig 1). The SE-US region [24] is represented by 4 population sample sites: New Smyrna Beach, Florida (NSB), Jacksonville, Florida (JAX), St. Marys, Georgia (SMA), and Sunbury, Georgia (SUN). For all populations, we collected between 30 and 50 samples which were either immediately placed in 100% ethanol or dried in silica gel (i.e. Drierite™) for DNA preservation. Additionally, we collected samples from two separate time points for one population (JAX2006 [24] and JAX2010, collected for this study) enabling us to estimate how genetic diversity changed over time. Although recent studies [55, 56] questioned whether *M. charruana* was native to Colombia, in this study we consider Colombia to be native given the genetic characterization presented in Gillis *et al.* [24] and the close proximity to the original species distribution [39].

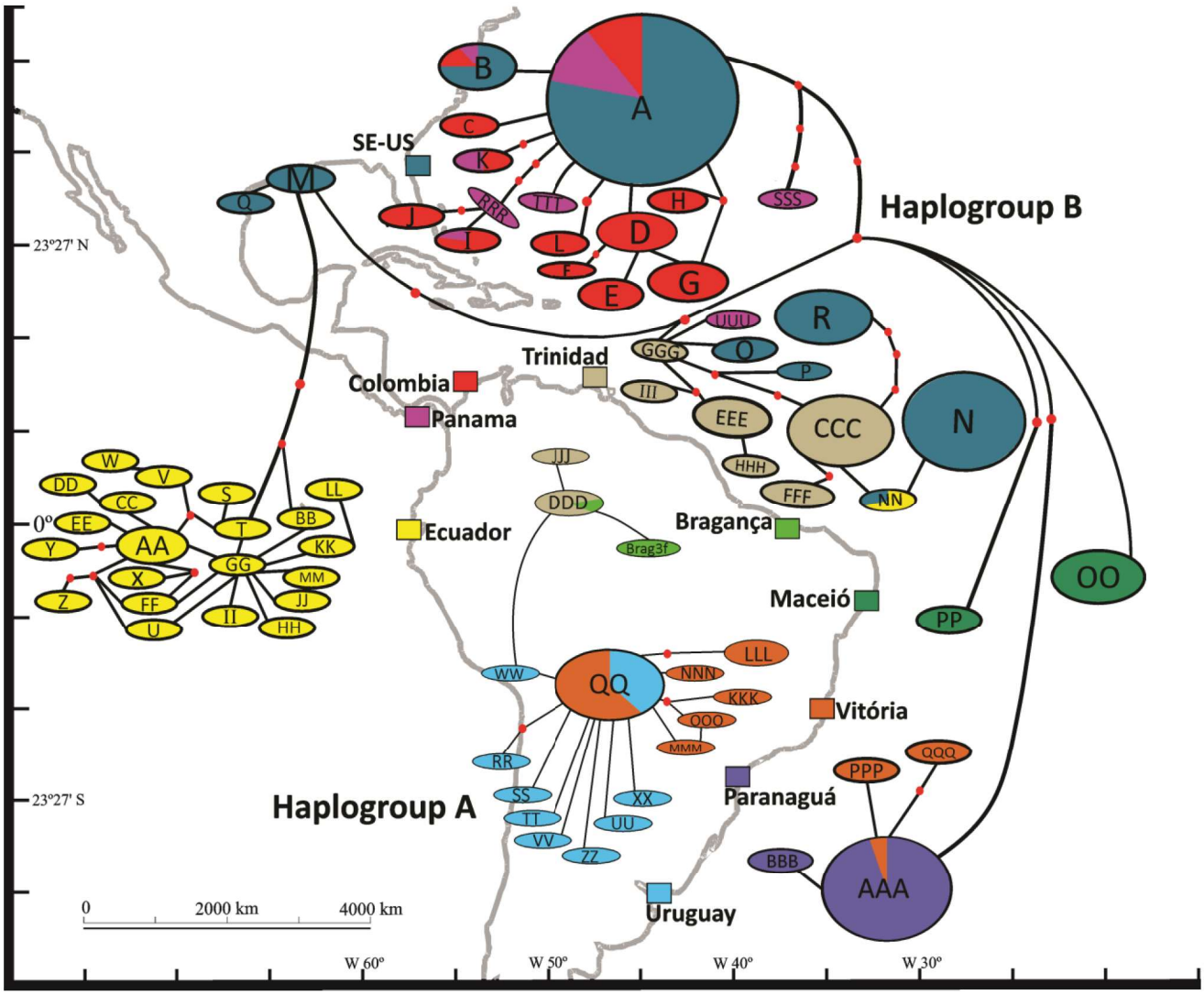


Figure 1-1 - Map of sample sites with overlay of haplotype network of the mtDNA COI gene. Haplotypes were labeled following the naming convention found in Gillis *et al.* [24]. Circles represent frequency of individuals per haplotype. Colors of site name and haplotype correspond to localities where haplotypes were found. Red nodes represent inferred haplotypes. Note that Lineage A and B haplotypes do not connect into a single network.

362 ## DNA extraction and amplification

We amplified a 722 base pair (bp) fragment of the mitochondrial cytochrome c oxidase
364 subunit I (COI) gene using polymerase chain reaction (PCR), with primers originally designed by Gillis *et al.* [24]. Genomic DNA was extracted from tissue samples of adductor muscle by
366 standard phenol–chloroform methods [57] or using QIAGEN© DNeasy Blood & Tissue Kit extraction kits following the manufacturer's protocol. PCR was performed using the following
368 final concentrations in a 20µL reaction: 2mM $MgCl_2$, 0.8mM dNTPs, 1x PCR buffer, 0.5 µM forward and reverse primers (novel primers [24]), 1 unit Taq DNA polymerase, and 50 - 100 ng
370 DNA template. Cycling conditions were: 95°C for 4 min followed by 35 cycles of 95°C for 30s, 45°C for 30s and 72°C for 30s; final extension period at 72°C for 7 min. PCR products were
372 checked for amplification and size on a 2% agarose gel. All PCR products were purified using exonuclease I and shrimp alkaline phosphatase (ExoSAP; USB, Cleveland, OH, USA) and were
374 submitted for sequencing in both directions to the University of Arizona Genetics Core (Tucson, AZ, USA).

376

Data analysis

378 Sequences were inspected and aligned in Sequencher 4.7 [58]. Open reading frames and changes in amino acids were verified in ExPASy [59] and all sequences were compared to those
380 of Gillis [24] for congruence. Sequences used in this study could not be directly compared to those of Souza [52] given minimal overlap of sequences. However, the overlap that exists enabled
382 us to determine whether our sequences fell into Lineage A or Lineage B of Alves [53] and our sequences are assigned to these groups.

384 To characterize phylogeographic patterns throughout native and introduced regions, we reconstructed the phylogenetic tree with unique COI haplotypes using the best-fit substitution

386 model and partitioning schemes identified by PartitionFinder v1.1.0 [60]. The phylogenic reconstruction was performed with MrBayes 3.2.6 [61] using two runs of four chains that were
388 sampled every 500 steps for 5,000,000 generations. For each run, the first 2,000 samples were discarded as burn-in. *Mytella guyanensis* was used as an out-group in the phylogenetic
390 reconstruction. Posterior probability values equal to or higher than 95% were considered statistically significant [62]. Because genetic differentiation is not always great enough to supply
392 strong nodal support when investigating intraspecific variation, we employed a statistical parsimony algorithm described by Templeton [63] to construct a 95% haplotype network in TCS
394 v. 1.21 [64].

To investigate the genetic structure of *M. charruana* from throughout the native and
396 invasive range, we estimated global and pairwise F_{ST} among all populations in Arlequin 3.5.2.2 [65]. Additionally, we estimated gene diversity (h) and nucleotide diversity (π) in Arlequin and
398 employed a *t*-test in R statistical package version 3.2.3 [66] to evaluate whether genetic diversity within native versus within invasive populations were significantly different. Further, we used
400 molecular analysis of variance (AMOVA) to estimate the differentiation among the four invasive populations and among the eight native populations as well differentiation between natives and
402 invasives. To determine whether native populations exhibited a pattern of isolation-by-distance, we ran a Mantel test with 1000 bootstrap simulations in IBDWS [67]. Geographic distances
404 between populations were estimated with Google Earth 7.1.5.1557 [68] by measuring the least-cost distance along the coastline.

406 To infer which native population(s) were the most likely source(s) of the introduced SE-US *M. charruana,* we used two approaches. First, we determined the number of shared haplotypes
408 between native and invasive populations. Second, we conducted pairwise contrasts between native and invasive populations via analysis of molecular variance (AMOVA). Here, we ran a series of
410 AMOVA analyses in which we grouped the introduced populations with populations from each

native region. If these population pairings revealed groupings that were not significantly differentiated between native and introduced populations, then that native population was inferred to be a putative founder population of the invasion. All AMOVA analyses were based on pairwise difference tests with 1000 bootstrap simulations to build confidence intervals for estimations using Arlequin 3.5.2.2 [65].

To address our second question, whether genetic diversity would change over time, we used AMOVA to compare the genetic composition of the 2006 SE-US introduced populations with that of the 2010 JAX population. Here, a significant F_{ST} value would indicate that genetic variation had changed between the two collection time points. Finally, we estimated gene diversity (h) and nucleotide diversity (π) in Arlequin for both time points of the invasive region to determine whether diversity indices had changed over time. Here, significance was determined if the 2010 estimates of genetic diversity were outside the 95% CI generated by the four SE-US populations in 2006.

Results

Sequence analysis

Our sample set comprised eight populations ranging throughout the native distribution, from Central to South America, as well as five populations from the introduced range along the SE-US (four from 2006 and one from 2010). The average number of sequences per population was 23, with MACO and MONT having the fewest (n = 14) and COLO the greatest (n = 41). A total of 320 sequences of *M. charruana* were analyzed using the mitochondrial COI gene. From those, 166 were new sequences and 154 were taken from literature [24]. All sequences were 722 bp long and belonged to the female mtDNA lineage because the novel primers developed by Gillis [24], and used in this study, only amplify COI matrilineal fragments [53]. The number of variable sites within populations ranged from 1 in PARA to 52 in VITO (Table 1). Among the native populations, ECUA and COLO presented the highest genetic diversity (h = 0.9) while PARA exhibited the least genetic diversity (h = 0.16; Table 1). The highest nucleotide diversity was found in TRIN and VITO (π = 0.0189) and was due to the occurrence of haplotypes from both female mtDNA lineages present in these populations (Table 1). Among the invasive populations, all haplotypes belonged to haplogroup B (Table 1). The highest gene diversity (h = 0.825) was found in SMA and the lowest (h = 0.702) in JAX2006. The nucleotide diversity ranged from π = 0.00738 (JAX2010) to π = 0.00904 (NSB). The entire data set is available in GenBank (Acc. No. MF074963 - MF075128).

446 **Table 1.1 Descriptive genetic parameters of *Mytella charruana* populations from western Atlantic and tropical eastern Pacific classified by realm, region and locality. #Haplotypes designates the total number of different haplotypes found in each**
448 **population. Lineage B designates the number of those haplotypes that belong to lineage B of Alves *et al.* [53].**

Realm	*Subtropical*					*Tropical*						*Subtropical*	*Temperate*
Region	Southeast United States					Pacific		South Caribbean		Brazil Northeast	Brazil Southeast	Brazil South	Southern America
Locality	New Smyrna,* FL# (NSB)	Sunbury GA* (SUN)	St. Marys GA* (SMA)	Jacksonville FL* (JAX2006)	Jacksonville FL* (JAX2010)	Guayaquil Ecuador* (ECUA)	Aguadulce Panama (PANA)	Cartagena Colombia* (COLO)	Trinidad & Tobago (TRIN)	Maceió AL (MACO)	Vitória ES (VITO)	Paranaguá PR (PARA)	Montevideo Uruguay (MONT)
Latitude Longitude	29°1'32.18"N 80°55'9.46"W	31°45'51.77"N 81°16'41.12"W	30°42'24.86"N 81°27'12.44"W	30°18'52.51"N 81°37'23.92"W	30°18'52.51"N 81°37'23.92"W	2°42'0.53"S 80°14'42.48"W	8°14'35.10"N 80°29'58.49"W	10°22'21.58"N 75°30'33.80'W	10°39'47.36"N 1°32'11.08"W	9°40'23.86"S 35°46'3.07"W	20°19'21.40"S 40°20'21.56"W	25°30'6.30"S 48°30'51.68"W	34°53'17.36"S 56°12'10.41"W
Number of sequences	17	16	16	34	31	30	27	41	35	14	21	24	14
Variable sites (polymorphic)	17	17	16	18	12	27	20	18	51	3	52	1	9
#Haplotypes (Lineage B)	6 (6)	5 (5)	5 (5)	5 (5)	5 (5)	22 (22)	24 (24)	12 (12)	8 (6)	2 (2)	9 (3)	2 (2)	9 (0)
h **(st. dev.)**	0.721 (0.087)	0.733 (0.079)	0.825 (0.052)	0.702 (0.052)	0.7462 (0.0434)	0.959 (0.026)	0.880 (0.035)	0.911 (0.020)	0.761 (0.054)	0.440 (0.112)	0.795 (0.077)	0.159 (0.094)	0.835 (0.101)
π	0.00904	0.00851	0.00895	0.0087	0.00738	0.00493	0.00631	0.00437	0.01877	0.00183	0.01896	0.00022	0.00178
	Invasive					**Native**							

*Data from Gillis *et al.* [24], *h* - Gene Diversity, *π* - Nucleotide diversity.

450

Phylogenetic relationship and population structure

The phylogenetic relationship was reconstructed via a Bayesian consensus tree which identified two well defined clades, haplogroup A and B, that had been previously identified by Alves *et al.* [53] (Fig 2). The best-fit substitution model identified in our analysis was the General Time Reversible model with invariable sites and gamma distribution (GTR + I + G; [69]) and each codon position [K80+I (COI1); F81 (COI2) and HKY (COI3)]. The clade differentiation between groups was well supported by major nodes exhibiting posterior probabilities > 0.95. Similarly, the 95% parsimony network showed haplotype relationships identifying two distinct lineages, with regional genetic differentiation among populations (Fig 1). Lineage B had more haplotypes than Lineage A and contained all haplotypes from the SE-US introduced populations. Additionally, the haplotype network revealed that all haplotypes from ECUA were grouped together, except one (NN) that was grouped with TRIN and SE-US. TRIN and VITO were the only populations that exhibited haplotypes from both Lineage A and B, and MONT was the only population with all haplotypes belonging to Lineage A. TRIN showed a higher number of haplotypes from Lineage B, while VITO had the opposite with more haplotypes from Lineage A. When testing for a relationship between genetic distance and least-cost-coastline distance, we found that native populations did not exhibit a pattern of isolation by distance (Mantel test; Z = 761.9835, p = 0.765; r = - 0.1576; Fig 3).

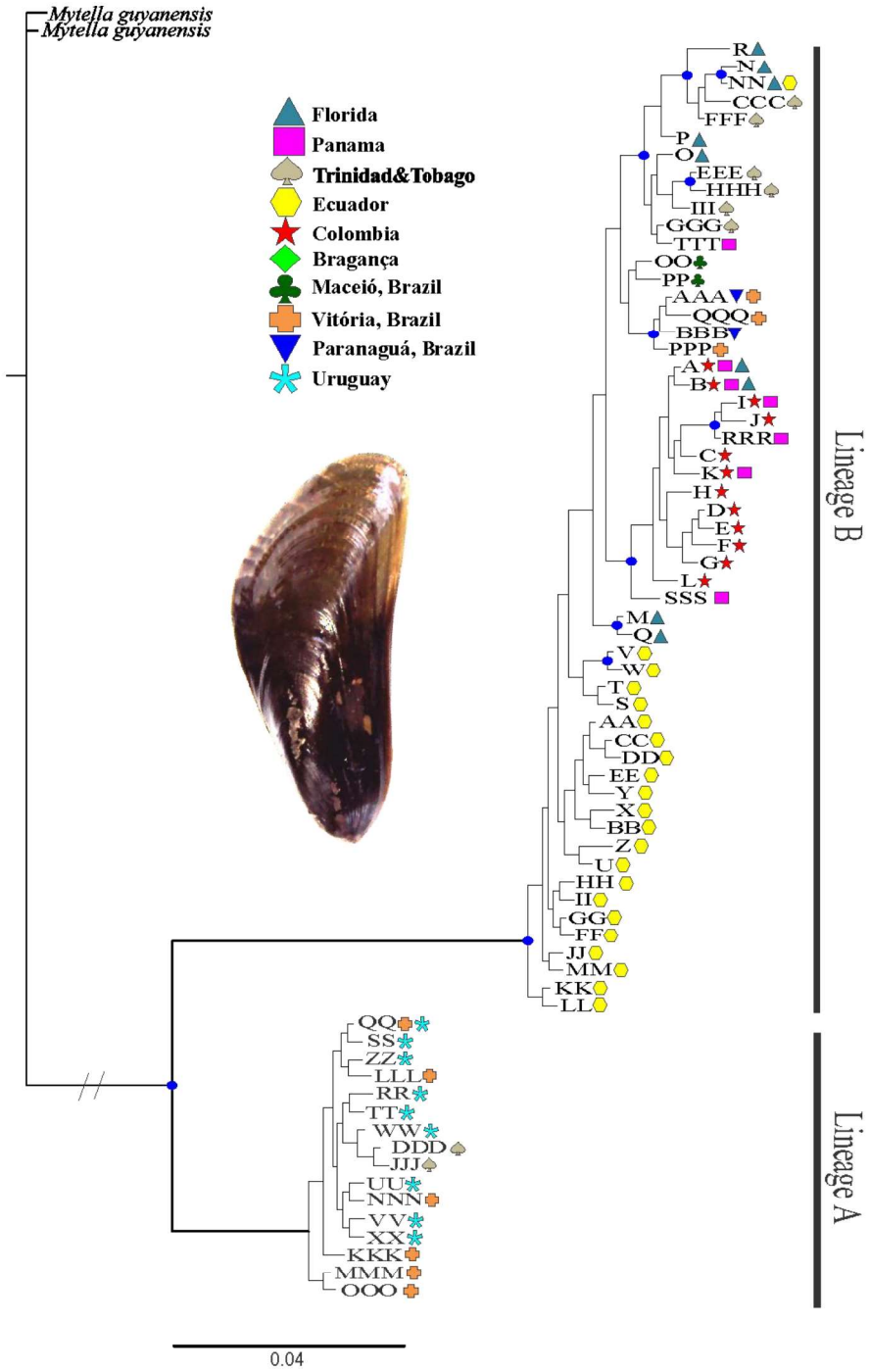
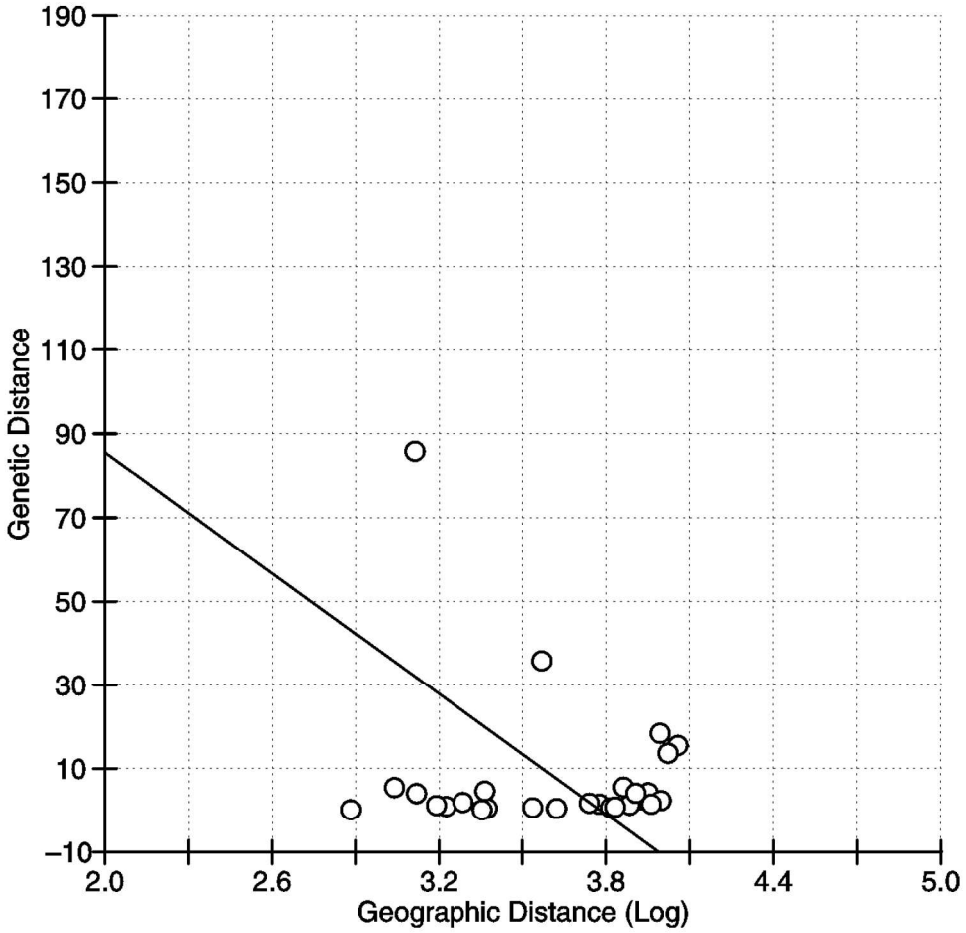


Figure 1.2 - Bayesian phylogenetic tree of Mytella charruana haplotypes using the mtDNA COI gene. The two in-group clades are designated Lineage A and Lineage B [52]. Blue dots designate Bayesian posterior probabilities greater than 0.95. Haplotypes icons are color coded by locality and names correspond to Fig 1.



476

478 **Figure 1.3 - Isolation by distance among native populations. Genetic distance (F_{ST}/1-F_{ST}) vs. log of least-cost over-water distance in km (Z = 761.9835, r = - 0.1576, p = 0.765).**

480

Genetic differentiation between the native and the invasive region showed significant

482 structuring (F_{ST} = 0.633; Fig 4). Specifically, comparisons between all native populations were marked by high F_{ST} values (global F_{ST} = 0.728), and a large component of variation among

484 localities (Fig 4 – Only Natives). Of 61 haplotypes found in native populations, only seven were shared among any eight native population (A, B, I, K, between PANA and COLO; AAA, between

486 VITO and PARA; QQ between VITO and MONT and DDD between TRIN and BRAG) and the remainder were private haplotypes and typically closely related to other haplotypes from the same

488 regions (Figs 1 and 2). In pairwise analyses, the lowest estimate of F_{ST} between native populations was between MONT and VITO (F_{ST} = 0.074), whereas the highest estimate of F_{ST}

490 was between PARA and MONT (F_{ST} = 0.988). In contrast, introduced populations were not

significantly differentiated from each other (global F_{ST} = -0.011, p = 0.602, Fig 4). Between introduced populations, pairwise F_{ST} estimates were predominantly negative with the highest value between JAX2010 and NSB (F_{ST} = 0.049). Values contrasting genetic diversity between native and invasive populations were not significantly different for either gene diversity (p = 0.791) or nucleotide diversity (p = 0.6187) (Table 1).

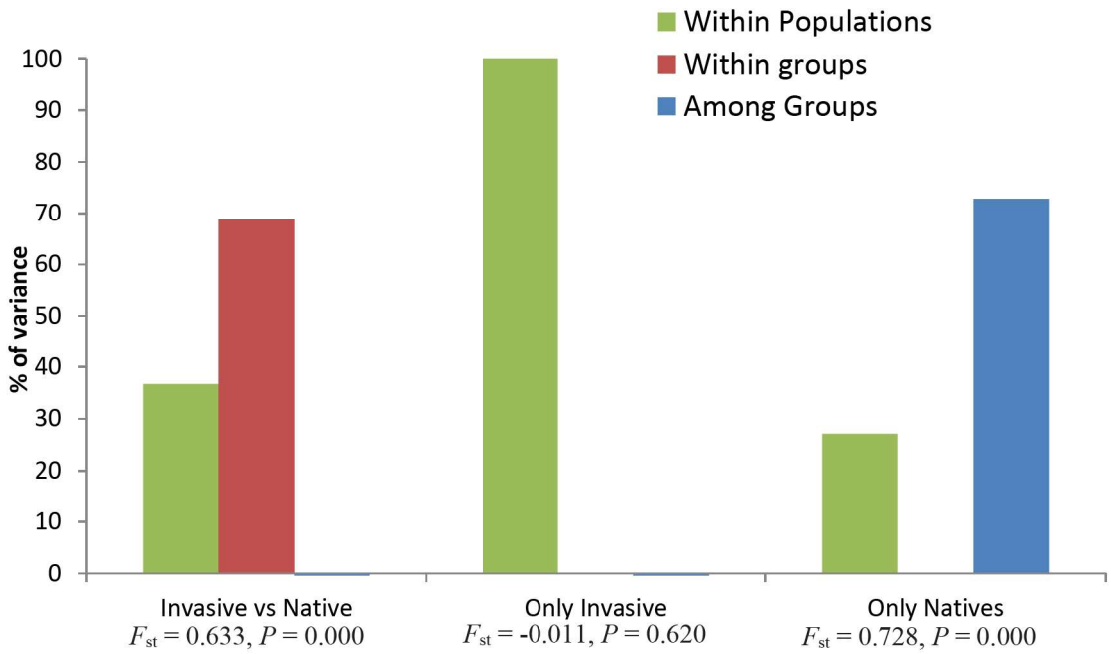


Figure 1.4 - Graphs of the results from three analyses of molecular variance (AMOVA). Each analysis is separated by populations included in the analysis (invasive versus native populations, invasive populations only, and native populations only). For each analysis, columns indicate the percent of variance explained. Below columns are global F_{ST} and p-values for a null hypothesis of no genetic structure.

Source of invasion and temporal changes

Given that native populations exhibited genetic differentiation from each other and that our collected samples spanned throughout the native distribution of *M. charruana*, we ran independent ΛMOVΛ analyses to determine the degree of differentiation between native and non-native populations, as a means to identify if any native population could be a potential founder of

the SE-US populations. We found that only a single native population exhibited a non-significant F_{ST} (PANA, F_{ST} = -0.06, P = 0.686; Fig 5) when contrasted with SE-US populations. All other native populations exhibited significant genetic differentiation relative to the SE-US populations (Fig 5). The percent of variance explained among regions in the AMOVA comparisons between native populations and the SE-US populations was smallest for PANA (as expected given the non-significant F_{ST} between these populations), then followed by the northern South American populations (TRIN, COLO, MACO; Fig 5). Other native populations exhibited significant variance explained by regions comparing the native versus non-native regions (Fig 5). Our direct comparisons of haplotype composition were largely congruent with the AMOVA comparisons. We observed nine distinct haplotypes in the SE-US populations (A, B, M, N, O, P, Q, R, NN) of which three (A, B, NN) were shared with native populations (PANA, ECUA and COLO).

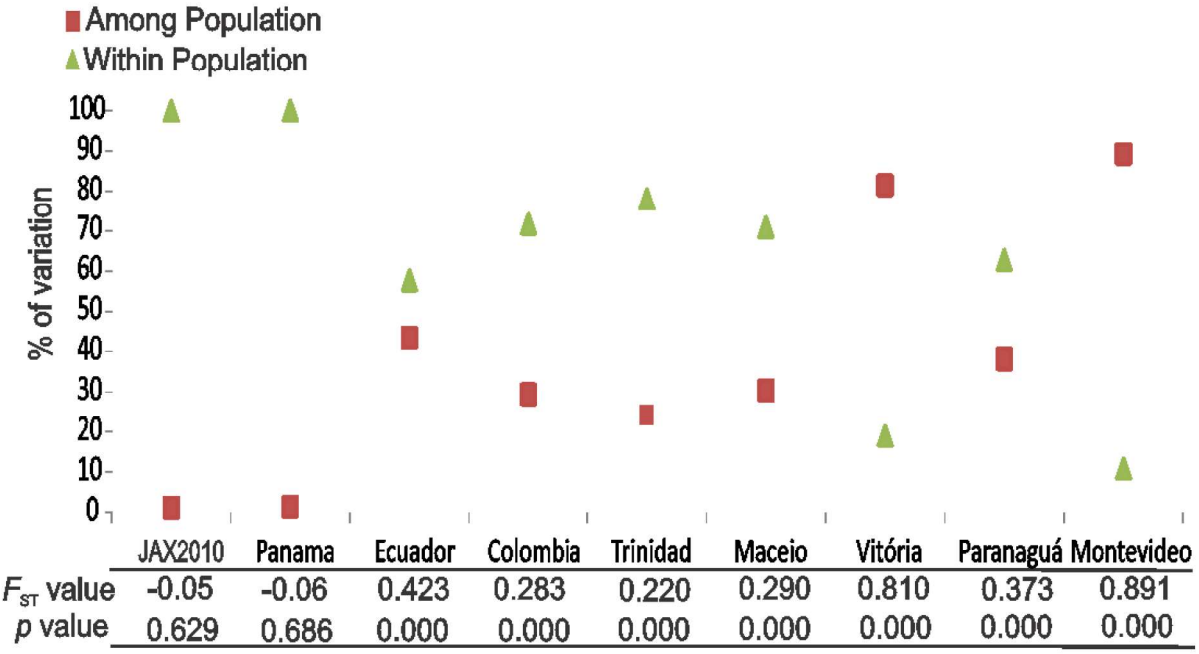


Figure 1.5 - Plots of multiple analyses of molecular variance (AMOVA) grouping Southeast-US 2006 with each native population and with the JAX2010 population. Below each plot are global F_{ST} and the p-values testing the null hypothesis of no genetic structure.

To determine whether there was a change in the genetic composition of populations over time in the invaded region, we ran an AMOVA contrasting the SE-US populations from 2006 with the JAX2010 population. This analysis revealed that the estimate of F_{ST} between these populations was low and not significant (F_{ST} = -0.05, p-value = 0.629), showing a lack of change over the four-year period (Fig 5). A comparison of haplotypes between sampling periods identified only a single haplotype (NN) which appeared in the JAX2010 sampling period, but was absent in SE-US populations from 2006. Moreover, there were four haplotypes present in 2006 that were absent in 2010 (M, P, Q, R; Fig 1 and 2). Similar to the genetic differentiation data, genetic diversity estimates exhibited little differentiation between sampling periods. Gene diversity for JAX2010 was 0.7462 and fell within the 95% CI of gene diversity from the 2006 SE-US populations (95% CI of h = 0.747 – 0.744), nucleotide diversity for JAX2010 was 0.0074 and fell slightly below the confidence interval found in 2006 SE-US populations (95% CI of π = 0.00881 – 0.00879).

Discussion

Our genetic assessment revealed a strong patterns of genetic structure throughout native *M. charruana* populations. In contrast, variation among invasive populations along the southeastern coast of the United States exhibited little differentiation, even when contrasted with temporally displaced samples on either side of a major population die-off. Diversity within native populations exhibited an order of magnitude in variation among populations, but this level of variation was driven by populations either fixed for a single haplotype group or populations that exhibited mingling of haplotype groups. The genetic diversity of introduced SE-US population remained high considering all invasive populations were composed of a single haplotype group. Overall, compiling genetic information from throughout the native range provided the necessary

data to determine that the introduced populations are likely closely related to Pacific Panamanian
552 populations. Likewise, occurrence of shared haplotypes between northern South America and the SE-US provides further evidence that the introduced range included samples of mixed origin.
554 Furthermore, admixture of haplotypes from Panama and southern Caribbean populations explains why within-population diversity is so high for the non-native populations.

556 Marine organisms have the potential to disperse throughout large areas where genetic isolation is generated with increasing distance [70, 71] and dispersal over large distances can
558 occur with sufficient time and stochastic events [72]. However, determining rates of connectivity and genetic structuring in the marine environment has proven to be challenging and more
560 complex than previously assumed. Although genetic structuring should reflect major biogeographic barriers (e.g. Isthmus of Panama Barrier (IPB); Amazon Barrier (AB); Mid-
562 Atlantic Barrier (MAB); and the Benguela Barrier (BB) [73]), barriers for dispersal do not affect all species equally [72]. There are examples of benthic species with long-range dispersal [73 - 77]
564 and mobile species that exhibit fine-scale genetic structuring [78, 79]. Likewise, long-term pelagic larval duration does not always cause species to exhibit a lack of structure over a large geographic
566 range [80]. Indeed, dispersal by marine organisms depends not just on pelagic larval duration but also on habitat requirements [81] and evolutionary history of a species [82]. Although the length
568 of pelagic larval duration is not directly related to genetic structuring for benthic invertebrates, long-range dispersal frequently occurs during the pelagic larval phase in benthic taxa [83, 84, 85].

570 Connectivity among populations of estuarine taxa would be expected to be reduced compared to marine and coastal taxa [86, 87]. In estuarine environments, structuring is dependent
572 on the process of migration from one estuary to another. Given that estuaries are often separated by open ocean segments, estuarine environments tend to restrict gene flow, driving populations to
574 local adaptation and boosting genetic differentiation among estuarine taxa [86]. Examples of estuarine taxa that exhibit significant genetic structuring include: amphipod (*Gammarus zaddachi*

576 [88]); gastropod mollusk (*Hydrobia ventrosa* [89]); catfish (*Cnidoglanis macrocephalus* [90]); echinoid (*Evechinus chloroticus* [91]); shrimp (*Macrobrachium nipponense* [92]); as well the 578 sister taxa to *M. charruana*, *M. guyanensis* [50]. *M. charruana,* as a typical estuarine mussel, exhibits a high degree of isolation throughout the native distribution, as demonstrated here.

580 In addition to the estuarine environment leading to differentiation, ocean currents are another feature along the South American coast that also likely contributes to differentiation 582 among populations [93, 94]. Ocean currents and associated differences in water temperature are known to impact genetic structure throughout the Atlantic (e.g. king weakfish, *Macrodon* 584 *ancylodon*, exhibit deep genetic divergence correlated with the northern flow of the Brazilian Current and the Malvinas Current, despite morphometric homogeneity [94]) and other oceans 586 (e.g. *Perna perna,* [95]). With regard to *M. charruana*, we can see the impacts of currents driving differentiation between the populations of PANA and ECUA where the intervening habitat is 588 ideal for *M. charruana* (e.g. mangroves and mud flats [96]). These populations are likely genetically differentiated owing to the flow of the Panama Current moving in a southward 590 direction and the Humboldt (Peruvian) Current moving Antarctic water northward. At the confluence, located between PANA and ECUA, the coastal water forms a constant vortex and the 592 current moves westward into the open ocean [97]. Similar to *M. charruana*, black mangroves (*Avicenia germinans*) exhibit high population genetic structuring between populations in the 594 northern and southern Pacific coasts of Colombia owing to these currents [98].

Given these barriers to natural dispersal found for *M. charruana* throughout the native 596 range, what processes likely brought *M. charruana* to the SE-US? Anthropogenic translocation by ship transportation (ballast water and/or hull biofouling) from South America appears to be the 598 most plausible vector for *M. charruana*. Many examples from other organisms support ballast water and ship hull fouling as efficient dispersal vectors for marine organisms [99-101], 600 especially considering the intense shipping traffic around Florida waters [102] and previous

investigations of the *M. charruana* SE-US invasion [24]. Given the characterization of genetic structure from throughout the native distribution conducted by this study, we were able to identify the likely source of the SE-US populations as being from, or near, the Pacific coast of Panama. Certainly new occurrence data from additional Caribbean populations could provide additional insight into the pathway(s) used by *M. charruana* to arrive in Florida from Panama.

Within the invasive range, there are both abiotic and biotic factors that can impact the spread of *M. charruana*. A recent study investigating temperature and salinity tolerances of *M. charruana* sheds light on how abiotic factors limit spread throughout the invaded range [103]. These researchers found that *M. charruana* survival was largely driven by temperature given that survivorship curves dropped off quickly at both low and high temperature extremes. With regard to biotic factors, *M. charruana* was found established on intertidal oyster reefs in the Indian River Lagoon, Florida [24]. Given that in the SE-US, *M. charruana* has only been found on hard substrates, further spreading may be directly related to oyster density. However, a recent study investigating competitive interactions between *M. charruana* and oysters (*Crassostrea virginica*) found that oyster spat survival and growth was reduced by the presence of *M. charruana* [104]. Native predators have also been found to limit invasive species distribution. For example, Dudas *et al.* [105] found that native crabs preferentially preyed upon non-native clams over native clams in choice experiments. Unpublished data has found that blue crabs (*Callinectes sapidus*) native to the SE-US equally prey upon *M. charruana* and the native ribbed mussel (*Geukensia demissa*) (L. Walters, unpublished data). Overall, there is a paucity of studies evaluating how biotic, abiotic, and the combination of these factors may impact the future spread of *M. charruana* throughout the SE-US. However, as climate change impacts North America, the range of invasive *M. charruana*, currently believed to be held in check by cold winter temperatures [45], will likely be able to expand into more northern latitudes.

Furthermore, by comparing genetic diversity and differentiation over a temporal scale that spanned the 2009/2010 [45] cold weather event, where the air temperature in Jacksonville, FL fell to 0°C or below for 12 of 13 days (2 January–14 January, 2010 [106]), we were able to address the ephemeral nature of the SE-US population. Our prediction was that the high summer occurrence data found by Spinuzzi *et al*. [45] could be caused by recurrent re-introductions leading to genetic change over time. Previous investigations of genetic change over time in other species have found mixed results, with some studies reporting genetic diversity to be static [29, 33] while others found that population genetic makeup does change over time [11,29,33,109]. However, our results were counter to our predictions in that we identified a persistent set of haplotypes belonging to the SE-US invasive population of *M. charruana*. These results indicate that, even during periods when *M. charruana* were not observed, some individuals were protected in stronghold locations and persisted to re-establish the coast when favorable conditions returned.

In sum, this study illuminates how an understanding of genetic differentiation from throughout the native range can provide insights into the spread and persistence of an invasive species. With regard to *M. charruana*, the differentiation identified among populations in the native range provided the necessary resolution to identify where the recent southeastern United States invasion originated. Moreover, this study identified an interesting contrast in dispersal patterns for *M. charruana*. Specifically, this species exhibits a pattern of minimal dispersal throughout the native range, yet is both persistent (this study) and spreading [45] throughout the invasive range. The life history characters that favor survival in the novel range (e.g. high salinity tolerance [35,108,109], high temperature tolerances [35,36], and the ability to change sex under stressful conditions [46]) cannot overcome the natural geographic barriers found throughout the native range. Synthesizing studies characterizing *M. charruana* in both the native and invasive range, we suggest that although *M. charruana* will not likely be transported long distances on its

own, anthropogenic transportation of this species can lead to foundation events in a variety of habitats that will likely be difficult to eradicate.

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970 # Chapter 2

Characterizing the *Perna perna* cryptogenic origin in Brazil.

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Abstract

Perna perna is a widespread mussel that occurs in warm/temperate regions of the Atlantic Ocean, Mediterranean Sea, and Indian Ocean, but current literature is inconclusive as to the origins of *P. perna* in the New World. Investigations of shell middens does not provide convincing evidence for the presence of *P. perna* in Rio de Janeiro middens dating back 2000 yr ago. Therefore, raised the possibility that *P. perna* have been brought to New World through the slave trade. After that, different studies did not come with a cogent clear conclusion. Here, we sought to investigate whether Brazilian *P. perna* is recently descended from North Africa via the slave trade approximately 500 yr ago. We genotyped 17 populations of *P. perna* at 10 microsatellite loci from three regions: North Africa (MR), South Africa (SA), and Brazil (BR). Seeking out to see if the Brazilian population clustered with other regions, we estimated number of clusters using structure and factorial correspondence analysis. Second, we estimated genetic diversity to determine if the Brazilian population contains reduced levels as might be indicative of a recent invasion. Last, to determine whether the Brazilian population was genetically similar to either of the three African clusters, we estimated pairwise F_{ST} for all populations and among clusters, and we tested for a pattern of isolation by distance. The three regions compose four genetic clusters: East South Africa (ESA), West South Africa (WSA), Brazil (BR), and North Africa (MR). Genetic diversity showed no general pattern of reduced or elevated levels within any region across loci. All regions are approximately equally genetically differentiated from each other and exhibit a significant pattern of isolation by distance. Our interpretation of these results is that they are entirely consistent with Brazilian populations of *P. perna* being a long-established native region.

Introduction

Biological invasions have become a major threat to biodiversity and marine vessels have been well established as the main vector for the spread of aquatic invasive species (Darwin, 1854, as cited in Bishop, 1951; Chilton, 1910; Visscher, 1928). During the past few decades, aquatic transportation has become the center of discussion for international organizations (Carlton, 1987; Norse, 1993; CBD, 1992; Williamson, 1996; IMO, 2001; Gollasch *et al.*, 2000; Seebens *et al.*, 2013) and many studies have identified vessels as a vector of bioinvasion by biofouling, ballast water, and sediment transport (Carlton, 1985; Carlton & Geller, 1993; Carlton, 1996; Leppäkoski *et al.,* 2002; Minchin *et al.,* 2003; WGBOSV, 2006).

The potential harm offered by biological invasions to public health, agriculture and biodiversity is well established (Eno, 1998; Wolff and Reise, 2002) and it is known that in coastal environments human activities increase the impact and the chance of invasion success (Grosholz, 2002). It is important to assess the characteristics of invasion success as a means to better comprehend what promotes the spread of non-native species and to develop management actions (Panov *et al.,* 2009). However, inferring processes after successful invasion is challenging, largely due to a lack of information about invasion history (Estoup & Guillemaud, 2010; Edelaar *et al.,* 2015). It can be difficult to determine the introduction patterns of invasive species because they are often detected years, decades, or even centuries after the introduction event (Kowarik, 1995; Martel *et al.,* 2004; Crooks, 2005).

Genetic data exhibit specific signatures of population origins that provide reliable evidence of population ancestry, interaction, and connectivity allowing us to track and determine the source of the invasive population by comparing the invasive and native populations (Holland, 2000; Sakai *et al.*, 2001; Lee, 2002). Knowing the source of introduced populations is important in assisting with a risk analysis that will implement certain actions to potentially eliminate or reduce

1062 the impact of the establishment of an invasive species (Stepien *et al.,* 2005). In the classic scenario, non-native populations have reduced genetic variation compared to native-range source
1064 populations due to founder effects (Nei *et al.,* 1975; Sakai *et al.,* 2001; Frankham *et al.,* 2002; Allendorf & Lundquist, 2003; Novak & Mack, 2005). Constraints generated by reduced
1066 population size, inbreeding depression, and vulnerability to natural selection in the non-native environment, reduce the number of founders as well as the genetic diversity of the non-native
1068 species (Fisher, 1930; Falconer & Mackay 1996; Newman & Pilson, 1997; Frankham & Ralls, 1998; Nieminen *et al.,* 2001;). In contrast to these classic scenarios, many introduced populations
1070 have been found to exhibit higher genetic variation than native populations typically owing to admixture of multiple source populations (Novak & Mack, 1993; Voisin *et al.,* 2005; Facon *et al.,*
1072 2003; Collins *et al.,* 2002; Kolbe *et al.,* 2004; Gillis *et al.,* 2009). Although there is no consensus of how genetic diversity in the founder population (high or low diversity / mixed or single source)
1074 affects invasiveness (Novak & Mack, 2005; Wares *et al.,* 2005; Roman & Darling, 2007; Edelaar, 2015), comparing patterns of genetic variability between native and introduced populations make
1076 it possible to unravel the relationship between putative source and non-native populations as well as elucidate the evolutionary history of the introduced population (Fonseca *et al*., 2000; Kolar &
1078 Lodge 2001; Lockwood *et al.,* 2007; Dlugosch & Parker 2008).

In Brazil, the marine mussel *Perna perna* is a key economic driver for people living along
1080 the coast as well as an important source of protein. Along the Atlantic coast of South America *P. perna* has been described as a natural species (Venezuela; Southeastern Brazil to Uruguay;
1082 Ihering, 1897, 1900; Klappenbach, 1965; Sidall, 1980; Vakily, 1989; Chung & Acuña, 1981; Rios, 2009). In addition to the Atlantic, *P. perna* naturally occurs across a wide-range throughout
1084 the old world, including the Red Sea, the Gulf of Aden, east and west coasts of Africa, and within the Mediterranean Sea (Ihering 1897, 1900; Klappenbach, 1965; Sidall, 1980; Vakily, 1989;).
1086 *Perna perna* has also been recorded as invasive in the Gulf of Mexico since the early 1990s

(Holland, 2001). Interestingly, recent studies have questioned whether *P. perna* is actually native along the Brazilian coast based on the lack of *P. perna* shells in ancient shell middens from Rio de Janeiro State (Moura-Neto, 2003; Souza *et al*., 2003; 2004; 2010; Fernandes *et al*., 2008; Cunha, 2014; Pierri *et al.,* 2016; Oliveira *et al.* 2017). The prevailing thought is that *P. perna* were introduced to the Atlantic coast of South America before modern history would have recorded their introduction, likely associated with the transatlantic human slave trade, circa 500 yr ago (Moura-Neto, 2003; Souza *et al*., 2003; 2004; 2010; Fernandes *et al*., 2008). In contrast, a recent genetic study has provided evidence supporting *P. perna* as native to South America (Pierri *et al.,* 2016).

Numerous political implications have been impacted based on the recent studies that suggested that *P. perna* was introduced along the South American coast, such as, in Venezuela, Pérez *et al.* (2007) included *P. perna* on a list of exotic species with cryptogenic origins. Additionally, in Brazil, the Ministry of Environment produced a data-sheet book listing *P. perna* as an established exotic species and the state of Paraná produced a standardization decree to regulate and control production of invasive species in the state, including *P. perna* (Lopes *et al.*, 2009; IAP, 2009, 2015). Moreover, additional publications have described the impact and strategies for *P. perna* control (Souza *et al.,* 2009; Machado *et al.,* 2009). Soon after the governmental position for the control of exotic species, including *P. perna,* opposing studies have highlighted the importance of *P. perna* as a natural environmental engineer along the rocky coastline and its use for human consumption (Freitas & Velastin, 2010).

The need to clarify the evolutionary history of *P. perna* along the South American coast has important social, economic, and political implications. Recent studies that used mtDNA markers to investigate this question have produced contrasting patterns (Pierri *et al.,* 2016; Oliveira *et al.,* 2017). Overall, this study seeks to clarify the question of whether *P. perna* is native to the western Atlantic (Brazilian) coast using microsatellite markers to assess patterns of

1112 diversity within populations, differentiation between populations and connectivity among African and South American populations. Owing to recent studies that have revealed the lack of ancient
1114 shell records (Souza *et al.,* 2003), the similarity between North African and South American populations (Wood *et al.,* 2007; Cunha *et al.,* 2014), and the possibility of admixture between
1116 Africa and South America (Oliveira *et al.,* 2017), we assumed a hypothesis that Brazilian *P. perna* was recently descended from Africa, primarily from North Africa, via the slave trade
1118 (approximately 500 yr ago). Therefore, we predicted that Brazilian *P. perna* should cluster more closely with North African populations. We also predicted that Brazilian populations would
1120 exhibit lower levels of genetic diversity than native African populations, with North African populations being genetically more similar to Brazilian populations or high genetic diverse by a
1122 mixed source formation. We also predicted that South America populations should exhibit fewer private alleles than old world populations, owing to the more recent founding of the Brazilian
1124 populations. Finally, we discuss how genetic structuring of populations can be used to better understand the evolutionary history of marine biological invasions.

1126 ## Materials and methods

Sampling collection and DNA analysis

1128 A total of 644 samples of the brown mussel, *P. perna,* were obtained from eight locations in Brazil (Cassino-RS, $n = 32$; Atlântida-RS $n = 39$; Palhoça-SC $n = 30$; Brava-SC $n = 38$;
1130 Matinhos-PR $n = 35$; Itanhanhém-SP $n = 20$; Vitória-ES $n = 44$; Macaé-RJ $n = 36$), three locations in Morocco (Essaouira $n = 30$; Imsouane $n = 30$; Mirleft $n = 30$), six locations along the
1132 coast of South Africa (Muizemberg $n = 11$; Hermanus $n = 48$; Plettenberg $n = 48$; Port Alfred $n = 47$; Port Eduard $n = 48$; Balito $n = 48$), and one location in Mozambique (Punta Ouro $n = 30$)
1134 (Tab. 2.1; Fig. 2.1). Mantle tissue (20 - 30 mg) was dissected from each individual, preserved in

92% ethanol and stored at -20°C until DNA extraction. Total genomic DNA extraction was performed using a standard Proteinase-K protocol adapted from (Sambrook *et al.,* 1989). We genotyped all 644 individuals from 17 populations of *P. perna* using a set of 10 microsatellite loci (Coelho *et al*., 2012) in three multiplex PCR reactions performed in a GeneAmp 9700 thermocycler (PE Applied Biosystems) with volumes of 10 µl containing ± 10 ng of DNA, 0.5 µM of each primer labelled with a florescent marker, 0.2 mM dNTPs (Bioline), 1.5 mM MgCl2, 3.0 µl of 5x PCR Buffer and 0.75 U of GoTaq Polymerase (Promega, Madison, WI). Cycling conditions consisted of an initial denaturing step of 5 min at 95°C, followed by 35 cycles of 30s at 95°C, 30s at 53 to 59°C as optimization of annealing temperature (Coelho *et al*. 2012), 40 s at 72°C, and a final elongation step at 72°C for 20 minutes, with subsequent separation of the PCR products using an ABI PRISM 3130xl DNA analyzer (Applied Biosystems) with Gene Scan Liz 500 as size standard (Applied Biosystems). Microsatellite raw allele sizes were manually scored in STRand 2.4.59 (Toonen & Hughes, 2001).

Table 2.1 - Number of samples per location and sample code.

Site	Pop. code	Sample (*N*)	Lat.	Long.
SF_Muizemberg	EB11	11	34° 6'39.67"S	18°28'5.95"E
SF_Hermanus	HMP48	48	34°24'52.32"S	19°15'19.50"E
SF_Plettenberg	PLP48	48	34° 3'40.93"S	23°22'51.83"E
SF_PortAlfred	PAP48	47	33°36'46.09"S	26°53'25.71"E
SF_PortEduard	PEP48	48	31° 2'44.83"S	30°13'44.30"E
SF_Balito	BAP48	48	29°26'50.21"S	31°17'21.04"E
MC_PuntaOuro	PDOP30	30	26°50'38.12"S	32°53'42.12"E
MR_Essaouira	ES30	30	31°30'28.28"N	9°46'28.34"W
MR_Imsouane	IM30	30	30°50'13.40"N	9°49'17.11"W
MR_Mirleft	ML30	30	29°35'23.59"N	10° 2'41.60"W
RS_Cassino	EA32	32	32°10'6.79"S	52° 5'35.81"W
RS_Atlantida	RS119	39	29°47'8.82"S	50° 1'26.05"W
SC_palhoca	SC34	30	27°38'28.73"S	48°38'3.48"W
SC_Brava	EC38	38	27°24'19.08"S	48°24'35.40"W
PR_Matinhos	PR143	35	25°50'38.81"S	48°32'2.65"W
SP_itanhanhem	SP192	20	24°11'19.52"S	46°47'26.96"W
ES_vitoria	VIT44	44	20°17'59.91"S	40°16'33.26"W
RJ_Macae	ED36	36	22°22'39.45"S	41°45'55.13"W
TOTAL		644		

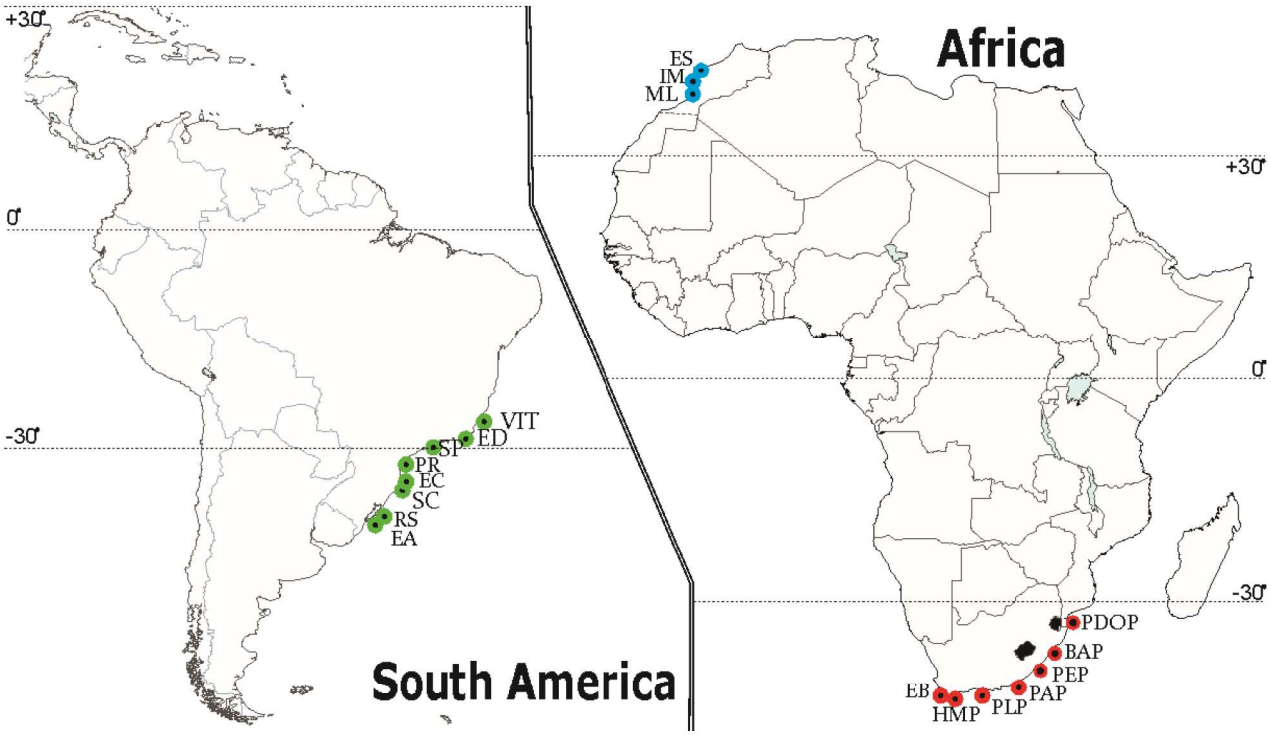


Figura 2.1 Map of localities with sample code where samples from Brazil is marked in Green, samples from Morocco in Blue, Samples from South Africa e Mozambique in Red.

Data Analysis

Microsatellite scoring errors, evidence of large allelic dropout, and null alleles at high frequencies were estimated based on the algorithm presented in Brookfield (1996) using the program MICROCHECKER (Van Oosterhout *et al.*, 2004). Hardy–Weinberg equilibrium (HWE) was estimated per population and locus with FSTAT (Goudet, 1995). Multiple contrasts of HWE probabilities were corrected according to the sequential Bonferroni procedure (Rice, 1989).

To determine whether the Brazilian population is genetically unique or composed of mixed ancestry from either the northern or southern African region, we used a Bayesian cluster analysis in STRUCTURE 2.2 (Prichard *et al.,* 2000). We ran the analysis assuming that all sampled sites were independent populations capable of forming their own clusters (K =1 to K = 18). The STRUCTURE analysis was set up with twenty replicate runs performed under the admixture and the correlated allele frequency model with 50,000 Markov Chain Monte Carlo

(MCMC) iterations after 500,000 burn-in generations. After the initial number of clusters was defined, we re-ran STRUCTURE for each initially described cluster to determine whether sub-structuring was present. Overall, we identified the best supported number of clusters via Evanno delta K on these successive analyses. Complementary to the STRUCTURE analysis, we used factorial correspondence analysis (FCA) to uncover population admixture in the absence of genetic assumptions in GENETIX Software v. 4.05 (Belkhir *et al*. 2004).

To determine whether patterns of genetic diversity in the Brazilian population were similar to those of the northern or southern African populations, we estimated allele frequencies, observed heterozygosity (H_O), expected heterozygosity (H_E), and Allelic Richness (AR) using FSTAT (Goudet, 1995). We used a two-way Analysis of Variance (ANOVA) with post-hoc Tukey test in R statistical package version 3.2.3 (R core, 2015) to test whether there were any significant differences among loci and between regions for levels of genetic diversity. Additionally, we estimated F_{ST} to characterize patterns of pair-wise differentiation in the program FSTAT (Goudet, 1995). To evaluate whether there were significant differences among regions, we employed a Kruskal-Wallis test comparing regional differences in average within-region pairwise-F_{st}. Moreover, to determine whether *P. perna* populations exhibit a pattern of natural genetic relatedness decaying with geographic distance we used the F_{ST} values (Weir & Cockerham 1984) in a pair-wise analysis test for isolation-by-distance (IBD) in IBDWS (Jensen *et al.* 2005) using a Mantel test with 10,000 randomizations of linearized F_{ST} (i.e. $F_{ST}/(1-F_{ST})$) with the least-cost distance along the shoreline distance between sampling locations (km) estimated in Google Earth Tools 7.1.5.1557 (Google®, 2016). To test for genetic similarity between the Brazilian population and putative source populations, we used a molecular analysis of variance (AMOVA) in Arlequin (Excoffier & Heidi, 2010) to estimate the differentiation within populations, among populations within regions, and among regions. Finally, to determine whether the Brazilian populations exhibited a pattern indicative of a recent invasion, we compared the

1192 mean frequencies of private alleles calculated in GenAlex v.6 (Peakall & Smouse, 2006) among all localities grouped by region.

1194 ## Result

All microsatellite loci were highly polymorphic among the 18 populations. The 644 1196 individuals returned 448 alleles, with an average of 45 alleles per locus, ranging from 5 (P02 - IM30 and ML30) to 57 (P20- PEP48). However, populations exhibited some loci with a deficit of 1198 heterozygotes relative to Hardy-Weinberg proportions. This pattern is unsurprising given that other studies have already described this pattern in molluscs in general, and especially in *P. perna* 1200 (Koehn *et al*., 1973; Zouros & Foltz, 1984; Borsa *et al.,* 1991; Coelho *et al.,* 2012; Zardi *et al.*, 2015). Following the methods in Zardi *et al.* (2015), we used null allele frequency as the basis of 1202 locus inclusion in downstream analyses. Tests for null alleles revealed that two loci (P11 and P16; Fig. 2.2) exhibited frequencies above 0.2, the threshold at which loci are characterized as 1204 consisting of high frequency null alleles (Selkoe & Toonen, 2006) and are subject to removal owing to false allele frequencies impacting results (Chapuis & Estoup, 2007). The remaining eight 1206 loci exhibited null allele frequencies below 0.2 and were thus used in all downstream analyses (Fig. 2.2).

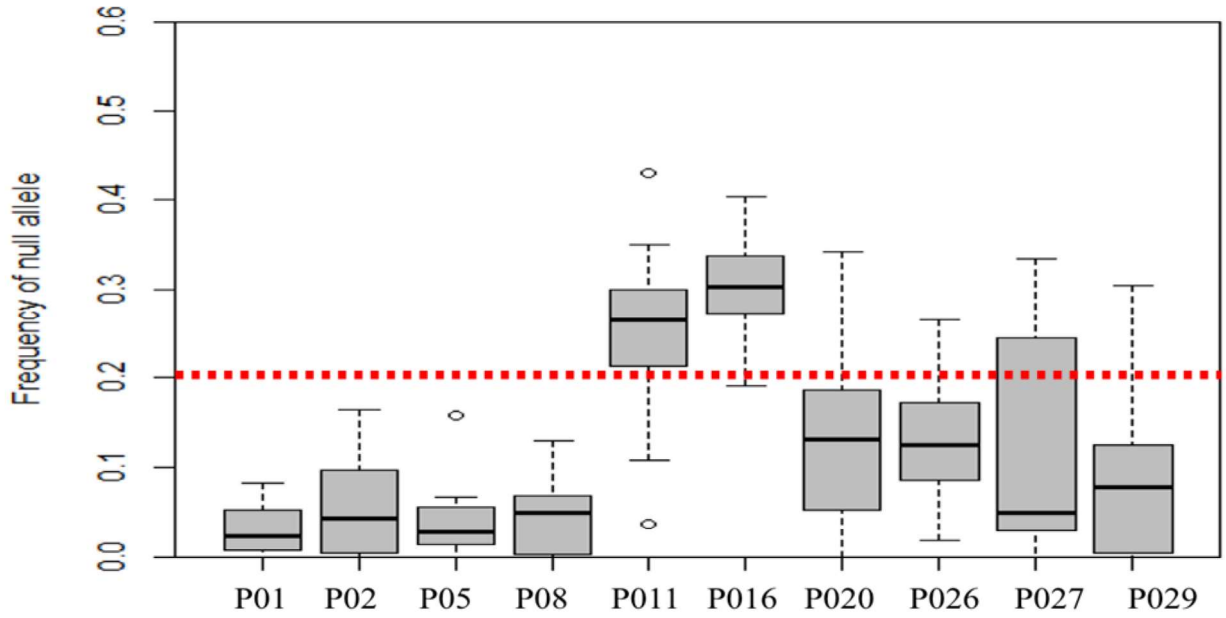


Figure 2-1 - Expected null allele frequencies calculated using the second method of Brookfield (1996). Locus P011 and P016 showing frequency of null allele above the acceptable limit of 0.2.

Table 2.2 - Measures of the deviation from Hardy-Weinberg heterozygote proportions FIS estimates per each loci per population.

Locus	SF_Balito	RS_Cassino	SF_Muizemberg	SC_Brava	RJ_Macae	MR_Essaouira	SF_Hermanus	MR_Imsouane	MR_Mirleft	SF_PortAlfred	MC_PuntaOuro	SF_PortEduard	SF_Plettenberg	PR_Matinhos	RS_RioGrande	RS_Atlantida	SC_palhoca	SP_itanhanhem	ES_vitoria
	BAP48	EA32	EB11	EC38	ED36	ES30	HMP48	IM30	ML30	PAP48	PDOP30	PEP48	PLP48	PR143	RGS15	RS119	SC34	SP192	VIT44
P01	0.0399	0.3833	0.0102	0.5575	0.4200	0.0353	0.4198	0.8087	0.1023	0.2239	0.1949	0.9575	0.8700	0.1102	0.3185	0.0000	0.1018	0.0177	0.1847
P02	0.2234	0.8992	0.4000	0.3828	0.0731	0.1293	0.0111	0.0633	0.0006	0.0046	0.0161	0.3756	0.0000	0.0034	0.0048	0.1048	0.3914	0.8363	0.0779
P05	0.0201	0.5007	0.0007	0.0734	0.7469	0.9014	0.0955	0.1507	0.3171	0.1219	0.4825	0.5042	0.0023	0.0258	0.9212	0.0347	0.0102	0.0387	0.1583
P08	0.0000	0.1896	0.4336	0.0195	0.0401	0.0000	0.0385	0.0350	0.0037	0.0033	0.1482	0.0123	0.0810	0.1692	0.6993	0.2130	0.1453	0.2273	0.0571
P011	0.0000	0.0000	0.0724	0.0000	0.0000	No	0.0000	No	No	0.0000	0.0675	0.0000	0.0000	0.0000	0.0000	0.0000	0.0259	0.0000	0.0000
P016	0.0000	0.0000	0.0021	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0389	0.0641	0.0000
P020	0.0075	0.0000	0.0071	0.0075	0.0210	0.0623	0.0000	0.0000	0.0780	0.0000	0.0666	0.2335	0.0000	0.0000	0.0000	0.0015	0.0000	0.0000	0.0000
P026	0.0000	0.0004	0.0091	0.0000	0.0223	0.0003	0.0051	0.7790	0.4328	0.0013	0.0170	0.0432	0.0314	0.0017	0.0573	0.0000	0.0262	0.0000	0.0000
P027	0.0000	0.0811	0.0000	0.0378	0.2968	0.2093	0.0000	0.3031	0.3199	0.0000	0.0000	0.0000	0.0000	0.0012	0.5721	0.3983	0.3089	0.2716	0.0090
P029	0.0000	0.3221	0.1702	0.0856	0.0937	0.0000	0.0000	0.0000	0.0000	0.0002	0.0312	0.2067	0.0000	0.0694	0.4910	0.8971	0.0066	0.6711	0.0256

The STRUCTURE analysis revealed four clusters ($k = 4$) for the 3 regions studied using the most likely posterior probabilities with the Evanno *et al.* (2005) criteria. These clusters included the three general regions (South America, North Africa and South Africa), with South

Africa split into eastern and western clusters (Fig.2.3). The results from the FCA corroborate the STRUCTURE results, revealing 4 primary population clusters with the three axes describing 50.31% of the total among-population variation (Fig.2.4).

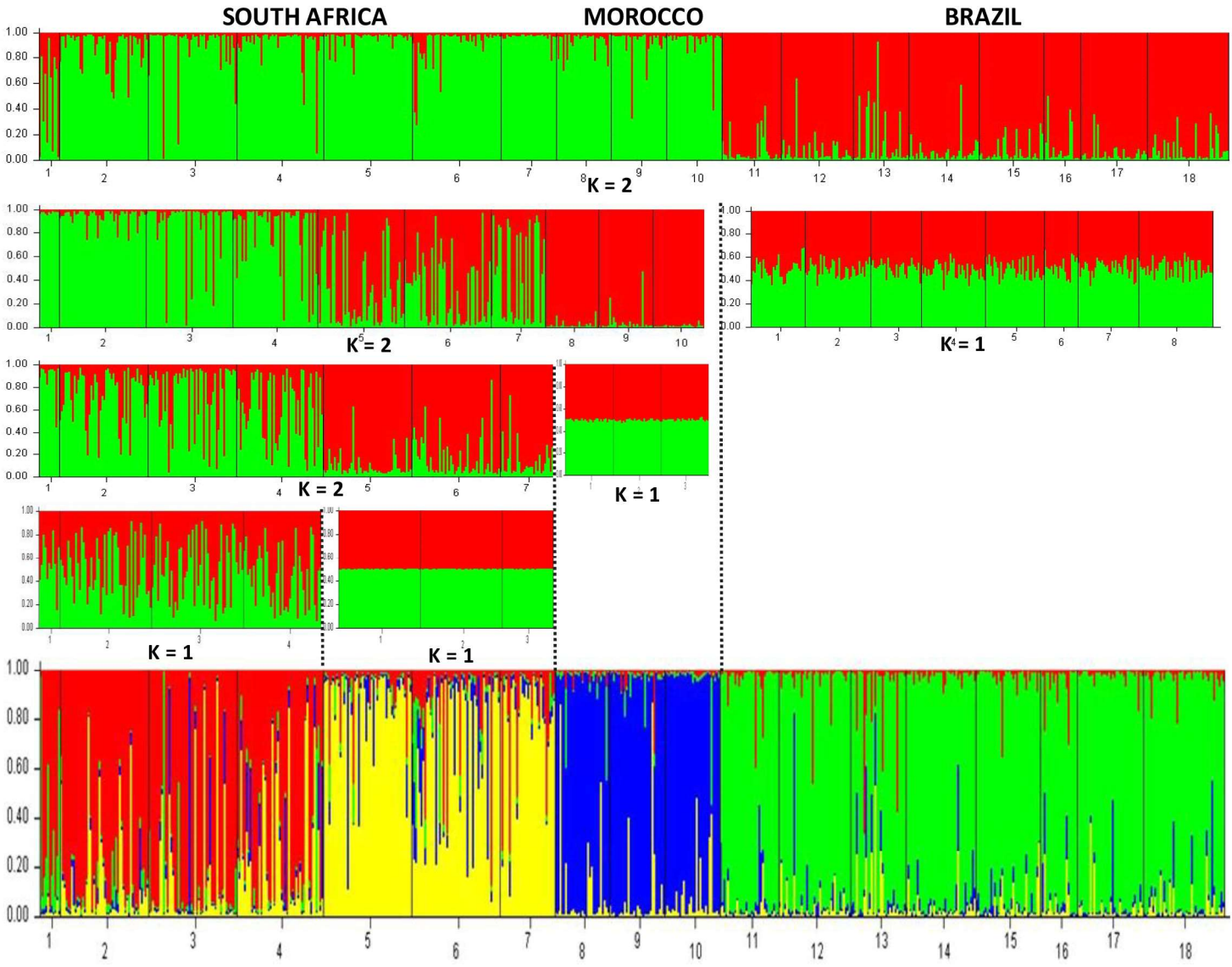


Figure 2.3 - Results of Bayesian population genetic structure for microsatellite data indicating four different groups of parent populations (K = 4) of *Perna perna*, shown in Red (West South Africa), yellow (East South Africa), in blue (Morocco) and in Green (Brazil). Individual probabilities of assignment are shown on the y-axis and are grouped by parent populations calculated by the admixture program in STRUCTURE 2.2.

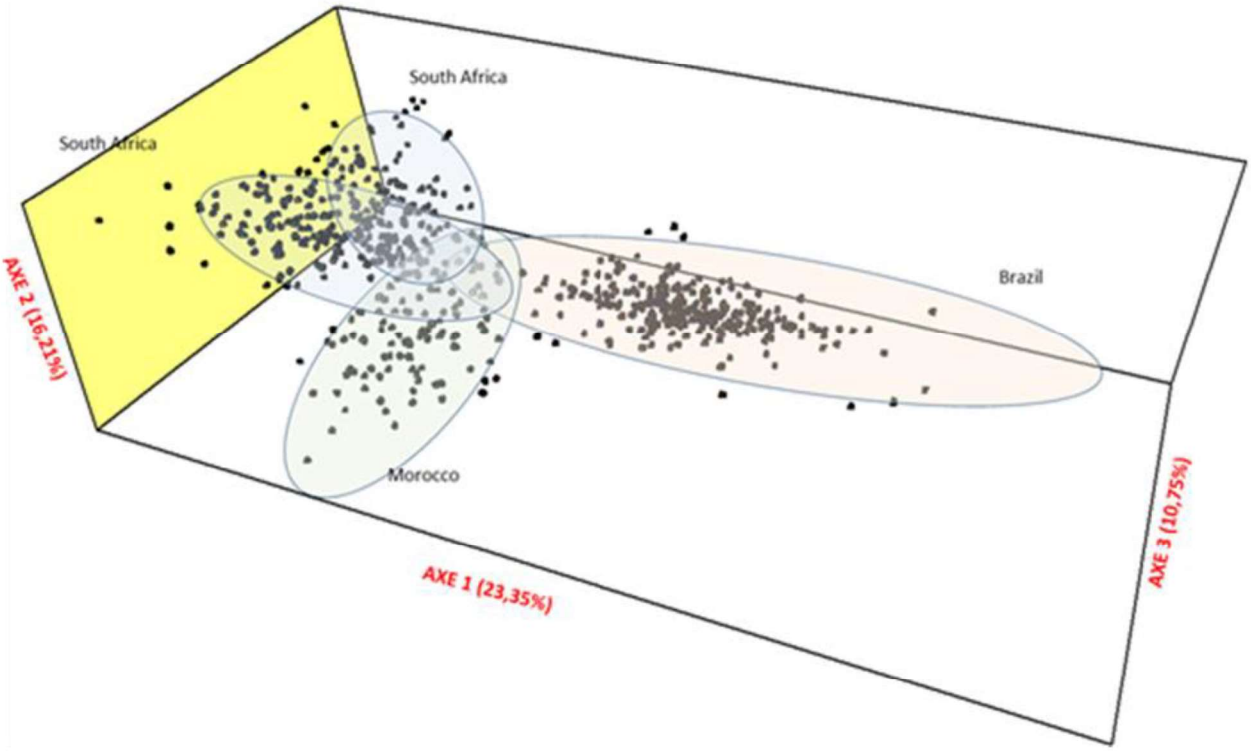


Figure 2.4 - Scatter plots of Factorial Correspondence Analysis (FCA) with four genetic group. Each dot correspond of one individual. The margins of the graphs represent the position of each gravity center with 23,35% of variation on axis 1, 16.21% on axis 2 and 10,75% on axis 3.

Population genetic diversity was high within all populations. Allelic richness (AR) and heterozygosity (H_E) varied between 2.38 - 12.87 and 0.2 - 0.99 respectively and the highest allelic richness was recorded at MC_PuntaOuro and MR_Essaouira (Table 2.3; 2.4; 2.5). Comparing differences in genetic diversity among the South American populations, the South African populations, and the North African populations, we found no general pattern of reduced or elevated levels of genetic diversity. Our analyses did reveal a significant effect of both Site and Locus as well as a significant interaction effect of site-by-locus for both measures of genetic diversity (ANOVA: Site $p < 0.001$, Locus $p < 0.001$, and interaction effect for H_E and AR, $p < 0.001$, Fig. 2.5). The significant interaction revealed that the pattern of genetic diversity is not driven exclusively by region or locus, rather, that different loci had different levels of diversity in different populations. Given the significant site effect, we ran post-hoc tests to determine whether

genetic clusters differed in genetic diversity measures. Tukey's post-hoc tests indicated that Brazil and Morocco are not differentiated in genetic diversity (i.e. *AR* and H_E) but both exhibit significantly less diversity than the South Africa region (post-hoc Tukey test, AR: $p < 0.001$; H_E: $p < 0.001$).

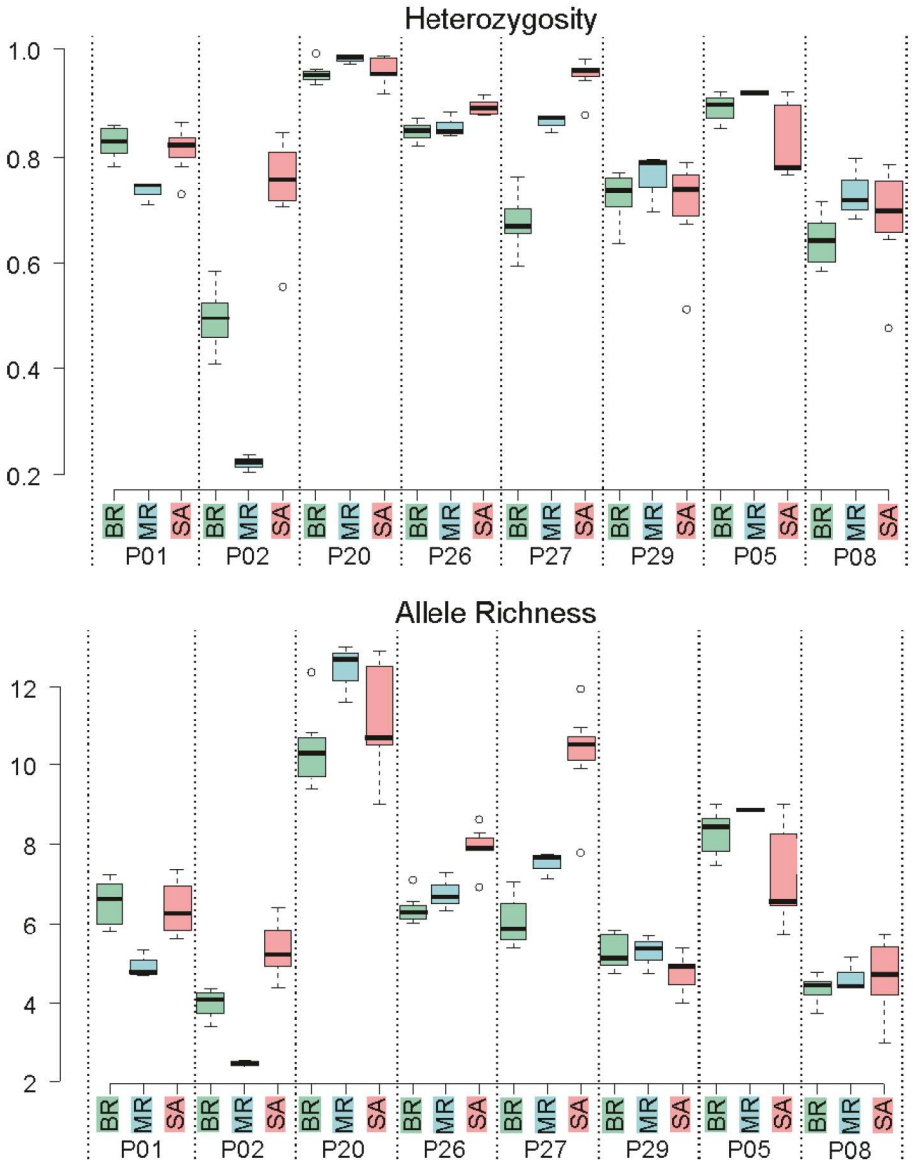


Figure 2.5 - Two-way Analysis of Variance (ANOVA) boxplot of heterozygosity (He) and Allelic Richness (AR). Where the axis y describe the genetic variance and on axis x present loci with the color highlighting localities (Green is Brazil, Blue is Morocco and Red South Africa).

Table 1.3 - Expected heterozygosity values - He. The collors identify the localities region, where red =West Soulth Africa; Yellow = East South Africa; Blue = Morocco; Green = Soulth America

	Expected heterozygosity – He																		All pop
P01	0.82	0.86	0.78	0.85	0.82	0.82	0.73	0.71	0.75	0.75	0.85	0.82	0.78	0.85	0.81	0.86	0.83	0.81	0.81
P02	0.84	0.8	0.76	0.81	0.56	0.7	0.73	0.24	0.2	0.22	0.53	0.41	0.49	0.52	0.5	0.47	0.45	0.58	0.55
P05	0.78	0.77	0.77	0.78	0.91	0.92	0.88	0.92	0.92	0.92	0.89	0.9	0.85	0.92	0.87	0.92	0.87	0.9	0.87
P08	0.48	0.79	0.78	0.73	0.64	0.7	0.67	0.8	0.68	0.72	0.64	0.67	0.58	0.59	0.68	0.72	0.62	0.65	0.67
P020	0.92	0.95	0.95	0.95	0.99	0.98	0.99	0.99	0.97	0.99	0.95	0.96	0.94	0.95	0.99	0.95	0.94	0.95	0.96
P026	0.89	0.88	0.88	0.88	0.91	0.9	0.9	0.85	0.84	0.88	0.83	0.86	0.85	0.87	0.84	0.85	0.82	0.84	0.87
P027	0.88	0.94	0.96	0.96	0.98	0.97	0.96	0.88	0.84	0.87	0.66	0.76	0.69	0.72	0.67	0.59	0.67	0.66	0.81
P029	0.51	0.74	0.76	0.77	0.67	0.7	0.79	0.79	0.79	0.69	0.71	0.7	0.75	0.75	0.72	0.64	0.77	0.77	0.72
Average	0.76	0.84	0.83	0.84	0.81	0.84	0.83	0.77	0.75	0.76	0.76	0.76	0.74	0.77	0.76	0.75	0.75	0.77	
	SF_Muizemberg	SF_Hermanus	SF_Plettenberg	SF_PortAlfred	SF_PortEduard	SF_Balito	MC_PuntaOuro	MR_Essaouira	MR_Imsouane	MR_Mirleft	RS_Cassino	RS_Atlantida	SC_palhoca	SC_Brava	PR_Matinhos	SP_itanhanhem	RJ_Macae	ES_vitoria	
Pop. Code	**EB11**	**HMP48**	**PLP48**	**PAP48**	**PEP48**	**BAP48**	**PDOP30**	**ES30**	**IM30**	**ML30**	**EA32**	**RS119**	**SC34**	**EC38**	**PR143**	**SP192**	**ED36**	**VIT44**	

Table 2.4 - Allelic Richness – AR The collors identify the localities region, where red =West Soulth Africa; Yellow = East South Africa; Blue = Morocco; Green = Soulth America

	Allelic Richness																		All Pop
P01	5.63	7.27	5.75	7.36	6.67	6.28	5.96	4.70	4.77	5.36	7.05	6.77	5.84	7.00	5.83	7.23	6.17	6.47	7.18
P02	6.24	5.48	5.10	6.42	4.38	4.77	5.23	2.59	2.38	2.48	4.37	3.42	4.06	4.11	4.14	3.59	3.87	4.38	5.04
P05	5.74	6.55	6.49	6.48	8.69	9.00	7.85	8.86	8.89	8.87	8.49	8.62	7.46	9.01	7.59	8.70	8.06	8.40	9.64
P08	3.00	5.73	5.56	5.23	4.25	4.72	4.18	5.17	4.40	4.41	4.45	4.53	4.24	4.47	4.77	4.55	3.74	4.18	5.01
P020	9.00	10.54	10.48	10.69	12.74	12.24	12.87	12.68	11.60	12.96	10.64	10.79	9.73	10.44	12.34	9.40	9.58	10.16	12.04
P026	6.94	7.86	7.87	7.92	8.63	8.00	8.28	6.32	6.70	7.29	6.41	6.55	6.40	7.09	6.04	6.07	6.16	6.19	7.54
P027	7.78	9.91	10.33	10.50	11.94	10.93	10.49	7.76	7.12	7.67	5.76	7.05	5.84	6.74	5.37	5.38	6.29	5.90	9.59
P029	4.00	4.94	4.96	4.93	4.10	4.77	5.39	5.38	5.70	4.75	5.08	4.81	5.84	5.09	5.21	4.76	5.60	5.85	6.12
Average	6.04	7.28	7.07	7.44	7.68	7.59	7.53	6.68	6.44	6.72	6.53	6.57	6.18	6.74	6.41	6.21	6.18	6.44	7.77
	SF_Muizemberg	SF_Hermanus	SF_Plettenberg	SF_PortAlfred	SF_PortEduard	SF_Balito	MC_PuntaOuro	MR_Essaouira	MR_Imsouane	MR_Mirleft	RS_Cassino	RS_Atlantida	SC_palhoca	SC_Brava	PR_Matinhos	SP_itanhanhem	RJ_Macae	ES_vitoria	
Pop. Code	**EB11**	**HMP48**	**PLP48**	**PAP48**	**PEP48**	**BAP48**	**PDOP30**	**ES30**	**IM30**	**ML30**	**EA32**	**RS119**	**SC34**	**EC38**	**PR143**	**SP192**	**ED36**	**VIT44**	

Table 2.5 - Per population average Heterozygosity and Allelic Richness. The collors identify the localities region, where red =West Soulth Africa; Yellow = East South Africa; Blue = Morocco; Green = Soulth America.

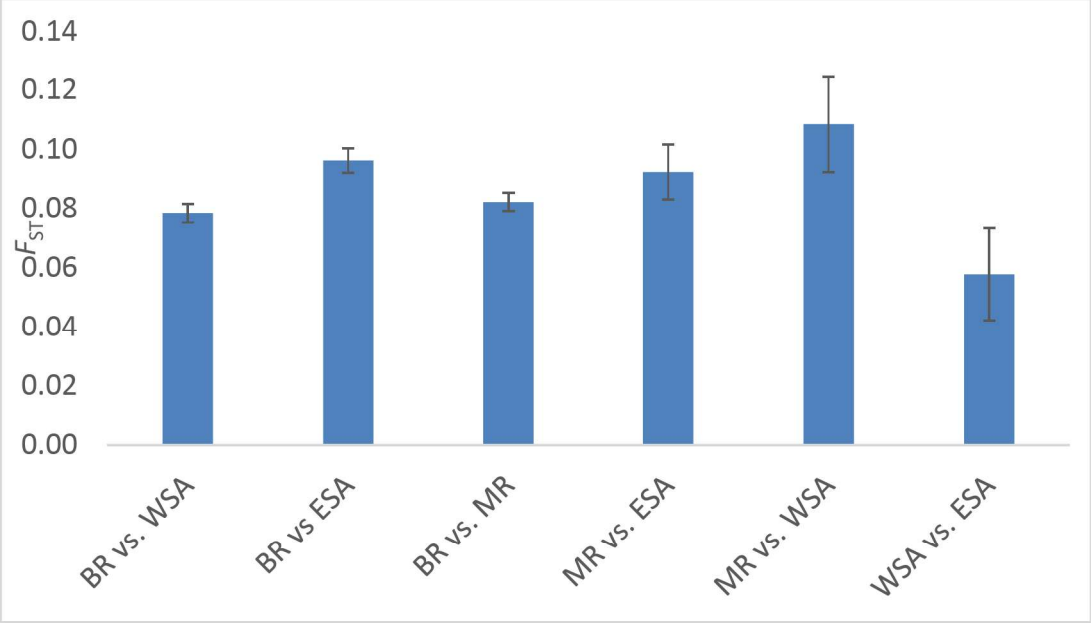
	EB	HMP	PLP	PAP	PEP	BAP	PDOP	ES	IM	ML	EA	RS	SC	EC	PR	SP	ED	VIT
Average H_E	0.76	0.84	0.83	0.84	0.81	0.84	0.83	0.77	0.75	0.76	0.76	0.76	0.74	0.77	0.76	0.75	0.75	0.77
Average AR	6.04	7.28	7.07	7.44	7.68	7.59	7.53	6.68	6.44	6.72	6.53	6.57	6.18	6.74	6.41	6.21	6.18	6.44

Genetic differentiation (i.e. pair-wise F_{ST}; Table 2.6) among regions indicated that significant differentiation occurs between regions ($p < 0.001$), with the following regional

differences: Brazil and Morocco (average F_{ST} = 0.08); Brazil and South Africa (average F_{ST} = 0.086). The level of genetic differentiation between regions showed that Brazilian *P. perna* populations exhibit equal differentiation between South Africa and Morocco. Whereas Morocco and South Africa appear slightly more differentiated (F_{ST} = 0.1). Comparing South America to each genetic cluster revealed that all clusters are approximately equally genetically differentiated from each other (Fig. 2.6) and exhibited a significant pattern of isolation by distance (Fig. 2.7). The analysis of molecular variance (AMOVA) showed weak but significant genetic differentiation among regions (Table 2.7; Global F_{ST} = 0.053, $p < 0.001$). The total amount of variation explained is characterized mostly by variation within populations sampled (94.6%), with among region variation only explaining 4.16% of the variation (Table 2.7). Finally, we found that the South American cluster exhibited the lowest frequency of private alleles: East Africa = 0.7; West Africa = 1.7; Morocco = 0.5 and Brazil = 0.3; Fig. 2.8).

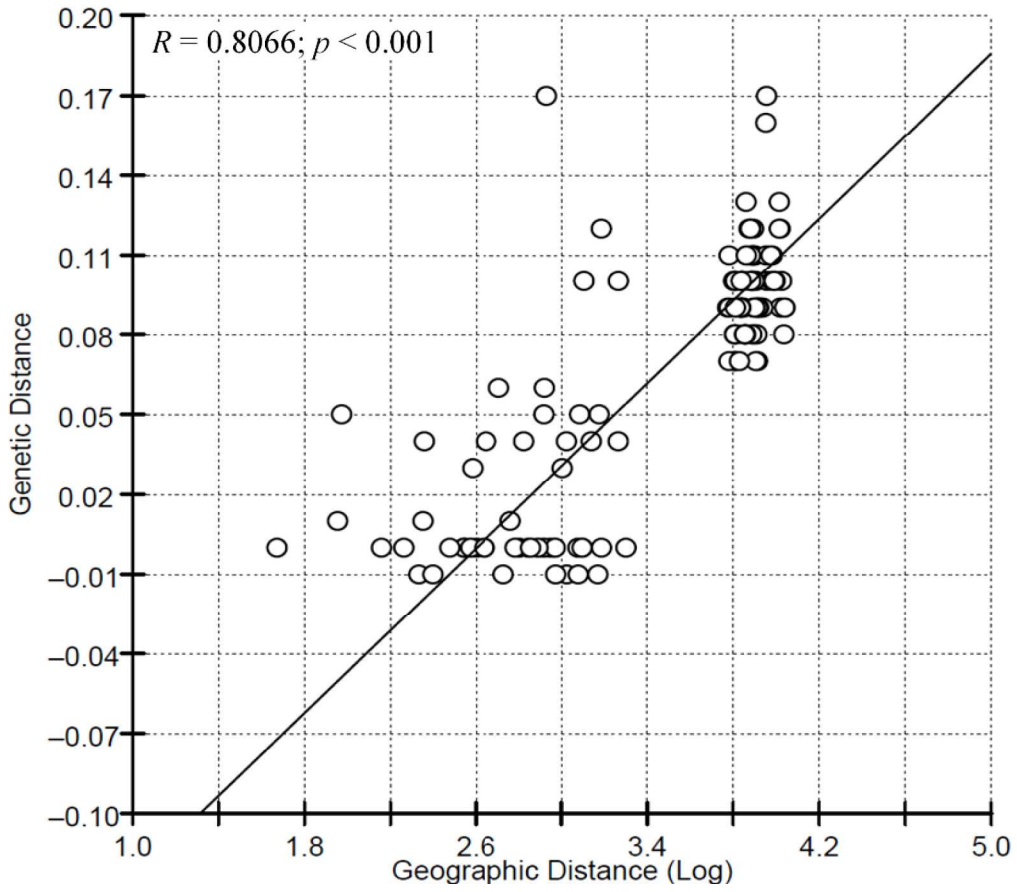
Table 2.6 - Pairwise F_{ST} as generated by FSTAT, geographic distance by least cost path between populations in km (i.e. shortest distance over water), bold indicates F_{ST} values that are not significantly different than 0; Colors indicate the type of pairwise relationship: Blue: Morocco and South Africa; Purple: Brazil and South Africa; Grey: Brazil and Morocco; Green: Only South Africa; Orange: Only Brazil; Red: Only Morocco

	SF Balito	RS Cassino	SF Muizemberg	SC Brava	RJ Macae	MR Essaouira	SF Hermanus	MR Imsouane	MR Mirleft	SF PortAlfred	MC PuntaOuro	SF PortEduard	SF Plettenberg	PR Matinhos	RS Atlantida	SC palhoca	SP itanhanhem	ES vitoria
	BAP48	EA32	EB11	EC38	ED36	ES30	HMP48	IM30	ML30	PAP48	PDOP30	PEP48	PLP48	PR143	RS119	SC34	SP192	VIT44
																	Geographic	Distance
BAP48	0	7964	1535	7835	7473	10603	1500	10550	10452	668	384	228	1056	7934	7815	7836	7797	7630
EA32	0.1002	0	6490	702	1537	8554	6523	8500	8408	7247	8258	7687	6916	891	350	637	1060	2000
EB11	**0.1069**	0.082	0	6400	6040	9011	94	8949	853	835	1844	1275	510	6484	6410	6374	6340	5940
EC38	0.1008	**-0.0024**	**0.0725**	0	925	7962	6384	7893	7810	7124	8156	7584	6786	183	350	47	400	1250
ED36	0.1096	**0.0044**	0.1006	**0.0023**	0	7000	6044	6947	6877	6781	7845	7233	6456	829	1196	937	610	350
ES30	0.0917	0.0822	0.1472	0.0796	0.0898	0	9129	90	225	9883	10971	10333	9566	8045	8180	8053	7775	7035
HMP48	0.0503	0.068	0.052	0.0672	0.0801	0.0954	0	9059	8957	780	1841	1218	437	6497	6446	6422	6410	6030
IM30	0.0814	0.0797	0.1389	0.0776	0.0893	**0.0079**	0.0924	0	144	8664	10951	10440	9541	7784	8122	7997	7715	7000
ML30	0.0853	0.0836	0.1484	0.0802	0.0916	**0.0081**	0.0962	**-0.0026**	0	9785	10847	10345	9460	7694	8027	7898	7616	6920
PAP48	0.036	0.073	**0.0546**	0.0733	0.0844	0.0906	**0.0028**	0.0801	0.0895	0	1009	444	375	7216	7154	7136	7135	6742
PDOP30	**0.0249**	0.0811	0.0888	0.0831	0.0964	0.0817	0.0379	0.0814	0.0775	0.0324	0	578	1378	8286	8240	8205	8170	7941
PEP48	0.0348	0.0972	0.0922	0.0996	0.116	0.1136	0.0445	0.1088	0.1087	0.0417	**0.0099**	0	830	7285	7589	7616	7573	7263
PLP48	0.0412	0.0854	0.0581	0.0856	0.0936	0.102	**0.0018**	0.0931	0.0984	**-0.0018**	0.0359	0.0473	0	6868	6835	6829	6800	6456
PR143	0.1019	**-0.0043**	**0.0898**	**-0.0043**	**-0.0017**	0.0746	0.0713	0.0767	0.0765	0.0781	0.0832	0.1011	0.0885	0	536	215	250	1200
RS119	0.103	**-0.0001**	0.0767	**-0.0048**	**0.002**	0.0672	0.0673	0.0698	0.0687	0.0743	0.0796	0.0978	0.0825	**-0.0076**	0	300	720	1480
SC34	0.1035	**-0.0027**	0.0888	**-0.0022**	**0.0013**	0.0894	0.0707	0.0847	0.09	0.0722	0.0852	0.1075	0.0839	**-0.0085**	**-0.0018**	0	437	1250
SP192	0.1026	**-0.006**	**0.0877**	**-0.003**	**-0.0039**	0.089	0.0647	0.0938	0.0936	0.0729	0.0852	0.1038	0.0799	**-0.0113**	**-0.0046**	**-0.0044**	0	942
VIT44	0.0939	**-0.0004**	0.0791	**-0.0033**	**-0.0017**	0.081	0.0655	0.0807	0.0815	0.0693	0.0789	0.0971	0.0792	**-0.0063**	**-0.0055**	**-0.0037**	**-0.0069**	0
F_{ST}	BAP48	EA32	EB11	EC38	ED36	ES30	HMP48	IM30	ML30	PAP48	PDOP30	PEP48	PLP48	PR143	RS119	SC34	SP192	VIT44



1292 **Figure 2.6 - Regional comparisons of pairwise F_{ST}. Were BR=Brazil, WSA=West South Africa, ESA=East South Africa, MR=Morocco.**

1294



1296 **Figure 2.7 - Isolation by Distance. Mantel test by genetic distance (F_{ST}/(1-F_{ST}) and log of shoreline distance between sampling locations (km).**

Table 2.7 - Analysis of Molecular Variance: South Africa x Brazil x Morocco.

Source of Variation	d.f.	Sum of squares	Variance components	% variation
Among Groups	2	30.36	0.0349va	4.16
Among Populations within Groups	15	22.67	0.0101vb	1.21
Within Populations	1266	1002.79	0.7921vc	94.62
Total	1283	1055.82		-
	Global F_{st} 0.053			p-value 0.000

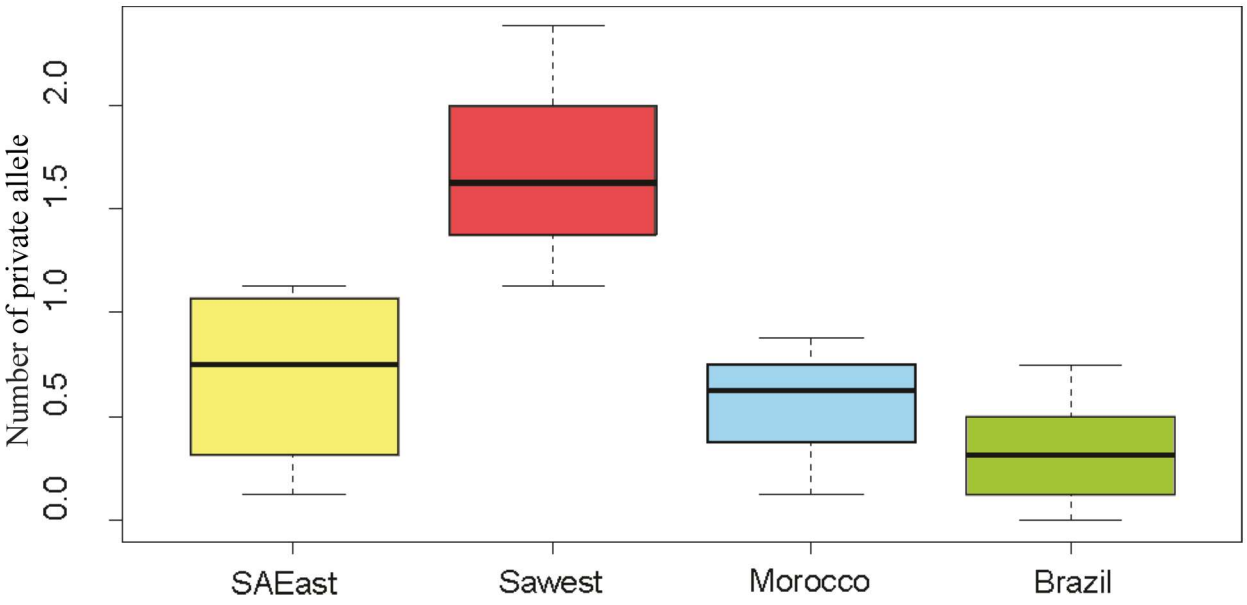


Figure 2.8 - Boxplots represent the number of private allelic genotypes patterns across regions.

Discussion

Our genetic assessment uncovered four genetic clusters (east South Africa, west South Africa, North Africa and Brazil) of *Perna perna* from the three regions evaluated in our study. Contrary to recent studies published that used mtDNA haplotype diversity (Cunha *et al*., 2014, Oliveira *et al*., 2017) to evaluate the evolutionary history of *P. perna*, our microsatellite analyses revealed that South American *P. perna* populations exhibited the characteristics of genetic

structure indicative of an established, isolated, and native population. Here, we discuss how our microsatellite results provide an interesting contrast with previous studies, how contrasting patterns of genetic diversity can arise, and how this provides insights into the evolutionary history of marine bioinvasive species.

The literature on the South American origin of *Perna perna* is complex. Different studies have reached different conclusions, even when using the same genes to estimate divergence dates between old world and new world populations (e.g. Wood, *et al.,* 2007; Pierri *et al.,* 2016). Various studies have found evidence to support the new world origins of *P. perna* having arisen from ancient natural dispersal, recent natural dispersal, and recent anthropogenic dispersal. Wood *et al.,* (2007), argued for an ancient natural origin via assessment of mtDNA variation supported by their conclusions that the occurrence of the most recent common ancestor of *P. perna* and *P. canaliculus* was likely to have occurred on the American continent during the middle to late Miocene (11-14 Ma ago) (Wood *et al.,* 2007). In contrast to this ancient origin, Cunha *et al.* (2014) analyzed mtDNA COX I variation between *P. perna* from South America and Africa and concluded that *P. perna* arrived in South America via a recent natural dispersal event, with the South American lineage originating an estimated 10 ka ago, with a range of 0 – 40 ka ago, from northern Africa. Complementarily to these genetic studies, carbon dating (C^{14}) of shells in Brazilian shell middens suggested that populations of *P. perna* were present as long ago as 720 ka ago (Pierri *et al.,* 2016). However, recent anthropogenic dispersal has also received a lot of attention and support with the apparent absence of *P. perna* records in Amerindian shell middens from Rio de Janeiro state dated to between 2 and 8 ka ago (Souza *et al*., 2003). Moreover, allozymes molecular markers have suggested that the Brazilian populations of *P. perna* were probably founded from multiple independent introductions from African stocks (Moura-Neto, 2003; Oliveira *et al*., 2017). It is from these studies that the idea of a recent introduction, via the human slave trade, brought *P. perna* to South America. However, our data do not provide support

for this hypothesis. Indeed, our data show levels of genetic diversity nearly equal among all regions (i.e. no evidence of a recent founding bottleneck or a mixed from different source), striking differences between South America and all African populations via Bayesian clustering, comparable levels of genetic differentiation among all regions, and an overall pattern of isolation-by-distance. Overall, our interpretation of these results is that they are entirely consistent with the description of Brazilian *P. perna* populations being a long-established population in this region.

The inference of an ancient origin of *P. perna* surmised in this study begs the question of why our study and previous studies have come to such a variety of conclusions regarding the new world origins of *P. perna*. There are multiple reasons for why such contrasting inferences have been made. First, the variety of methods used among different studies can be a root cause for arriving at different conclusions from studies of the same species. Historically, mtDNA studies would provide the data used to make conclusions relative to the evolutionary history of a taxa. However, mutation rate differences between mtDNA and microsatellite markers, with microsatellites evolving more quickly, suggested that microsatellite studies likely provided more accurate patterns of contemporary gene flow. Moreover, next generation sequencing techniques enabling whole genome sequencing will likely be able to provide the most accurate data for the interpretation of evolutionary history (Cristescu, 2015). The second and third reasons why studies could arrive at such contrasting results would be differences in sample size and sample locations among studies, as reviewed in Muirhead *et al.* (2008). With *P. perna*, for example, Wood *et al.* (2007) used relatively few samples from few localities with broader species coverage than Cunha *et al.* (2014) (who used more samples from Africa) or Oliveira *et al.* (2017), (who employed a larger sampling scheme from throughout South America). In general, different studies that utilize different subsets of sampling are more capable of arriving at different conclusions, than studies that incorporate large-scale sampling. Fourth, the degradation of physical materials (e.g. from shell middens) can also lead to contradictions of results. Although fossil ancient samples or even

fossils can be used as clear indicators of presence (or calibration for molecular dating), the absence of such physical evidence needs to cautiously be used to provide evidence for species absence. Specifically with regard to this study, one explanation for the absence of *P. perna* from shell middens is that natural degradation is more likely to impact the fragile shells of *P. perna* more so than hardier shells of oysters or cockles found in the same area (Pierri *et al.,* 2016, apud Kneip, 1994). Similarly, degraded shells are difficult to identify within archeological sites and researchers have made errors in identifying the actual species of shell fragments and the age of those fragments (Souza *et al.* 2003). An ideal approach incorporates better geographic representation with good sampling, the use of a more informative gene markers, and the presence of verifiable physical evidence (Hartl & Clark, 1997, Davies *et al.*, 1999).

Implications related to whether *P. perna* is defined as native versus invasive along the South American coast may influence public policies for biological invasions and management of aquaculture production (Pérez *et al*., 2007; Lopes *et al*., 2009; IAP, 2009, 2015). Given that Brazil has considerable mariculture production (88,967 tons in 2010), and an expectation that the total aquaculture production will grow by 104% from 2012 to 2025 (FAO, 2012), the primary need and concern is with the development and consolidation of regulations that may impact aquaculture with licenses and regulations by the public policies (Ostrenky & Borghetti, 2007). When we consider that *P. perna* is one of the main cultivated organisms, just after fish (tilapia, carp, and tambaqui) and white shrimp (*Litopenaeus vannamei*) (FAO, 2012), then policy changes may have a great impact on the future expansion in *P. perna* aquaculture considering it an exotic invasive or a main native resource. Especially if we consider that non-native species should be subject to management and control (CDB, 1992; Souza *et al.* 2009; Machado *et al.* 2009). This case, highlight the need for the discussion of the usage and interpretation of scientific information through the development of social policies and environmental management with interaction with academic findings. Public policies have already listed *P. perna* as non-native, but this non-native

1386 status should be subject to management and control according the most up-to-date scientific findings. If these findings are unclear, then they warrant further study to assess the species status. 1388 Regardless of the control of non-native species, our study shown that *P. perna* appears to be a native species and it is well to consider that it should not be impacted by public policy regulating 1390 invasive species.

Although previous studies have suggested that *P. perna* was likely introduced to South 1392 America just before historical records were kept, likely associated with the human slave trade from Africa, our data fail to validate this hypothesis. Considering not only one and not in only one 1394 place authors have described *Perna perna* on sambaquis (Piazza, 1969; Hurt, 1974; Rohr, 1984; Kneip, 1994); Wood *et al*. (2007) have found a 170kyr divergence in COI; Cunha *et al.* (2014) 1396 have estimated the Most Recent Common Ancestor - MRCA between *P. perna* in Brazil and Morocco to be at 10 [0-40] kyr; Pierri *et al*. (2016) have estimated with Carbon-14 dating shell 1398 older than 690 years; and the present study found with microssatelites that Brazilian population presents a divergent (FCA), separated (Structure), genetically distance to others populations 1400 (pairwise), with similar genetic diversity (He, Ar, table 4), with an overall pattern of isolated by distance (IBD) and presence of uniqueness genetic characteristics in Brazil. All of these are 1402 indicatives that South American *P. perna* are indistinguishable from an established population and exhibiting genetic characteristics usually expected for native populations. Considering all this 1404 together, it is plausible to assume that the set of evidence we can gather with previous studies such as those found in this evaluation leads us to reject the hypothesis that the *P. perna* was 1406 brought to Brazilian coast by the slave rout navigations. This would only not be real if the mutation rates in Brazil population be very high or a bottleneck effect connects Brazil population 1408 to another African population not sampled in this study. With the vast number of marine bioinvasions, genetic studies have provided power tools to help evaluate source populations for 1410 species of unknown origin (Blakeslee *et al.,* 2008). As more studies incorporate better tools and

more thorough sampling, population genetic and genomic research proves to inform public policy and management decisions as more species with unknown origins continue to be resolved.

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Final Considerations

In the past few decades, with the advent of molecular genetic techniques, it has become possible to address questions associated with the evolutionary history of non-model species. In this thesis, I used different molecular markers to answer similar questions associated with native versus invasive populations revealing their characteristics of invasiveness and their origin. Specifically, I used mtDNA to identify potential source populations of the southeastern United States invasion of *Mytella charruana* and microsatellites to address whether Brazilian populations of *Perna perna* exhibit genetic patterns associated with a recently founded invader or a long-standing natural population. The general methods applied here was useful to investigate evolutionary processes and identify potential dispersal pathways for invasive species as for conservation biology. Genetic patters between populations allow us to understand species patterns of divergence and evolutionary relationships among species and populations, within-species diversity, and also rates and patterns of migration which helps unravel the evolutionary history of species and their populations. Overall, this study contributes to the general knowledge we have on the population genetics of molluscs and our understanding of the management of invasive species. In the broader aspect, my thesis contributes to the description of basic processes of genetic drift and gene flow, and how they relate to the genetic structure of natural populations and invasive species using two species of mussel as models. This study also affirms how these biological characteristics may have changed associated with the human maritime trade activities.

The oceans are known for their continuities and connectivity, allowing circumnavigation, which could be a viable factor to break the physical barriers for dispersal of marine organisms. However, studies such as the present one, have shown that organism dispersal is also related to the biological characteristics of the species, their evolutionary history and their ability to interact with environmental variations. For both models species in the studies characterized here it was possible to

observe a clear genetic structuring in the native populations evaluated; high genetic variation in the markers used and population subdivisions.

Marine bivalves are recorded as invasive organisms in various parts of the world (Streftaris & Zenetou *et al.*, 2006; Lourenço *et al.*, 2012) influencing the structure of local ecological communities (Galimany *et al.*, 2016). My thesis helped our understanding of the evolutionary history and dispersal patterns of the mussels *Mytella charruana* and *Perna perna*. In this way, we could better understand the ecological and biogeographic scenarios using the genetic composition of each species.

This thesis identified traces of the origin of population from both evaluated species. I looked for the signs of connectivity between populations considered invasive and native. Following that, my findings indicated differences in genetic structure, identified differences in genetic diversities and provided insights into the spread, persistence and development of invasive species. In Chapter 1, with a genetic characterization of large area in natural occurrence extension was possible to identify evidence of admixture in the invasive population with a native population as the most probable main source. We identified that over time the invasive population did not undergo modifications in its genetic composition, despite a fluctuation population sizes. Among other characteristics it was possible to understand that *M. charruana* has an extreme ability to survive environmental variations, even in new environments, maintaining its high genetic variability. While in Chapter 2 it was possible to clarify the questions of the origin of *P. perna* mussel on the coast of New World in which it was a natural dispersion or a recent unintentional introduction by navigation activities. Despite several indications that may lead to believe that this species would be a recent invader, we have identified that South America populations has its own genetic characteristics, similar to those natural established population. All regions evaluated were equally genetically differentiated from each other and without interaction, with the data entirely consistent with Brazilian populations of *P. perna* being a long-established and isolated group.

Uncovering the evidence of the origin of the studied mussels we observe an interesting contrasting dispersal patterns between *M. charruana* and *P. perna*. Where *M. charruana* presented a high level of genetic variance between regions, while the genetic divergence of *P. perna* was more representative within regions; besides the variance, both species presented conspicuous genetic differentiation between natural regions, with high level of diversity. The genetic traits that could identify the origin of the population was only found with *M. charruana* whereas with the *P. perna* it was only possible to corroborate that the population of South America is descended from African populations. With all these, our data provide insights illuminating new possibilities for studies of understanding genetic differentiation and reconstruction of evolutionary history from throughout the native range and into the spread and persistence of invasive species.

The use of different methodologies to elucidate similar questions to unravel the origin of the non-native population provides interesting insights into how different methodologies can provide differing conclusions, even if the overall results seem to suggest similar genetic patterns. However, the two chapters presented in this thesis highlight the contrast on interpretation of genetic data. In Chapter 1, there was no doubt about the exotic status of *Mytella charruana* in SE-US, and the use of a slowly evolving genetic marker should not be a problem for identify the allelic composition, On the o ther hand, it would not be appropriate to observe changes on the genetic composition in just a few years generated by mutation or selection. However, when the question on issue is to evaluate whether population densities fluctuations over time is influenced by intermittent events of populations declines followed by the constant re-introduction of new mussels it should not be a problem to use a slowly evolving genetic marker. In Chapter 2, the oringin of *Perna perna* is unknown, being considered cryptogenic species on the new world. Previous studies have already used slowly evolving genetic markers (e.g. COI) but results were not conclusive on the definition of its status in Soulth America. The use of a more variable genetic marker (i.e. higher mutation rate) has aleady identify possible multiple source introduction on the *Perna perna* invasion on the Gulf of Mexico (Holland,

2001). Thus, a more accelerated genetic marker (i.e. microsatelities) was used here on the attempt to offer any indicative of the South American population formation. On the present case the more variable genetic marker identified that the South American population was constituted of a genetic composition correspondent or expected for a native population.

The results generated in these assessment have the potential to influence public policy with regard to the environmental management of biological invasions and for aquaculture production of marine taxa of cryptogenic origin. Exciting and productive advances in ballast water and in environmental management can be reached if population biology and the coastal management are more integrated, especially with regards to biological invasion. The knowledge that *M. charruana* is able to withstand environmental variations and to recompose its population with high diversity even without constant propagule pressure and repeated reintroductions, raise concerns for the monitoring of the dispersal of species among different estuaries in the USA. Additionally, if these regions wish to fight off secondary introductions, such knowledge becomes useful for the Caribbean regions that receive transatlantic ships from Florida. While with the recognition that Brazilian populations of *P. perna* are likely native, it may be necessary to re-evaluate the list of recognized invasive species in Brazil.

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