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Filogeografia e diversidade genética de *Lineus sanguineus* (Rathke, 1799) e *Prosorhochmus belizeanus* (Maslakova, 2008) na costa brasileira

Phylogeography and genetic diversity of *Lineus sanguineus* (Rathke, 1799) and *Prosorhochmus belizeanus* (Maslakova, 2008) on the Brazilian coast

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GENERAL INTRODUCTION

1.1 NEMERTEANS

The phylum Nemertea is part of the Lophotrochozoa, as supported by several phylogenomic studies (Dunn et al., 2008; Struck and Fisse, 2008; Hejnol et al., 2009; Kocot et al., 2017; Laumer et al., 2019), showing classical characteristics of the spiralian cleavage in their early development (Henry et al. 1998). Characterized by its eversible proboscis, this group contains approximately 1,300 described species (Kajihara et al., 2008). Their soft-body is unsegmented and the representatives are found in a variety of sizes, from the longest metazoan recorded, *Lineus longissimus*, which can measure more than 30 m (McIntosh, 1873–1874), to small organisms from meiofauna, such as *Ototyphlonemertes* and *Cephalothrix* genera (Norenburg, 1988). These animals are mostly marine and occur in almost all benthic ecosystems, but conquered successfully the terrestrial environment. Due to their carnivore or scavenger habits, the animals from the phylum Nemertea can have a strong impact on their ecosystems (Kuris, 1991; Thiel, 2001). Currently Nemertea is composed by three main classes: Pilidiophora, Hoplonemertea and Palaeonemertea (Figure 1) (Andrade et al., 2014, Strand et al., 2019). Some Palaeonemertea and Hoplonemertea representatives can have offsprings with direct development characteristics, such as juvenile-like appearance and minor or none metamorphosis events (Maslakova and Hiebert, 2015). In contrast, the Pilidiophora clade,

composed by the orders Heteronemertea and Hubrechtiformes, has a very distinct developmental strategy: the unique pilidium larvae (Thollessen and Norenburg, 2003; Maslakova, 2010). This type of larvae seems to be an innovation from this clade and its characterized as a long-lived and hat-shaped planktotrophic larvae (Giribet, 2020). An important aspect of this larvae is its distinctive and rapid metamorphosis, where the juvenile erupts and feeds from the larval enclosure (Lacalli, 2005; Maslakova, 2010).

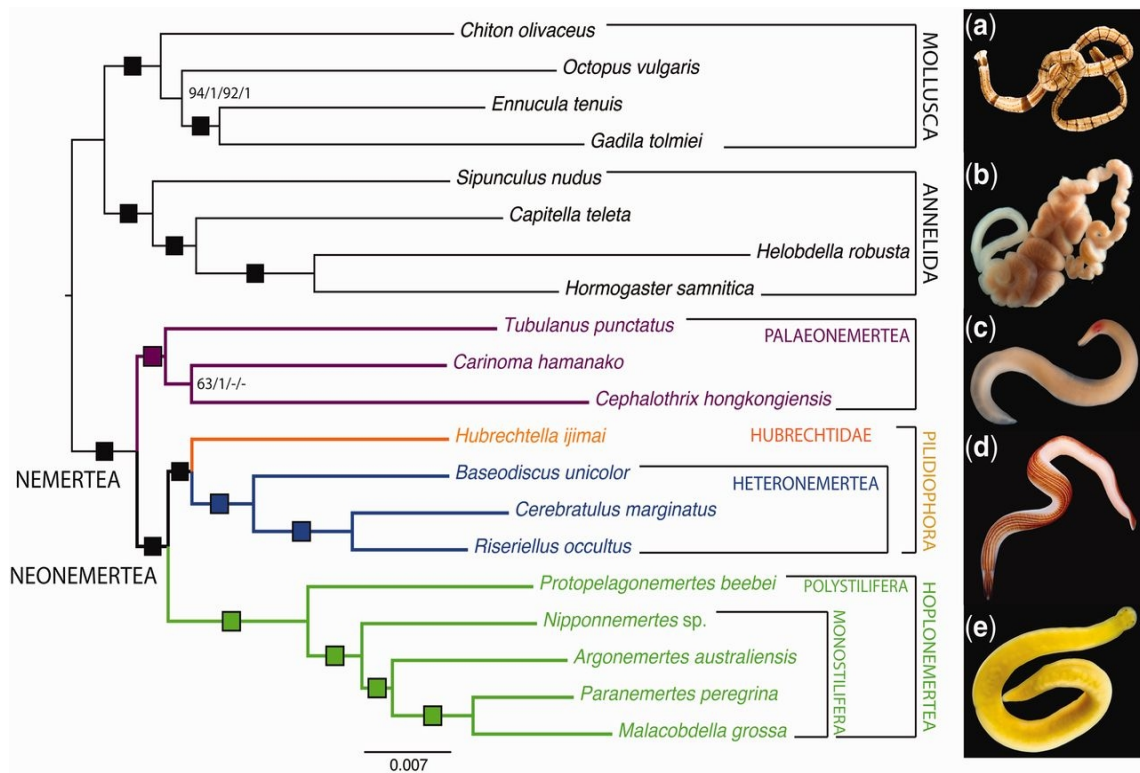


Figure 1: Nemertean phylogenetic hypothesis from Andrade et. al, 2014. Nemertean main lineages shown in color/shades. Photos are of representatives of the different lineages: (a) Palaeonemerteza (*Tubulanus rhabdotus*), (b) Hubrechtidae (*Hubrechtella ijimai*), (c) Heteronemerteza (*Cerebratulus leucopsis*), (d) Hoplonemerteza, Polystilifera

(*Drepanophorus spectabilis*), and (e) Hoplonemertea, Monostilifera (*Tetrastemmatidae* sp.).

The nemertean species *Lineus sanguineus* (Rathke,1799) and *Prosorhochmus belizeanus* (Maslakova et al, 2008) present distinct developmental modes and distribution on Brazilian coastline, being interesting representatives to study nemerteans genetic diversity and phylogeography along the Brazilian coastline. *Lineus sanguineus* is a cosmopolitan heteronemertean species that occurs in South and North America, Europe, Asia and Oceania (Figure 2, Coe, 1943; Riser, et al. 1994; Runnels, et al. 2013; Ament-Velásquez et al. 2016; Xu and Sun, 2020; Zattara et al. 2022; Figure 2). Until recently, it was thought to reproduce exclusively asexually, due to their ability to reproduce by fissiparity (Caplins and Turbeville, 2011). Their larvae was reported by Sagorny and Döhren (2022), explaining the high connectivity found on diversity studies on this species so far (Runnels, 2013; Ament-Velasquez et al 2016). *Lineus sanguineus* have a slender and slightly dorsoventrally flattened body ranging from 0.5 to 20 cm in length (Coe 1943; Riser 1994) (Figure 3A). They present long cephalic grooves along each side of the head, two to eight pairs of ocelli and body colors varying from olive, red, green and brown, which is suggested to be a consequence of their diet (Coe 1943; Riser 1994) (Figure 3B).

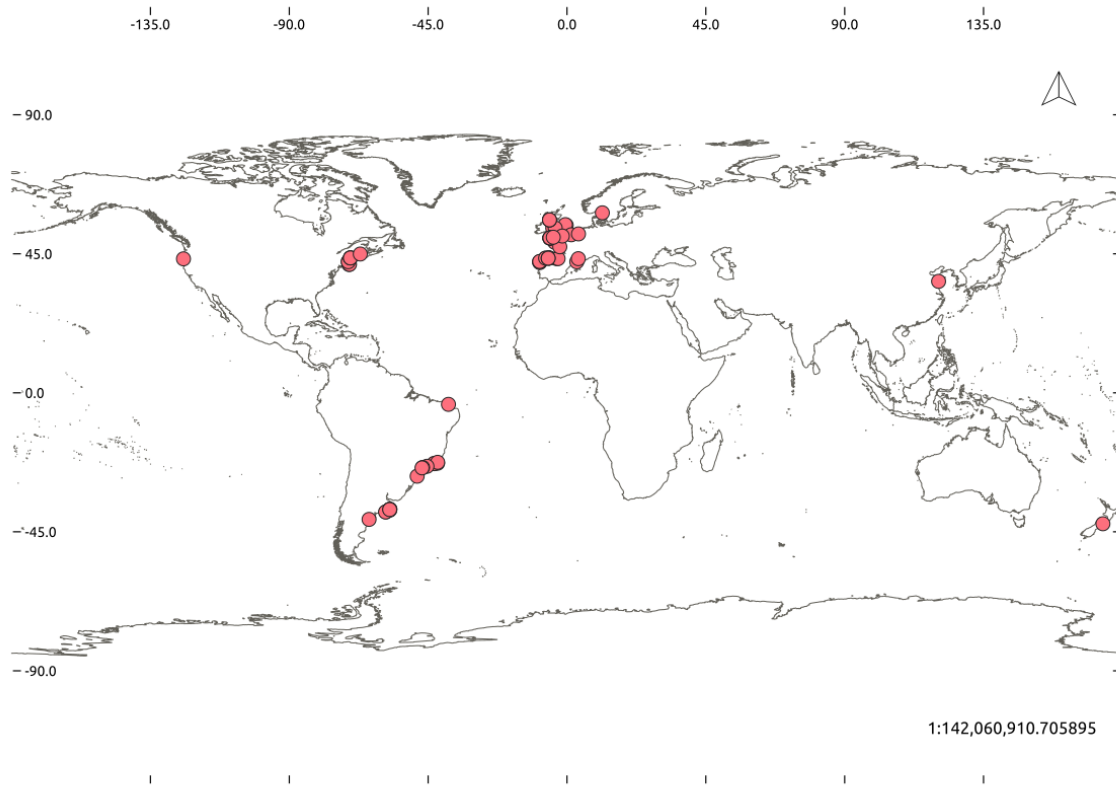


Figure 2: *Lineus sanguineus* world distribution (pink dots). Scale used is numeric, where 1 cm of the map represents 142,060,910.7 cm (1,420.609 km) in the real landscape. Data obtained from GBIF, Ament-Velásquez et al. (2016) and Xu and Sun, (2020).

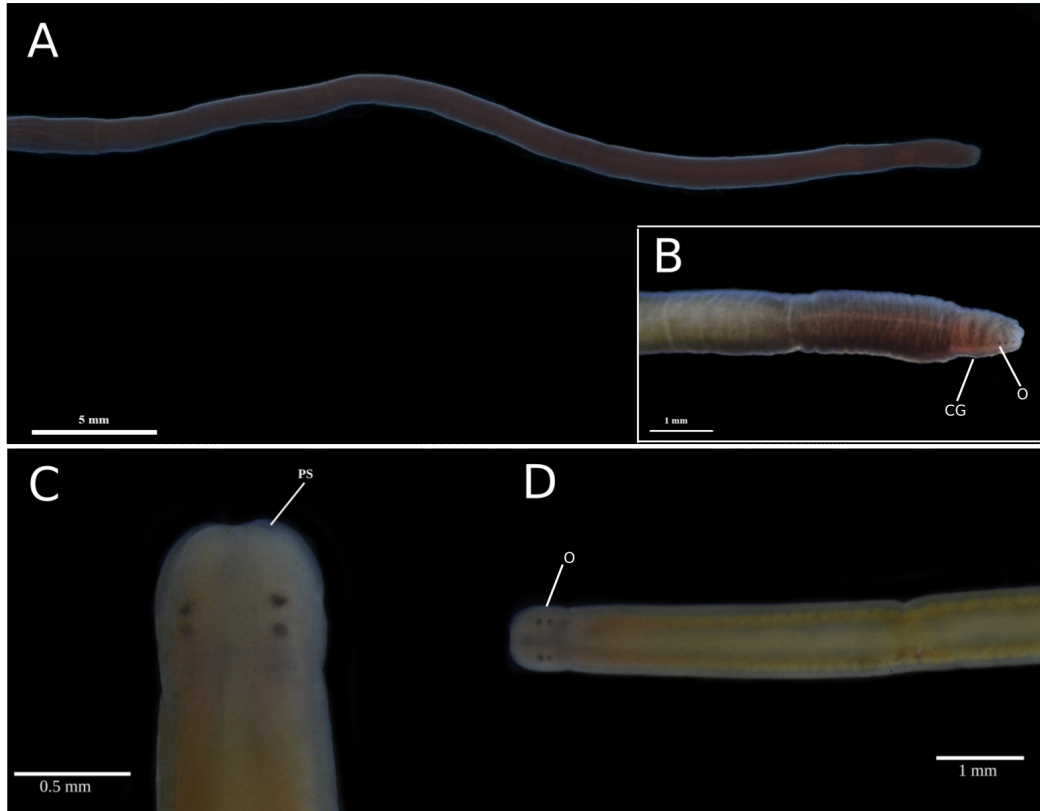


Figure 3: Photomicrographs of *Lineus sanguineus* and photomicrographs of *Prosorhochmus belizeanus*. A: *Lineus sanguineus* dorsal view of an adult. B: *Lineus sanguineus* dorsal view of anterior region detail, showing the pairs of ocelli (O) and cephalic grooves (CG). C: *Prosorhochmus belizeanus* anterior detail on dorsal view showing prosorhochmid “smile” (PS). D: *Prosorhochmus belizeanus* dorsal view showing the two pairs of ocelli (O). Images from Cecili B. Mendes.

On the other side, *P. belizeanus* are hoplonemertean described as oviparous and gonochoric (Maslakova et al., 2008). These animals are only recorded for North and Central Atlantic (Figura 4, Maslakova et al., 2008) and present the prosorhochmidae

smile, a characteristic of their family, Prosorhochmidae, attributed due to a dorsal epidermal fold on the bilobed head of their members (Figure 3C and D). To the best of our knowledge, there is no study so far on the genetic diversity and phylogeography of this species.

Lineus sanguineus as well as *P. belizeanus* live on intertidal regions of rocky shores, being found among oysters, mussels and barnacles (Maslakova et al., 2008; Hiebert and Hunt 2015). A deep knowledge of genetic diversity of both species can be of great value to understand nemertean and rocky shore invertebrates evolutionary biology.

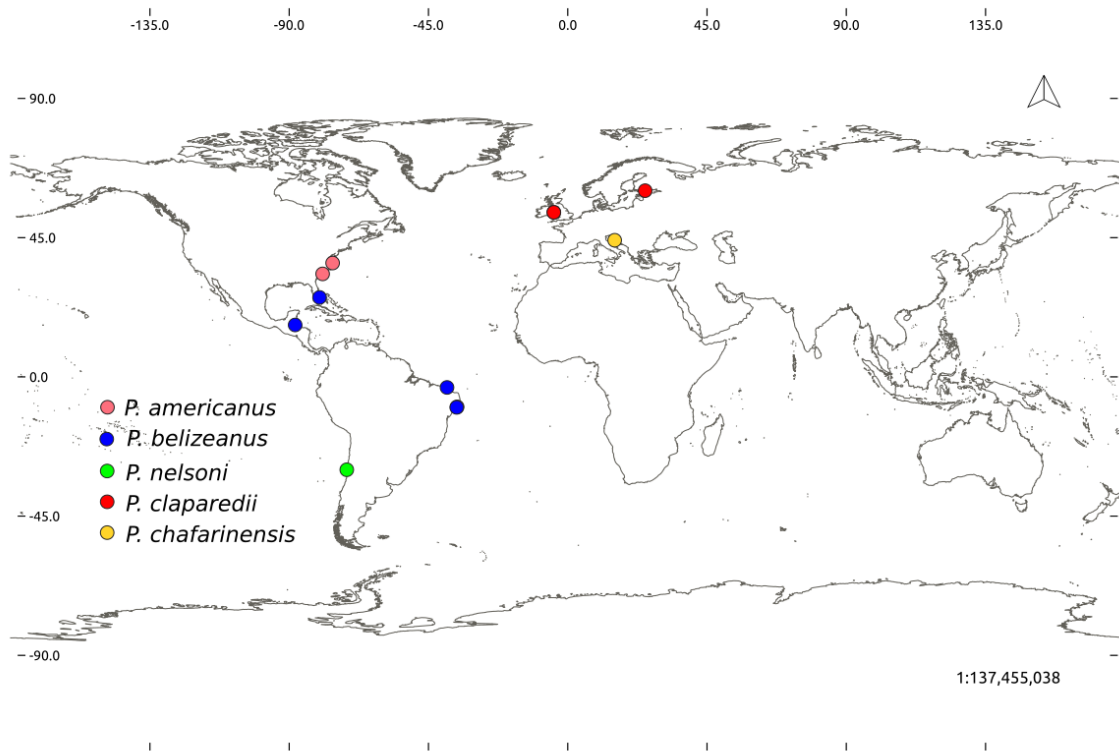


Figure 4: *Prosorhochmus* genus world distribution. *P. americanus* is represented by pink dots, *P. belizeanus* by blue dots, *P. nelsoni* by green dots, *P. claparedii* by red dots and *P.*

chafarinenses by yellow dots. Scale used is numeric, where 1 cm of the map represents 137,455,038 cm (1,374.55 km) in the real landscape. Data obtained from GBIF.

1.2 PHYLOGEOGRAPHIC STUDIES IN MARINE ENVIRONMENT

Geographical and demographical factors are decisive to understand dispersion and genetic connectivity among organisms. On terrestrial environment, the geographical barriers are determinant to dispersion patterns, gene flow and speciation. On marine environment such boundaries are subtle, varying according to species or groups (Martins et al., 2022). For such environment, ecological limits, life traits and developmental characteristics of organisms can play an important role on species evolutionary history and diversification (Kinlan et al., 2005; Bowen et al., 2013).

A prevalent and highly debated hypothesis is that lower levels of genetic differentiation should be found between populations of species with high planktonic larval duration (Riginos and Liggins, 2013; Haye et al., 2014). Many studies have shown brooders and species with short-lived planktonic larvae with low gene flow and higher differentiation among populations (Weersing and Toonen 2009; Kelly and Palumbi 2010; Riginos and Liggins, 2013; Haye et al., 2014). Nonetheless, there are some studies showing poor and weak relation between planktonic larval duration and genetic differentiation among populations (Weersing and Toonen 2009; Riginos et al. 2011;

Selkoe and Toonen 2011; Riginos and Liggins, 2013). Therefore, the relationship between larval duration and genetic differentiation is still in active debate, and more studies on this area can help to elucidate these diversity patterns.

Besides the advantages of genetic approaches as a way to understand demographic processes and gene flow dynamics in marine systems, those methods can still exhibit many challenges, such as difficulties in markers development and difficulties of the theoretical models to explain the data (Selkoe et al., 2008). Among the different available markers, presenting relatively rapid evolution and therefore a high level of polymorphism, the mitochondrial DNA (mtDNA) is commonly used for demographic history analyses (Uthicke and Benzie, 2003; Lee and Boulding, 2007; Crandall et al., 2007; Baker et al., 2008; Liu et al., 2012). mtDNA has a smaller effective size, and for this reason it is expected to be more strongly affected by genetic drift, showing a greater degree of population structure when compared to nuclear loci (Avice, 1994; Karl et al., 2012).

Another common marker used on phylogeography and population genetics study is the internal transcribed spacers (ITS), which lies between the 18S, 5.8S and 28S subunits (Källersjö et al., 2005). This region evolves very fast, due to their non-coding nature and low selection pressure, accumulating substitutions very quickly. It can be very useful for distinguishing between closely related species or intrapopulation studies

(Cruickshank, 2002). Then, the association with ITS and mtDNA markers can be a powerful tool to unravel phylogeographic and demographic patterns.

Molecular markers can be used on biodiversity studies in species delimitation analyses (Gaytán et al., 2020; Magoga et al., 2021). Such analyses are composed of different methods and processes for determining if the sampled individuals belong or not to the same species (Queiroz, 2007). Currently, species delimitation methods based on molecular data rely on (1) distance estimates, such as the Automatic Barcoding Gap Discovery (ABGD; Puillandre et al., 2012), and the Assemble Species by Automatic Partitioning (ASAP; Puillandre et al., 2020); and (2) phylogenetic and coalescent-based data, such as the Generalized Mixed Yule Coalescent model (GMYC; Fujisawa and Barraclough, 2013; Pons et al., 2006), GMYC Bayesian implementation (bGMYC: Reid and Carstens, 2012), the Poisson Tree Process (PTP; Zhang et al., 2013) and PTP multi-rate extension (mPTP; Kapli et al., 2017).

Along with methods using genetic markers to elucidate phylogeographical and population genetics patterns, there are also some tools that rely on ecological and geographical major changes in the planet. Ecological Niche Modelling (ENM) analyses, for example, combine ecological and distribution data to predict the present, past and future potential distribution of the species (Rödder, 2010; Valencia-Rodríguez, 2021).

1.3 OBJECTIVES

The main objective of the present study was to characterize the unknown genetic diversity and phylogeographical patterns of *L. sanguineus* and *P. belizeanus* along the Brazilian coast, contributing to the knowledge from Brazilian rocky shores environment genetic diversity. For such general objective we defined two specific objectives:

- 1) Estimate the intra and inter population genetic diversity of the two species (*L. sanguineus* and *P. belizeanus*) in order to understand their population structure and phylogeographic distribution.
- 2) Unravel the migration and demographic history of the two species.

1.4 DISSERTATION STRUCTURE

This manuscript starts with a general introduction about the context and main concepts of the project, followed by two chapters. The first chapter is about *L. sanguineus* phylogeography, genetic diversity, population patterns and paleodistribution. The second one is about the first record of the genus *Prosorhochmus* in Brazil and geographical expansion of *P. belizeanus*, discussing the species genetic diversity and migration patterns. The manuscript is concluded by some final considerations summarizing the study main discoveries.

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5. RESUMO

As espécies de nemertinos *Lineus sanguineus* (Rathke,1799) e *Prosorhochmus belizeanus* (Maslakova et al, 2008) vivem em costões rochosos em ambientes entremarés e apresentam distribuição distinta na costa brasileira. Nesse estudo, pesquisamos a filogeografia dessas duas espécies na costa brasileira. Para *L. sanguineus*, usamos as sequências moleculares da subunidade I do citocromo c oxidase (COI), RNA ribossomal 16S (16SrRNA) e o espaçador interno transcrito I (ITS-1) de indivíduos de nove localidades. Nas redes de haplótipos e árvore filogenética não foi observado nenhum padrão geográfico, sendo que o ITS-1 apresentou maior diversidade intrapopulacional que 16SrRNA e COI. A análise de AMOVA evidenciou estruturação genética moderada, com a maior parte da variação genética contida dentro das localidades. F_{ST} par a par mostrou alta estruturação genética entre o Nordeste e algumas localidades do Sul e Sudeste, um padrão que sugere papel importante das correntes oceânicas brasileiras na conectividade dessa espécie. Esses resultados indicam que a reprodução sexual e a dispersão larval tem papel central na conectividade dessas populações, em conjunto com variáveis ambientais. Nesse trabalho, também foi apresentado o primeiro registro de *P. belizeanus* no Brasil em conjunto com a análise da diversidade genética dessa espécie nessa região. Usando COI e 16SrRNA, não encontramos evidência de estruturação genética, padrão geográfico e sinais de retração ou expansão demográfica, indicando a colonização da costa brasileira por um número pequeno de linhagens mitocondriais. Entretanto, as análises de migração evidenciaram certa influência da Corrente Brasileira na dispersão dessa espécie. Esses resultados em conjunto aumentam o conhecimento e ajudam o entendimento sobre filogeografia e diversidade genética de invertebrados marinhos de costões rochosos no Brasil.

6. ABSTRACT

The nemertean species *Lineus sanguineus* (Rathke, 1799) and *Prosorhochmus belizeanus* (Maslakova et al, 2008) live in intertidal environment of rocky shores and have distinct distribution on Brazilian coastline. In this study, we survey the phylogeography of the two species along the Brazilian coast. For *L. sanguineus* we used the molecular markers of cytochrome c oxidase subunit 1 (COI), 16S ribosomal RNA (16SrRNA) and Internal transcribed spacer 1 (ITS-1) of individuals from nine localities. No clear geographic patterns was found in the haplotype network and phylogenetic tree, also ITS-1 showed more intrapopulation diversity than mtDNA. AMOVA evidenced moderate genetic structuring, with most of genetic variation within localities. Pairwise F_{ST} shows some high genetic structuring between **Northeast** and some **localities** from **South and Southeast**, a pattern that suggests that Brazilian oceanic currents play an important role on the species connectivity. These results indicate that sexual reproduction and larval dispersion play a key role in the connectivity of these populations along with environmental and oceanographic variables. Also, we presented the first record of *P. belizeanus* in Brazil along with its genetic diversity analysis for this region. Using COI and 16SrRNA, we found no genetic structuring among localities, no clear geographical pattern and no sign of demographic retraction or expansion, suggesting colonization by a small number of mtDNA lineages followed by population stability. In addition, migration analysis evidenced some influence of Brazilian Current in dispersion. Together, those results increase the knowledge and helps to understand the phylogeography and genetic diversity of brazilian marine invertebrates from rocky shores.

4. FINAL CONSIDERATIONS

To the best of our knowledge, this is the first phylogeographic study about *Prosorhochmus belizeanus* and the first study using mitochondrial and nuclear markers and Ecological Niche Modelling for *Lineus sanguineus* across Brazilian coast. In the present study we were able to characterize the intra and inter localities genetic diversity of both species, infer the paleodistribution of *L. sanguineus* and expand the geographical range of *P. belizeanus*.

Lineus sanguineus seems to be a true cosmopolitan species, with incredibly high genetic connectivity among continents. Historically this species was thought to only reproduce by fissiparity, but a recent study was able to record its larval stage. This finding elucidates the patterns of high connectivity found in this study and other recent studies with *Lineus sanguineus* in North America and Europe. Still, in Brazil, there were some signs of genetic structure between Northeast localities and some South and Southeast sample localities, mainly explained by the Brazilian currents, as they act as gene flow barriers. Besides that, as *Lineus sanguineus* is found among fouling fauna of rocky shores, it is possible that human activity may influence their dispersal, as they can travel through ballast water and fouling fauna of ships hulls, as already hypothesized and demonstrated for other organisms.

In this dissertation, along with new findings about *Lineus sanguineus*, we brought the first record of the genus *Prosorhochmus* in Brazil, the species found corresponds to *Prosorhochmus belizeanus*, previously registered only in Florida and Belize. This species presented no geographic genetic structure and seems to colonized the Northeastern coast

with a small number of mtDNA lineages. Although there is a lack of genetic structuring, migration analyses were able to detect more exchange of migrants between samples from Maceió (AL) and Barra de São Miguel (AL), and also a high flow of migrants from Paracuru (CE) to the other two localities. This pattern can be partially explained by the bifurcation of the South Equatorial Current, where South Brazilian current can be isolating Paracuru (CE) from receiving migrants.

Thereby, using genetic, environmental and geographic data, this dissertation sheds lights on phylogeographic and population genetics studies of Brazilian rocky shores and nemerteans, helping to elucidate the patterns of dispersion of non model organisms and animals of different reproduction strategies.