

Cecili Barrozo Mendes

Nemertopsis bivittata (Hoplonemertea), *Lineus sanguineus* (Heteronemertea) e *Perinereis ponteni* (Polychaeta): VALIDADE DAS ESPÉCIES, FLUXO GÊNICO E DIVERSIDADE GENÉTICA NA COSTA BRASILEIRA

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Orientadora: Sônia Cristina da Silva
Andrade

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Dedicatória

Às cientistas brasileiras que apesar de todas as dificuldades, sociais, financeiras e governamentais, continuam lutando para fazer do mundo um lugar melhor.

Epígrafe

But also when I am active *scientifically*, etc. – an activity which I can seldom perform in direct community with others – then my activity is *social*, because I perform it as a man. Not only is the material of my activity given to me as a social product (as is even the language in which the thinker is active): my own existence is social activity, and therefore that which I make of myself, I make of myself for society and with the consciousness of myself as a social being.

Karl Marx, *Reflections of a Young Man* (1835)

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General Introduction

The marine biodiversity and their taxonomy

The marine environment is the Earth's most extensive habitat. However, the marine biodiversity is not equally distributed, being the coastal region more densely populated and better known to science (APPELTANS et al., 2012; WEBB; BERGHE; O'DOR, 2010). The complete marine diversity, nonetheless, is still unknown. The average number of named marine species is ~410,000, of which ~390,000 are animal species, being ~330,000 of these invertebrate species ("WoRMS - World Register of Marine Species", 2021/11/22). According to taxonomists, the estimate number for the total of Eukaryote marine extant species is approximately four times this number, reaching 700 – 970 thousand species (APPELTANS et al., 2012).

Many of the unknown invertebrate species are part of cryptic species complexes that are not yet recognized or delimited. Nonetheless, the cryptic species complexes have been more easily recognized due to the use of molecular tools that can differentiate species of similar morphology, which helped clarify the real distribution of many species believed to be cosmopolitan (JÖRGER et al., 2012; PUILLANDRE et al., 2012; SUNDBERG; KVIST; STRAND, 2016). This is especially important for animals of simpler morphology, and sometimes for animals with more complex morphology, for which the morphological characterization traditionally relies on histological preparations or other difficult to observe characters, such as wormlike animals as nemerteans, nematodes, peanut worms, among others (JOHNSON et al., 2016; JÖRGER et al., 2012; KAWAUCHI;

GIRIBET, 2010; MENDES et al., 2018; STRAND; SUNDBERG, 2011; SUNDBERG et al., 2009, 2016; SUNDBERG; KVIST; STRAND, 2016). However, genetic data information use is better suited when coupled with morphological, ecological, environmental and behavioral data. In addition, the utilization of a single gene in species delimitation studies might bias the conclusions, since the gene history might not be the same as the species history (KNOWLES; CARSTENS, 2007; MURPHY et al., 2015). One of the important causes for this bias is the Incomplete Lineage Sorting, when the lineages do not coalesce into a recent ancestor due to, e.g., a rapid diversification process (MADDISON, 1997; PAMILO; NEI, 1988). This can have meaningful implications to phylogenetic studies, and the most common way to overcome it is to add more or longer markers in the analysis (HODEL et al., 2020; NICHOLS, 2001; WANG et al., 2018). The use of new genetic tools, such as the fairly recent genomic approaches to understand the population structure can ensure that the species delimitation will not be artificial or influenced by a single locus unique or divergent history.

Gene flow in animal marine populations

The gene flow among marine populations is usually directly linked to the species reproduction mode. Usually, species with direct development will have more structured populations than species with indirect development. Among the indirect developers, usually species with planktotrophic larva (i.e. larva that feeds in the plankton, taking a longer time to settle) will have better connectivity than species with lecithotrophic larva (i.e. larva that do not feed in the plankton, using only the yolk already present in the egg, staying for shorter times in the water column until settle) (BOHONAK, 1999; HELLBERG et al., 2002; KINLAN; GAINES; LESTER,

2005; PALUMBI, 1994; RIESGO; TABOADA; AVILA, 2015). In addition to the time that the larvae stay in the plankton, their behaviour in the water column also affects the distance they can reach in this period. Most larvae cannot change their horizontal position, but they can travel vertically in the water column, and some do daily migrations to escape predators and UV-light. These movements can change the speed and direction of their dispersion due to the different ocean currents in the different depths (BECKER et al., 2007; COWEN; SPONAUGLE, 2009; LEVIN, 2006).

Other factors like adult dispersion, local population extinction and geographic barriers also have an important influence in the population connectivity and might explain possible pattern deviations (ANDRADE; NORENBURG; SOLFERINI, 2011; FERNÁNDEZ et al., 2015). Another important factor to the population connectivity is the natural selection, which can act very differently in distinct environments, leading to differentiation between populations and limiting the gene flow. Such process can happen even among species with high dispersion rates if the local selection is strong enough (NOSIL; FUNK; ORTIZ-BARRIENTOS, 2009; VIA, 2002). Therefore, the study of patterns of connectivity between populations must take into account all possible influences, like the species biology, recent and ancient environmental changes, and possible geographic barriers (BANKS et al., 2007; BERNATCHEZ et al., 2019; SELKOE; TOONEN, 2011).

With the advent of DNA amplification and sequencing, these studies could have a better resolution and, especially among marine populations, unravel patterns of connectivity and differentiation rather unexpected (e.g. AMENT-VELÁSQUEZ et al., 2016; ANDRADE; NORENBURG; SOLFERINI, 2011; BOWEN et al., 2006; CARD

et al., 2016; MENDES et al., 2018; NUNES et al., 2021; SELKOE; TOONEN, 2011). Most population genetic and phylogeography studies use mitochondrial markers, as they are haploid, non-recombinant and probably neutral (HELLBERG et al., 2002). However, some questions of fine scale structure still could not be resolved with the use of only mitochondrial regions. Multiple genes, both nuclear and mitochondrial, microsatellites and especially SNPs (Single Nucleotide Polymorphisms) can also be used for population studies, showing a better resolution in most cases. The cheapening of genomic scale sequencing enabled a broader use of SNPs, and a new strategy combining SNPs and environmental features was developed (LIGGINS; TREML; RIGINOS, 2019). This approach is called landscape genomics when used to understand the role of the environment and geography on the terrestrial populations connectivity and seascape genomics, when focused on marine populations. In both cases, the patterns of connectivity and differentiation among populations are studied taking the environment effect into account, which allows for the discovery of possible loci under selection due to environment adaptation (SELKOE et al., 2016). The seascape genetics approach has allowed researchers to better understand connectivity patterns and evolution of well-known species, like abalones, oysters, lobsters and clown fish (BERNATCHEZ et al., 2019; SAENZ-AGUDELO et al., 2015; SANDOVAL-CASTILLO et al., 2018; SINGH et al., 2018). However, the application of seascape genetics approach in non-commercial and non-model species is still scarce.

The studied species

To better understand how the reproductive mode can shape population connectivity and local adaptation, one can compare species with different

reproductive modes, but inhabiting the same environment. Along the Brazilian coast oyster and barnacle beds are quite common, and many different animal groups inhabit this environment. Among those, we have nemerteans and annelid polychaetes. Nemerteans are mostly benthic marine worms that possess a muscular proboscis housed in a coelomic cavity, the rhynchocoel, which characterizes the phylum. Three classes compose the phylum Nemertea, Palaeonemertea, Pilidiophora and Hoplonemertea (STRAND et al., 2019). They are morphologically distinguished by the position of the mouth and armature of the proboscis. Among palaeonemerteans and pilidiophorans the mouth opens ventrally, separately from the proboscis pore, and the proboscis is unarmed. Among hoplonemerteans the mouth shares an opening with the proboscis pore in the tip of the head, and the proboscis is armed with one or many chitinous stylets (ANDRADE et al., 2012; THOLLESSON; NORENBURG, 2003). The larval development is also different between these three classes; most pilidiophorans have a hat-shaped planktotrophic larva that can stay up to several weeks in the plankton, feeding upon microalgae; the palaeonemerteans also present a planktotrophic larva, but these are planuliform and feed upon other invertebrate larvae; the hoplonemerteans also have a planuliform larva, but their development is only known for a few lecithotrophic species (BIRD; VON DASSOW; MASLAKOVA, 2014; MASLAKOVA; HIEBERT, 2014; MASLAKOVA; VON DÖHREN, 2009). However, previous population genetic studies on Hoplonemertea species indicate high connectivity between populations hundreds of kilometers apart, indicating that these species might have a long lived larva (ANDRADE; NORENBURG; SOLFERINI, 2011; LEASI; ANDRADE; NORENBURG, 2016; MENDES et al., 2018; TULCHINSKY; NORENBURG; TURBEVILLE, 2012). In

addition, these species have eggs smaller than 100µm, a strong indicative of a planktotrophic larva (MASLAKOVA; HIEBERT, 2014).

Polychaeta is paraphyletic class of the phylum Annelida characterized by the presence of cephalic and locomotor appendages, the parapodia (AMARAL; RIZZO; ARRUDA, 2005; ANDRADE et al., 2015; ZRZAVÝ et al., 2009). Based on current knowledge Annelida is recognized as the two major clades of Errantia (polychaete families) and Sedentaria (that includes the traditional Clitellata, and Echiura, and polychete families). The two clades together form Pleistoannelida. Besides, there are more five basal branching lineages Sipuncula, Amphinomida, Chaetopteridae, Magelonidae, and Oweniidae (WEIGERT & BLEIDORN, 2016).

They have a well demarcated head, with a prostomium and peristomium, and sensorial and feeding appendages, as antennae, tentacular cirri and palps, may be present. Along the body they present parapodia and chaetae, that may show variation in shape, development, and number, respectively. They can also present gills, retractable or not (AMARAL; NONATO, 1996; ROUSE & PLEIJEL, 2001). These animals are mainly free-living, and most species are benthic and solitary. Other few species are planktonic and use their parapodia as paddles (AMARAL; NONATO, 1996; AMARAL; RIZZO; ARRUDA, 2005). The benthic polychaetes can live in many environments, like algae mats, oyster, mussel and barnacle beds, beach rocks, from the intertidal to the deep sea, etc. These species can be omnivorous, carnivores, scavengers, suspensivorous or herbivores (FAUCHALD; JUMARS, 1979; JUMARS et al., 2015). Some species are an important part of the diet of fishes and crustaceans of commercial value (PAIVA, 2006). Polychaete species present both planktotrophic and lecithotrophic larvae, and usually the larval types are consistent within each

family, with a few exceptions (AMARAL; NONATO, 1996; PAIVA, 2006; ROUSE, 2000; ROUSE & PLEIJEL, 2001).

Taking that into consideration, the polychaete species *Perinereis ponteni* Kinberg, 1865, and the nemerteans *Lineus sanguineus* (Rathke, 1799) and *Nemertopsis bivittata* (Delle Chiaje, 1841) seem to be a good choice for a comparison study aiming to understand how the environmental conditions along the Brazilian coast influence the intertidal invertebrate populations. All three species live in the intertidal zone, usually among *Brachidontes* sp. mats, barnacle and oyster beds (Fig. 1), from Northeast to South Brazil. The polychaete *P. ponteni* (Fig. 2A) is an important generalist in these communities. This species present epitoky with a planktonic stage and might have a lecithotrophic larva, as most Nereididae do (BAKKEN et al., 2018; ROUSE, 2000). The nemerteans *L. sanguineus* (Fig. 2B) and *N. bivittata* (Fig. 2C) are predators. *Lineus sanguineus* usually preys upon syllid polychaetes (CAPLINS; TURBEVILLE, 2011; RUNNELS, 2013), while *N. bivittata* prefers acorn barnacles (CAPLINS; NORENBURG; TURBEVILLE, 2012). In both cases, they use toxins to paralyze their prey, and can feed upon other invertebrates. Their reproduction strategies, however, are quite dissimilar, as *L. sanguineus* is a Pilidiophoran, which presents both sexual and asexual reproduction strategies. Some authors observed gonads under development, but gamete spawn was never observed, while asexual reproduction through fission is commonly observed (COE, 1899; MORETTO; BRANCATO, 1997; RUNNELS, 2013). The reproduction of the hoplonemertean *N. bivittata* is also not completely known, but their egg size smaller than 100µm. From the three studied species, this is the one with less information about their development available, since we do not have information on the development of any close related species.

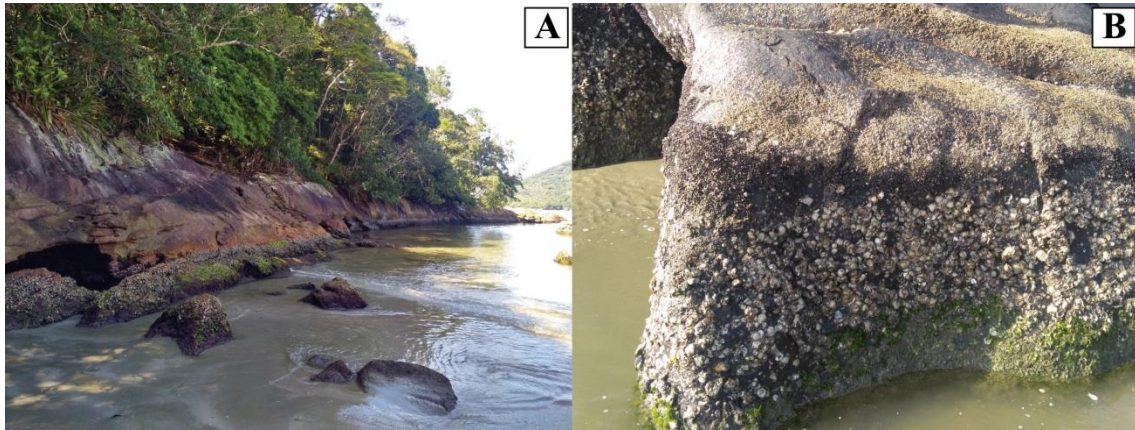


Figure 1. The environment where the species were collected. A: general view; B: zoom in the cluster of barnacles and oysters in the lower half, and mussels in the upper half.

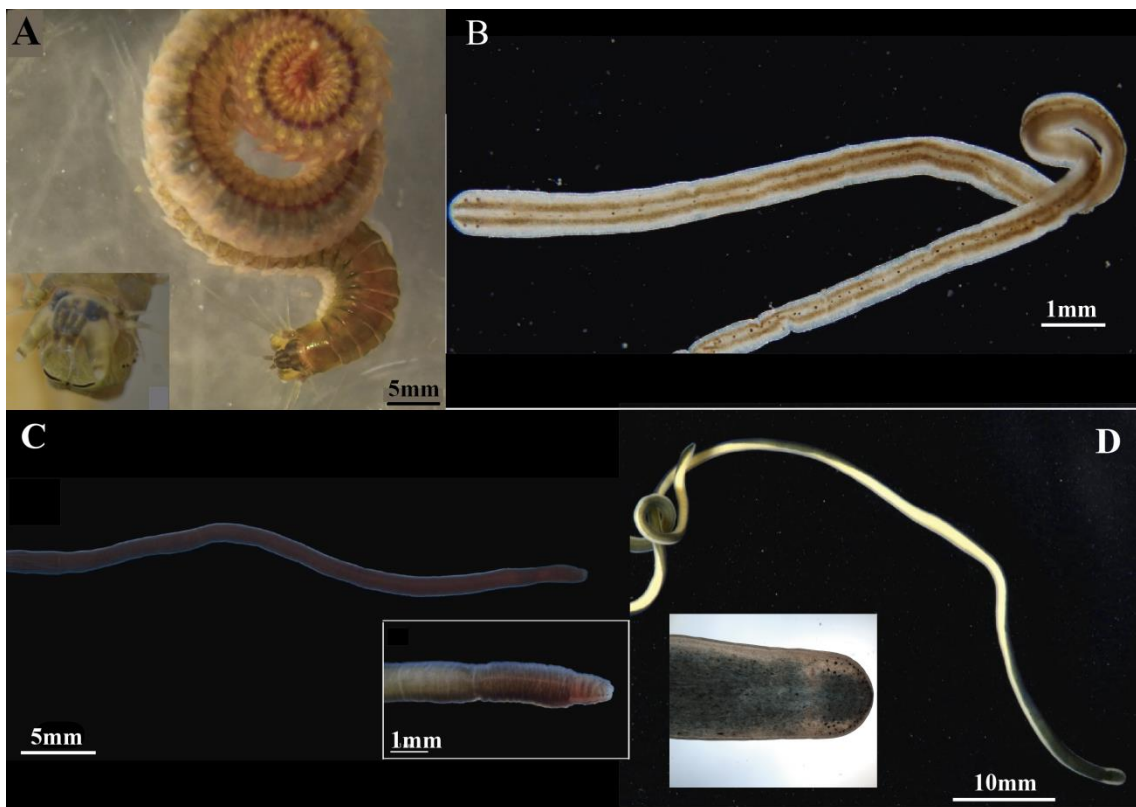


Figure 2. The four studied species. A: *Perinereis ponteni*, detail of everted pharynx, dorsal view. B: *Nemertopsis bivittata*. C: *Lineus sanguineus*, detail of the head in lateral view. D: *Emplectonema viride*, detail of the head in dorsal view.

Nemertopsis bivittata is a species of the family Emplectonematidae, being closely related to *Emplectonema viride* Stimpson, 1857, a very common species in the Northeastern Pacific ocean, present from Alaska to California, living in a similar environment as *N. bivittata* in the Brazilian coast (MENDES et al., 2021). Therefore, investigating *E. viride* larval development, one can have better insights about the larval development of *N. bivittata*. The development of *E. viride* is also not well known, but different sized larvae were observed in plankton samples along the Oregon coast (HIEBERT, 2016; MENDES et al., 2021). These larvae were also occasionally observed feeding upon barnacle nauplii (VON DASSOW et al. in prep). Reproductive individuals of *E. viride* can be easily found among barnacle clusters in the coast of Oregon in winter months, and can reproduce in captivity, being a good choice as a model to understand the hoplonemertean larval development and behaviour. Our initial hypothesis is that *E. viride* and *N. bivittata* both have planktotrophic larvae, being indirect developers. We also expect that the populations of the indirect developers (*N. bivittata* and *P. ponteni*) will be better connected than the populations of the partial asexual one (*L. sanguineus*).

This thesis is divided in four chapters: the first encompass the larval development of *E. viride* with notes in its larval behaviour and feeding; the second presents the taxonomic revision of *N. bivittata* along the Brazilian coast, with descriptions of three new species; the third chapter deals with the seascape genomics of *P. ponteni* populations in Brazil; and the fourth chapter comprehends a comparative seascape genomics of the nemertean species present in this study.

Objectives

The main objective of this work is to understand if there are genomic regions that might be involved with the response to environment in species with different reproductive and development modes. In addition, to identify what are the most important environmental variables influencing such response, and if there are congruencies among these species. We compared three species, two with sexual reproduction and indirect development (being one with lecithotrophic larva, and another with possibly planktotrophic larva), and one with partially asexual reproduction. To do so, we have the following specific objectives:

1. Describe the hoplonemertean larval development using *Emplectonema viride* as a model;
2. Genetically characterize, evaluate and compare the populations of *Nemertopsis bivittata*, *Lineus sanguineus* and *Perinereis ponteni* along part of the Brazilian coast;
3. Estimate their migration patterns and demographic history along the Brazilian coast;
4. Detect and identify potential loci associated with environmental factors that might indicate local adaptation.

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General Conclusions

In the present study we evaluated the validity of the three selected species (*Lineus sanguineus*, *Nemertopsis bivittata* and *Perinereis ponteni*), their populational structure along the Brazilian coast, as well as their past demography and possible responses to their environment.

Our first results show that *N. bivittata* is a complex of cryptic species comprehending three different species in Brazil, *N. berthalutzae*, *N. caete* and *N. pamelaroeae*. From those three species, only two seem to be present along the entire sampling range, *N. berthalutzae* and *N. pamelaroeae*, being *N. caete* restrict to the coast of Alagoas state.

The hoplonemertean larval development was not yet well known, except for a few species with direct development. However, many populational results, as well as some anecdotal observations of larval growth in the plankton, indicated indirect development. Therefore, we investigated the hoplonemertean development using *Emplectonema viride* as a model species. The observation of *E. viride* larval development indicated a long planktonic stage of around 120 days, with feeding starting as soon as the stylet developed, at four days old. These results, as well as the ones described in von Dassow *et al.* (in press) indicated that planktonic larval development is well spread among the hoplonemerteans. The long planktonic stage observed is compatible to the populational results found for other hoplonemertean species that have very well connected populations, despite the low motile adults.

The population results from all four species (*P. ponteni*, *L. sanguineus*, *N. berthalutzae* and *N. pamelaroeae*) reaffirm the importance of developmental modes to the population dynamics. Species with longer larval stage (i.e. the two

planktotrophic *Nemertopsis* species) have more connected populations than species with shorter larval stage (i.e. the two lecithotrophic *L. sanguineus* and *P. ponteni*), despite having similar habits during the adult life. Nonetheless, our results also shed light in other factors that also shape the population dynamics, particularly the past demography and the present environment.

The demography results for the *Nemertopsis* species are not informative, due to the uneven sampling. However, both our results for *P. ponteni* and *L. sanguineus* suggest a demographic expansion from the Southeast region, where the populations show higher levels of diversity. These levels of diversity are most likely consequence of this area being a refuge for the species during the Last Glacial Maximum (~20ky), when the Brazilian coast was more exposed than nowadays and the suitable habitats for coastal species were rarer.

Currently, the temperature seems to be main environmental factor that influence all studied species. They all show SNPs candidates to be under selection related directly or indirectly to temperature variation. The SNPs are related to many different genes in the different species. Nevertheless, genes related to DNA repair and immunity are present in all cases – *ZBRAN3* and *PARP9* among *P. ponteni* populations; *TMP2L*, *NRDE2*, *USP9X*, *SACS*, *CRHBP*, *CD9* and *BLNK* in *L. sanguineus*; *T184B*, *TENSI*, *MACF1* and *DYH7* amongst *N. berthaltutzae* populations – suggesting a common response to the challenging environment in all studied species, despite their differences. The increase in temperature favors primary production and microorganism proliferation, which can increase the likelihood of disease in environments already highly impacted by human interventions. In addition, fouling communities provide a propitious microhabitat for accumulation of organic matter that also favors such microorganisms. Besides the local effect of temperature

increase, the changing in the ocean temperature can also have a long term effect in these populations, due to the alteration in the oceanic circulation. We cannot yet affirm how this alteration will affect the populations. However, the temperature difference is a very important factor to the direction and velocity of ocean currents, and a warmer ocean is likely to have slower currents, isolating the animal populations that use the present ocean circulation for connection. It is interesting to note that even a species with such a high gene flow still responds to biogeographic barriers, highlighting the importance of biotic and abiotic factors shaping population connectivity. It is also worth noting the importance of temperature in shaping these populations, indicating that future climate change and ocean warming can have huge impacts on coastal communities.

Resumo

Entender quais são os principais fatores que influenciam a conectividade entre populações animais marinhas tem sido objeto de diversos estudos há décadas, sendo o a mobilidade dos indivíduos reconhecida como um dos fatores determinantes. Espécies com longo desenvolvimento larval (e.g. planctotróficas) usualmente apresentam populações melhor conectadas que espécies com desenvolvimento direto ou curto desenvolvimento larval (e.g. lecitotróficas), devido sua maior capacidade de dispersão. Entretanto, outras características bionômicas e o ambiente também tem grande influência na conectividade. Além disso, a percepção da conectividade populacional também sofre interferência devido a presença de espécies crípticas, uma vez que tais espécies podem ter extrema semelhança morfológica. Assim, o presente trabalho avaliou como a heterogeneidade ambiental influencia a conectividade de espécies com diferentes modos reprodutivos, através de técnicas de genômica de paisagem comparativa. Para tanto, utilizamos as espécies *Perinereis ponteni* (Polychaeta, de reprodução sexuada e larva lecitotrófica), *Lineus sanguineus* (Heteronemertea, de reprodução assexuada e sexuada, e larva lecitotrófica) e *Nemertopsis bivittata* (Hoplonemertea, de reprodução sexuada, com desenvolvimento larval não completamente conhecido, mas presumidamente planctotrófica). De forma a garantir a monofilia das espécies, as populações estudadas foram avaliadas taxonomicamente. Nesse sentido, amostramos populações ao longo da costa brasileira. Os animais coletados foram utilizados na extração de DNA para prospecção de SNPs, a partir da técnica de *Genotyping by Sequencing* (GBS). Ainda, acompanhamos o comportamento e desenvolvimento larval do hoplonemertíneo *Emplectonema viride* como proxy para o desenvolvimento de *N. bivittata*, uma vez que ambas são da família Emplectonematidae e vivem em ambientes extremamente similares. Contudo, a espécie *N. bivittata* é na verdade um complexo de espécies, com três unidades evolutivas distintas na costa brasileira. Essas foram descritas como, *Nemertopsis berthallutzae*, *Nemertopsis pamelaroeae* e *Nemertopsis caete*, a última estando presente apenas na costa de Alagoas. A observação do desenvolvimento larval de *E. viride* indicou a presença de larvas planctotróficas, com longo desenvolvimento, levando em média 120 dias para metamorfose bastante sutil (notada principalmente pela mudança na ciliação e no comportamento). As larvas se alimentam primariamente de nauplius e ciprídes de cracas. Nossas análises de paleodistribuição e demografia de *P. ponteni* apontaram a presença de um refúgio na região Sudeste durante o Último Máximo Glacial, de onde as populações expandiram. Quanto às análises de genômica de paisagem, observamos que as espécies com desenvolvimento larval mais curto tendem a apresentar populações mais estruturadas, porém ainda com significativo fluxo gênico. Entretanto, fatores ambientais semelhantes afetam a conectividade em todas as espécies, sendo a temperatura e precipitação os mais comuns. Esses fatores estão associados à presença de SNPs candidatos à seleção natural em três das quatro espécies estudadas. Tal achado reforça quão influente as condições ambientais locais e globais são na conectividade e, por conseguinte, na diversidade genética das espécies.

Abstract

Understanding the main factors influencing connectivity among marine animal populations has been the subject of several studies for decades, with the mobility of individuals being recognized as one of the determining factors. Species with long larval development (e.g. planktotrophic) usually have better connected populations than species with direct or short larval development (e.g. lecithotrophic), due to their greater dispersal capacity. However, other biogenic features and the environment also have a major influence on connectivity. In addition, the perception of population connectivity can also be affected by the presence of cryptic species, since such species can have extreme morphological similarity. Thus, the present work evaluated how environmental heterogeneity influences the connectivity of species with different reproductive modes, through comparative landscape genomics techniques. For this purpose, we used the species *Perinereis ponteni* (Polychaeta, with sexual reproduction and lecithotrophic larvae), *Lineus sanguineus* (Heteronemertea, with asexual and sexual reproduction, and lecithotrophic larvae) and *Nemertopsis bivittata* (Hoploneurtea, with sexual reproduction, and presumably planktotrophic larvae). In order to guarantee the monophyly of all species, the populations studied were taxonomically assessed. In this sense, we sampled populations along the Brazilian coast. The collected animals were used in DNA extraction for SNPs prospecting, using the Genotyping by Sequencing (GBS) technique. Furthermore, we followed the behavior and larval development of the hoploneurtean *Emplectonema viride* as a proxy for the development of *N. bivittata*, since both are from the Emplectonematidae family and live in extremely similar environments. However, the species *N. bivittata* is actually a complex of species, with three distinct evolutionary units on the Brazilian coast. These were described as, *Nemertopsis berthallutzae*, *Nemertopsis pamelaroeae* and *Nemertopsis caete*, the latter being present only on the coast of Alagoas. The observation of *E. viride* larval development indicated the presence of planktotrophic larvae, with long development, taking an average of 120 days for quite subtle metamorphosis (noticed mainly by changes in ciliation and behavior). The larvae feed primarily on barnacle nauplius and cyprids. Our palaeodistribution and demography analyzes indicate the presence of a refuge in the Southeast region during the Last Glacial Maximum, from where the populations expanded. As for landscape genomic analyses, we observed that species with shorter larval development tend to have more structured populations, but still with significant gene flow. However, similar environmental factors affect connectivity in all species, with temperature and precipitation being the most common. These factors are associated with the presence of SNPs candidates for natural selection in three of the four species studied. These findings reinforces how important local and global environmental conditions are for animal connectivity and, therefore, for the genetic diversity of species.