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INTERAÇÃO ENTRE ANFÍPODES HERBÍVOROS E ALGAS: A IDENTIDADE DA ALGA HOSPEDEIRA INFLUENCIA A DIVERSIDADE GENÉTICA E MORFOLÓGICA DE POPULAÇÕES DE HERBÍVOROS?

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Resumo

A interação entre plantas e seus herbívoros no ambiente marinho representam um sistema interessante para realizar estudos em múltipla escala, os quais podem ajudar a compreender as relações ecológicas que ali ocorrem. Em costões rochosos, anfípodes se destacam entre os crustáceos, sendo os anfípodes ampitoídeos um dos grupos de herbívoros mais frequentes. Esses animais possuem hábito de vida tubícola, são sedentários e possuem desenvolvimento direto (i.e. não possuem estágio larval), apresentando limitada capacidade de dispersão, usando macroalgas como alimento e habitat. Uma vez que macroalgas hospedeiras afetam a aptidão desses animais, espera-se que as diferentes hospedeiras variem quanto a sua contribuição para a sobrevivência e encontro de parceiros sexuais dos anfípodes. Nesse trabalho, a espécie *Cymadusa filosa* Savigny,1816 e suas algas hospedeiras foram utilizadas como modelos para testar a hipótese de que esses animais, em escala local, são estruturados geneticamente de acordo com a hospedeira e apresentam variação morfológica devido as diferentes características entre as algas. Além disso, foi investigado se as populações de diferentes costões rochosos são diferentes entre si devido à baixa dispersão dos animais. Para responder essas questões, foram utilizados marcadores microssatélites para acessar a diversidade genética, e morfometria geométrica para acessar a diversidade morfológica. Como resultados gerais, foi observado que tanto as algas como as diferentes localidades geográficas não são fatores estruturadores da população. Indivíduos aparentam ser altamente móveis localmente ou possuírem uma dispersão baseada nos juvenis, além de serem capazes de se dispersar entre localidades por *rafting*. Apesar das indicações de fluxo gênico, a ocorrência de grupos morfológicos distintos provavelmente ocorre devido condições ambientais de cada local, caracterizando uma metapopulação com divergência fenotípica.

Abstract

Marine plant-herbivore interactions represent an interesting natural system to perform multiple scales approaches in order to have a better comprehension of their ecological interactions. Amphipods are an abundant group of crustaceans in rocky shore environments, and the ampithoid amphipods are one of the most frequent herbivores. These animals are tubicolous and sedentary, they are direct developers (i.e. they do not have a larval phase), presenting a limited dispersion capability, and they use macroalgae as food and shelter. Macroalgae can affect the fitness of these animals, so is expected that different macroalgae species vary in how they contribute to survival and mate encounter of amphipods. Here, the species *Cymadusa filosa* Savigny,1816 and its host macroalgae were used as models to test the hypothesis that amphipods are genetically and morphologically structured in fine scales because of differences in host traits. Furthermore, it was tested if there are differences among populations from distinct rocky shores because of limited dispersion in amphipods. Microsatellites were the molecular markers used to access the genetic diversity, along with geometric morphometric analyses to access morphological diversity. Our results indicate that host macroalgae or locations are a factor that has a role on the genetic diversity. Individuals seem to be highly mobile in local scales or to have a juvenile based dispersal, and they seem capable to disperse among shores by rafting. Even though there are indication of intense gene flow, the occurrence of distinct morphological groups occurs probably because of environmental conditions of each location, characterizing a metapopulation structure with phenotypic divergence.

Sumário

Introdução Geral

Uma questão central em biologia é compreender a ligação entre a ecologia e evolução das espécies. Nesse contexto, a escala em que um estudo é realizado tem grande importância, pois pode revelar padrões que são resultados de processos que atuam em diferentes escalas (Levin, 1992; Chave, 2013). Entretanto, não há uma escala única em que um estudo deve ser conduzido, mas devemos ter a premissa de que os sistemas naturais estão organizados no tempo e espaço de forma complexa, sendo que as explicações dos padrões não são capazes de extrapolar a escala na qual este foi observado (Wiens, 1989).

A escala, por sua vez, acaba sendo um conceito arbitrário e depende do observador, sendo escolhidas baseadas em nossa percepção, limitações logísticas ou tecnológicas (Steele, 1978). Deste modo, estudos que utilizam múltiplas escalas são interessantes, pois permitem fazer inferências que conectam aspectos ecológicos e evolutivos de processos locais e globais para explicar padrões. A busca ativa de locais específicos para assentamento em escala local e a dinâmica de correntes oceânicas quando exploradas em conjunto, por exemplo, esclarecem o padrão de distribuição de larvas de invertebrados marinhos (Butman, 1987). Em outro caso, o gastrópode *Littoraria flava* apresenta estruturação moderada ao longo de 4000 km na costa brasileira (Andrade *et al.*, 2003), porém subpopulações geneticamente distintas quando os indivíduos de uma mesma localidade são comparados (Andrade & Solferini, 2007). Para esse gastrópode, a baixa diferenciação genética entre localidades distantes está relacionada à sua dispersão larval, enquanto que em poucos metros de distância os indivíduos podem estar sob força de seleção diversificadora devido a heterogeneidade ambiental.

Assim, a maneira como cada organismo percebe e interage com o ambiente está intimamente ligado a sua história natural, uma vez que características exclusivas é que geram padrões espaciais de distribuição. Nesse sentido, as maneiras com que as espécies dispersam e o seu tipo de desenvolvimento estão intimamente ligados ao fluxo gênico, o qual tem um papel importante em elucidar as escalas em que a diferenciação entre populações pode ocorrer (Slatkin, 1985)

Os táxons marinhos podem apresentar diferentes potenciais de dispersão, dependendo do seu tipo de desenvolvimento. Organismos que apresentam desenvolvimento indireto, ou

seja, que apresentam uma fase larval dispersora, são associados a uma dispersão de longas distâncias mantendo uma baixa diferenciação entre populações (Ayre *et al.*, 1997). Por outro lado, animais com desenvolvimento direto, que não apresentam uma fase larval, são associados a maior diferenciação genética entre populações e altas taxas de endocruzamento (Ellstrand & Ellam 1993; Knowlton & Jackson 1993; Thornhill 1993; Frankham 1995). Entretanto, trabalhos têm mostrado que nem sempre uma associação direta entre o tipo de desenvolvimento e a capacidade de dispersão pode ser feita, fazendo com que investigar as escalas em que as espécies se dispersam seja interessante para se realizar inferências de como elas interagem com seu habitat, respondem a distúrbios e evoluem (Roughgarden *et al.* 1988, Hanski 1999).

Além disso, classicamente se admite que a intensidade do fluxo gênico está relacionada a um possível surgimento de variações adaptativas no fenótipo dos organismos (Slatkin, 1987). Isso ocorre porque uma baixa taxa de migração entre populações, por exemplo, pode fazer com que ocorra adaptação relacionada às condições ambientais locais uma vez que não há homogeneização da diversidade genética. Por outro lado, se o fluxo gênico for mais intenso que a força de seleção natural, fenótipos divergentes podem não surgir (Garant *et al.*, 2007). Dessa forma, tanto as características de história natural (e.g. tipo de desenvolvimento) quanto a variação das características entre os ambientes podem influenciar na diversidade genética e morfológicas de espécies marinhas (Palumbi, 1992; Crispo & Chapman, 2008).

Dentro desse contexto, a interação planta-herbívoro em ambientes marinhos representa um modelo de estudo interessante para se testar diferenciação entre populações em diferentes escalas, especialmente porque macroalgas marinhas podem desempenhar um papel fundamental na evolução de pequenos herbívoros, assim como as plantas em ambientes terrestres (Ehrlich & Raven, 1964; Futuyma & Moreno, 1988; Arrontes, 1999). Em escala local, macroalgas não apenas representam um recurso alimentar, como também oferecem um refúgio contra predadores (Hay *et al.* 1987; Buschmann, 1990). Além disso, essa interação também está sujeita aos mecanismos contra herbivoria que macroalgas podem apresentar, as quais ocorrem na forma de defesas morfológicas e químicas (Hay, 1996; Pereira & Gama, 2008; Paul *et al*, 2011). Por outro lado, em maiores escalas, os organismos marinhos são limitados pela sua capacidade de dispersão (Palumbi, 1992).

Em costões rochosos, a comunidade de produtores primários é dominada pelas macroalgas (Eston *et al*, 1990; Nyberg *et al*, 2012). Nesses ambientes marinhos, os herbívoros são classificados em macroherbívoros e mesoherbívoros devido ao seu tamanho, mas também por causa dos diferentes aspectos biológicos que permeiam as interações herbívoro-alga nesse sistema (Little *et al*, 2009). Os macroherbívoros (e.g. peixes, ouriços e grandes gastrópodes) são capazes de percorrer relativamente grandes distâncias, forragear em diversas manchas de algas e são menos susceptíveis a predação devido a maior capacidade de locomoção, no caso de peixes, e também defesas físicas, como ouriços e gastrópodes (Hay *et al*, 1987). Por outro lado, os mesoherbívoros são aqueles organismos menores que 2,5 cm (e.g. anfípodes, poliquetas, pequenos gastrópodes), geralmente são mais sedentários, e que vivem associados às macroalgas em altas densidades (Leite *et al*, 2007; Cunha *et al*, 2013).

Os mesoherbívoros possuem uma relação íntima com as macroalgas, pois é nelas que encontram abrigo e alimento, sendo comparados aos insetos terrestres e chamados de organismos 'insect-like' por alguns autores (Hay *et al*., 1987; Poore *et al*., 2008). Dentre as características em comum, podemos ressaltar o tamanho reduzido em relação ao hospedeiro, altas densidades locais, forte dependência da planta e o impacto que podem causar sobre a comunidade de produtores primários (Hay *et al*., 1987). Dentre os mesoherbívoros, os anfípodes são um dos grupos mais representativos em abundância que ocorrem em associação com macroalgas (Leite *et al*, 2007).

Para esses pequenos animais, assim como para outros crustáceos (Dunham, 1978), o encontro de parceiros sexuais é mediado pela liberação de feromônios sexuais espécie-específicos que atraem indivíduos do sexo oposto (Borowsky, 1984; Borowsky, 1985; Borowsky & Borowsky, 1987). Os feromônios são produzidos por processos fisiológicos que sintetizam novas moléculas a partir da alimentação. Considerando que anfípodes herbívoros podem ocorrer em mais de uma espécie de macroalga (Tararam *et al*. 1986), geralmente alimentando-se da alga que habitam (Taylor & Steinberg, 2005), e que espécies de macroalgas podem apresentar concentrações e perfis químicos diferentes (Hay & Fenical, 1988), é esperado que haja produção de feromônios distintos entre organismos que habitam algas de espécies diferentes.

Por exemplo, em bioensaios com a espécie *Eogammarus confervicolus*, que ocorre em diferentes substratos biológicos em estuários, foi encontrado que indivíduos que habitam e se alimentam de algas do gênero *Fucus* produzem um feromônio sexual diferente daquele

produzido pelos indivíduos de outros substratos (Stanhope *et al*., 1992a). Esse ferômonio diferente é uma modificação do composto já existente produzido pela espécie, ou seja, as fêmeas que habitam *Fucus* produzem tanto o feromônio padrão, que é reconhecido pelos outros indivíduos da espécie, como também tem a capacidade de modificá-lo de forma que apenas os machos que habitam e se alimentam de *Fucus* são capazes de diferenciá-lo do padrão, respondendo a esse estímulo de forma acentuada (Stanhope *et al*., 1992a). Isto é, para a espécie *Eogammarus confervicolus* ocorre uma produção de um tipo específico de feromônio sexual que está associado à alimentação do animal. Tais resultados indicam um potencial de que a dieta possa modular o metabolismo de diferentes feromônios nos anfípodes herbívoros. Assim, dentro de uma mesma população, isso pode levar a limitação do encontro de parceiros sexuais, sendo essa ditada pela capacidade de um organismo reconhecer o feromônio produzido por outro (Landolt & Phillips, 1997; Stanhope *et al*, 1992b; Via, 2001).

Além disso, diferentes itens alimentares estão diretamente ligados a distintos padrões de sobrevivência, fecundidade e crescimento. Em experimentos com criação de quatro espécies de anfípodes para os quais eram oferecidos apenas uma espécie de alga, um mix delas ou itens animais, Cruz & Rivera (2000) demonstraram que as diferentes dietas tinham influência em aspectos biológicos das espécies. Dessa forma, as macroalgas e suas características específicas devem atuar como uma força seletiva, pois a escolha da alga hospedeira está ligada ao encontro de parceiros sexuais, assim como ligada a sobrevivência e parâmetros reprodutivos.

Entre os anfípodes herbívoros, a família Ampithoidae se destaca por ser rica em espécies, apresentando 106 espécies distribuídas nas zonas temperadas e tropicais (Barnard & Karaman, 1991). É comum que esses animais construam tubos, ou seja, são sedentários, e que apresentem baixa mobilidade (Appadoo & Myers, 2003). Devido a isso, esses animais normalmente se alimentam das algas que habitam (Poore *et al*, 2008; Taylor & Steinberg, 2005) e, portanto, apresentam uma estreita relação com a alga hospedeira. A espécie *Ampithoe longimana*, por exemplo, parece apresentar adaptação local em relação à macroalga parda *Dictyota* no litoral sudeste dos Estados Unidos (Sotka & Hay, 2002; Sotka *et al*, 2003). A porção norte desse litoral não apresenta a macroalga, que possui grandes quantidades de metabólitos contra herbivoria, enquanto que esta ocorre nas porções mais ao sul. Experimentos em laboratório indicaram que as populações de *Ampithoe longimana* simpátricas a *Dictyota* apresentam forte preferência alimentar por essa alga e maior tolerância

aos metabólitos produzidos em comparação às populações de herbívoros de regiões em que a alga não ocorre (Sotka & Hay, 2002; Sotka *et al*, 2003). Além disso, *A. longimana* apresenta uma rápida mudança quanto ao uso de macroalgas como alimento ao longo de poucas gerações (Sotka & Reynolds, 2011).

Existem estudos em relação à interação de outros organismos com diferentes tipos de substratos biológicos que fornecem exemplos de outras possíveis consequências que podem surgir entre anfípodes e macroalgas hospedeiras. Por exemplo, em ambientes marinhos, o caranguejo da espécie *Pachycheles monilifer* possui diferentes tamanhos e fecundidade quando comparados indivíduos que habitam briozoários e banco de poliquetas (Leone & Mantelatto, 2015); o anfípode *Perampithoe hystrix* apresenta diferentes fenótipos de coloração entre os hospedeiros (esponjas, briozoários, corais), cuja análise genética revelou tratar-se de um complexo de espécies que estavam relacionadas ao habitat do indivíduo (Schnabel & Hebert, 2003). Exemplos com insetos terrestres também ocorrem, como a borboleta *Heliconius eratus*, que apresenta morfologia de asa distintas dependendo da planta-hospedeira da qual a larva se alimentou (Jorge *et al.*, 2011). Outros trabalhos têm avaliado a associação de anfípodes e macroalgas hospedeiras (Stanhope *et al*, 1992a,1992b; Poore& Hill, 2006; Poore *et al*, 2008), mas não há conhecimento sobre avaliações genéticas e morfológicas em pequena escala de como essa interação ocorre.

Estudos em maiores escalas, por outro lado, muitas vezes reforçam o que é predito pelo modo de reprodução dos anfípodes. Por esses animais serem um grupo o qual apresenta desenvolvimento direto, carregando os jovens em bolsas incubadoras chamadas marsúpio, espera-se que suas populações sejam subdivididas, com baixo fluxo gênico e apresentando um padrão de isolamento por distância (Kane *et al*., 1992; Duan *et al*., 2000; Baird *et al*., 2011). Por exemplo, estudos na Antártica com os anfípodes dos gêneros *Eusirus* relatam alta diferenciação genética entre localidades distantes 150 km (Baird *et al*., 2011), enquanto que *Orchomenella* apresentou um padrão de isolamento por distância que pode ser explicado pela dispersão de indivíduos ocorrendo entre locais adjacentes (Baird *et al*., 2012).

No litoral do estado de São Paulo, ocorrem com freqüência espécies da família Ampithoidae, dentre elas *Cymadusa filosa* (Leite *et al*, 2000). Esse anfípode é um herbívoro generalista e tubícola, apresentando baixa mobilidade, e que ocorre em diferentes macroalgas, como as dos gêneros *Sargassum*, *Padina* e *Dictyota* (observação pessoal). Tais características, aliadas a curtas gerações, fazem com que a associação de *C. filosa* com suas

algas hospedeiras ofereça um modelo adequado para o estudo sobre a diferenciação das populações em diferentes escalas. Há um potencial para que haja divergência ecológica nesse sistema. Consequentemente, avaliar a variabilidade genética e morfológica em escalas pequenas de populações simpátricas de *C. filosa* que habitam espécies diferentes de algas poderá trazer informações novas a cerca desse tipo de interação. Além disso, como os outros anfípodes, *C. filosa* é uma espécie com limitada capacidade de dispersão e, portanto, sujeita a diferenciação entre os diferentes costões rochosos.

No presente trabalho, investiguei a relação entre o anfípode herbívoro *Cymadusa filosa* Savigny,1816 e suas algas hospedeiras, abordando essa interação em diferentes escalas. Especificamente, avaliei a diversidade genética e morfológica de indivíduos de *C. filosa* que habitavam três diferentes espécies de algas hospedeiras. O sistema de estudo permitiu testar tanto a questão da dispersão e diferenciação dos indivíduos entre as espécies de algas hospedeiras de um mesmo local, quanto entre os diferentes costões rochosos, abordando a questão em diferentes escalas espaciais. Aqui, testei as hipóteses de que: 1) em escalas locais, as algas hospedeiras são fatores estruturadores das populações de herbívoros, ou seja, em um mesmo costão rochoso, as populações que habitam as diferentes espécies de algas são subpopulações geneticamente distintas; 2) as algas hospedeiras, devido às particularidades de cada espécie, criam condições distintas capazes de gerar fenótipos diferentes nos herbívoros, fazendo com que os indivíduos que habitam cada espécie de hospedeira sejam morfologicamente distintos; 3) devido ao desenvolvimento direto, *C. filosa* forma populações geneticamente estruturadas entre os diferentes costões rochosos por ser uma espécie que apresenta desenvolvimento direto.

Essa dissertação está dividida em dois capítulos, escritos em forma de manuscrito. No capítulo 1, apresento a caracterização de marcadores moleculares microssatélites que foram desenvolvidos para a espécie *Cymadusa filosa* a fim de serem utilizados para responder a questão proposta neste trabalho. No capítulo 2, apresento o trabalho desenvolvido utilizando marcadores microssatélites e morfometria geométrica para testar as hipóteses apresentadas anteriormente.

Capítulo 1

Capítulo 1

*Manuscrito submetido como short communication no periódico Marine Biodiversity

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Development and characterization of novel microsatellites loci for the amphipod *Cymadusa filosa*.

Introduction

Microsatellites are highly variable DNA sequences of tandem repetition of 1-6 nucleotides with codominant inheritance that are found at high frequency in the nuclear genomes of most organisms (Tautz 1989). These molecular markers have emerged as one of the most popular choices for population genetics and molecular ecology studies and, although microsatellites are facing a transitioning to the use of single nucleotide polymorphisms (SNPs), they are still useful tools for answering a wide range of questions especially species fine-scale genetic structure (Gardner *et al*. 2011; DeFaveri *et al*. 2013) and parentage relationships (Jones *et al*. 2010). Despite all the uses and advantages of microsatellites, they also have several challenges (Selkoe & Toonen 2006). For example, the need of development of species-specific markers when there are no primers available for a given species or for a related species. In order to use microsatellites as a marker in these cases, an enriched library must be built and novel primers developed.

The amphipod *Cymadusa filosa* is an algae dwelling species that occur in shallow waters associated with their host macroalgae (Nelson 1979; Jacobucci & Leite 2014). The distribution of this species is considered pantropical, occurring in Australia, Mediterranean Sea, Africa, India and Brazil (Barnard & Karaman 1991; Peart 2004). As other species of Ampithoidae family, *Cymadusa filosa* is a herbivorous and tubicolous amphipod. These animals present direct development, brooding juveniles in a ventral pouch called marsupium (Havermans *et al*. 2007). They are very abundant in rocky shores, occurring in high densities (Cunha *et al*. 2013), representing a relevant component in this environment. Additionally, amphipods are an important link between benthic and pelagic system because fish feed on them (McCurdy *et al*. 2006). Besides these ecological importance, as other amphipods, *Cymadusa filosa* can be used as a model organism for toxicological studies (Neuparth *et al*. 2002).

Here, we describe novel microsatellites for the amphipod *Cymadusa filosa* Savigny, 1816 that will be very useful in future studies of taxonomy, genetic diversity and conservation. To this point, there were no sets of microsatellites developed for the Ampithoidae family, and in this work we present the first microsatellite markers for this group.

Materials and Methods

We used six *Cymadusa filosa* individuals from the Ubatuba region (southeastern Brazil) to build a microsatellite-enriched library according to Billote *et al*. (1999). Genomic DNA was extracted from the entire individuals following a modified salt-extraction protocol according to Aljanabi & Martinez (1997) and digested with *Afa*I. DNA fragments were ligated to the double-strand adapters 5'-CTCTTGCTTACGCGTGGACTA-3' and 5'-TAGTCCACGCGTAAGCAAGAGCACA-3'. The enrichment was performed by using a hybridization-based capture with $(CT)_8$ and $(GT)_8$ biotinylated primer probes and StreptavidineMagneSphere Paramagnetic Particles (Promega). Selected DNA fragments were amplified by PCR and then cloned into a pGEM-T easy vector (Promega). Competent XL-1 Blue cells were transformed with the recombinant plasmids and cultivated on agar medium containing 100 μg/mL ampicillin and 50 μg/mL of X-galactosidase. Following the overnight incubation at 37ºC, single colonies were transferred onto microplates for long-term storage at -70ºC.

We sequenced 96 positive clones from the library in which 134 repeated motifs were identified using SSRIT (http://archive.gramene.org/db/markers/ssrtool). The following selection parameters were used to design microsatellite primer pairs: primer length between 18 - 22 pb; melting temperature (Tm) between 45ºC – 65ºC; maximum difference in Tm between primer pairs of 3ºC; GC content above 35%; no complementarity within and between primer pairs. A total of 42 primer pairs complementary to sequences flanking the repeat motifs were designed using PrimerSelect (DNASTAR). To each forward primer we added a M13 tail (5'-CACGACGTTGTAAAACGAC-3') at its 5' end, which enabled the fragments to be scored on 6.5% polyacrylamide gels on Li-Cor 4300 DNA Analyser (Li-Cor Biosciences).

For the validation step of microsatellite loci, all the 42 primers pairs were tested for amplification on 6 DNA samples of *C.filosa.* PCR amplification were performed in a final volume of 10uL or 15 uL, depending on the locus (PCR1 and PCR2, respectively; Table 1 and 2). Each PCR contained1.5 ng of DNA template; 1X PCR buffer; 3mM magnesium chloride;

4ug BSA; 0.2 uMof each dNTP; 0.1 uM of each primer; 0.1 uM of 700 or 800 nm infrared dyes (Li-Cor Biosciences) and 1 U*Taq* DNA polymerase. All loci were amplified using touchdown PCR, according to the following thermocycling conditions: 94ºC for 4 min; 10 X [94ºC for 45s, 65 or 57 or 52ºC (-0,5ºC/cycle) for 1 min and 72ºC for 1 min 15s]; 25 X [94ºC for 45s, 50ºC for 1 min and 72ºC for 1min 15s]; and 72ºC for 10 min. Primer pairs were discarded if they failed to amplify or led to multiple none specific fragments. From the initial 42 primer pairs, 22 were successfully validated.

Polymorphism level of each microsatellite locus was evaluated in 30 *C. filosa* individuals from the Ubatuba region. Conditions and characteristics of microsatellite loci are provided in Table 1 and 2. For polymorphic loci, we calculated the number of alelles (A) and the observed (H_O) and expected (H_E) heterozygosities using GeneAlEx 6.5 (Peakall & Smouse 2006; Peakall & Smouse 2012). Loci adherence to Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between all loci pairs were tested using GENEPOP 4.2.2 (Rousset 2008), with 10000 dememorizations, 1000 batches and 10000 iterations per batch. Bonferroni corrections for multiple tests were applied at the significance level of 0.05 (Sokal & Rohlf 1995). The frequency of null alleles (F_{NA}) was tested using FreeNA (Chapuis & Estoup 2007).

The sampling of *C. filosa* was authorized by Instituto Chico Mendes de Conservação da Biodiversidade (SISBIO/ICMBio; license 15053).

Results and Discussion

Of the 22 validated microsatellite loci, 12 were monomorphic and 10 were polymorphic. Characteristics of polymorphic and monomorphic loci are indicated in Table 1 and 2. For polymorphic loci, the number of alleles ranged from 2 to 26 alleles per locus. No significant deviation from the HWE or LD was found. The frequency of null alleles (F_{NA}) ranged from 2% to 11%, which is considered negligible to moderate (Chapius & Estoup 2007), but for most of the loci they were not detected. H_O ranged from 0.033 to 0.933 and H_E ranged from 0.033 to 0.943, revealing some highly variable loci and others with moderate variation levels.

The results obtained in this current study were similar to the ones found in previous papers, in which the average of polymorphic microsatellites for amphipods was 9 and the observed heterozygosity (H_O) ranged from 0.04 to 0.941 (Westram *et al*. 2010; Baird *et al*.

2012; Gemmer & Gergs 2013; Pavesi *et al*. 2013). None of these studies used amphipods from Ampithoidae family, however, their results are in agreement with the ones presented here. This work represents the first microsatellites loci for this family.

In summary, we developed and characterized several microsatellite loci for *C.filosa*, whose application will be useful for analyzes of the genetic population structure of this amphipod, providing impactful information regarding its ecology and behavior. Finally, we tested these markers using *C.filosa* from from the southeast of Brazil, but we encourage testing them on populations from other locations.

Table 1. Characteristics of ten polymorphic microsatellite loci developed for *Cymadusa filosa.* Primer sequences; repeat motif; TD, range of temperature for touchdown PCR amplification; size range including M13 tail; A, number of alleles; H_E, expected heterozigosity; H_O,observed heterozigosity; PCR reaction used; P, P-value for deviation from Hardy-Weinberg equilibrium or NI indicating insufficient information to compute estimates and/or confidence intervals; $F_{NA,}$ frequency of null alleles; GenBank accession number

Locus	Primer Sequence (5' - 3')	Repeat Motif	TD(ºC)	Range (bp)	A	H_E	H_O	PCR	P	F_{NA}	GenBank
Cym01	F: ACGAGCCCAAGCTTCATACA R: GTGGAGACTAGAAAGGGCGG	$(GT)_{12}$ $(GT)_4$	57 – 52	137 – 176	26	0.943	0.933	PCR1	0.4453	-	KX389907
Cym02	F: CCGTTGGATTCTGTTTAGTTC R: TAATAAGGACTCGGATTTTGAT	$(AT)_3$ $(GA)_3$ $(GA)_4$	57 – 52	287 – 292	3	0.649	0.733	PCR2	1.0	-	KX389908
Cym03	F: TAGTTTCATAATTGCTGTGGTG R: ATTGTATTTTGTCCCTTTTGAC	$(AT)_4$	52 – 47	164 – 179	4	0.429	0.467	PCR1	1.0	-	KX389909
Cym04	F: TGTGTTCATACTCCATTGCTAA R: TGGCAAGGAAAGTCAAAT	$(CT)_4$	52 – 47	213 – 222	4	0.127	0.133	PCR2	0.7690	-	KX389910
Cym05	F: TTCTTCCTAATCAAAAGCATCA R: TCTTTATCCTGACTGGGTGTC	$(AT)_3$	52 – 47	170 – 172	2	0.206	0.233	PCR2	0.0170	0.11	KX389911
Cym06	F: TTCACAAAGAAGTGGGGAAA R: TCTCATTGGTTCAGGAAAAA	$(AG)_3$ $(AT)_3$	57 – 52	244 – 248	3	0.096	0.033	PCR2	NI	-	KX389912
Cym07	F: ATTCATGCATAATCATCTGGTG R: TCCAAGTGTTTTTAATCAATCG	$(AG)_3$	65 – 60	156 – 175	4	0.186	0.133	PCR2	NI	-	KX389913
Cym08	F: CTAGGAGAGGGAGAACCAG R: CTCTTAAATTCTGCAACACTTC	$(GA)_3$	52-47	159 – 160	2	0.033	0.033	PCR2	NI	-	KX389914
Cym09	F: ACGCCATCCTGTTATTCACG R: AGAGATCAACCACCCTGTCCAC	$(TG)_3$	57-52	184 – 187	2	0.033	0.033	PCR2	0.1497	0.08	KX389915
Cym10	F: TAGTTCTGTTTGTCCTCGTGAT R: ACTGTTTGCCGGTTGTAGC	$(TC)_3(TTG)_4$	52-47	149 – 150	2	0.033	0.033	PCR1	0.1481	0.02	KX389916

Table 2. Characteristics of twelve monomorphic microsatellite loci for *Cymadusa filosa.* Primer sequence; repeat motif; TD, range of temperature for touchdown PCR amplification; size range including M13 tail; PCR reaction used; GenBank accession number.

Locus	Primer Sequence (5'-3')	Repeat Motif	TD(ºC)	Length	PCR	GenBank
Cym11	F: TCCGTGATACTCGTGACC R: ATGGAAATGTATTGCTGCTA	$(TA)_3$	52-47	176	PCR2	KX389917
Cym12	F: TGCAGCACAAATAGTCATCCAC R: AAGTATCCCATCGAAGAGAAGG	$(TC)_3$	52-47	208	PCR2	KX389918
Cym13	F: TCAAATTATCGAGAGCAACAAG R: ACATTTAATTTGTTCGCACCTA	$(TA)_3$	52-47	200	PCR2	KX389919
Cym14	F: TGGCATCATTTGGACTGAGA R: CAAGAAATCGTGGCAACCTATT	$(TG)_3$	52-47	236	PCR2	KX389920
Cym15	F: CGACCACAGGAGACTAAAATAA R: GAAGTGCTAATGAAGACAGAAC	$(AT)_3(AT)_3(AAT)_3$	57-52	274	PCR2	KX389921
Cym16	F: GATAAAAAGTTGCCTGCTGC R: GGAGCCAGTTTTATGTCACTAA	$(TA)_3$	57-52	192	PCR2	KX389922
Cym17	F: CACCTTCACCATGTCGGAA R: GCTAGTTGGGGAATCATTGAA	$(TA)_3$	54-49	186	PCR1	KX389923
Cym18	F: TGATACCTTGGCGTTATTCTTA R: AATGCTCACAGTTTCTTCCCT	$(TA)_3$	54-49	134	PCR1	KX389924
Cym19	F: AAGTTTTGCTCAATGGGTTAC R: CATTGAACTCATCTTGGGTATT	$(AG)_3$	52-47	200	PCR2	KX389925
Cym20	F:AAGCGGTATTTGGTAGATTAGG R: GGTCTGTTTTCTCATTTTATCC	$(AT)_3$	52-47	239	PCR2	KX389926
Cym21	F: TTGATCAGCTTTCCCGAG R: TTTTCTATCGACCCACTCATTT	$(GT)_3(GA)_3$	52-47	241	PCR2	KX389927
Cym22	F: TGGGATTATTTTTCGTTGAG	$(AT)_3$	52-47	230	PCR1	KX389928

R: CTCAATGTGGGCTTATCTTATC

Capítulo 2

Capítulo 2

Multiple scale approach reveals a metapopulation structure with phenotypic divergence in a direct developer marine invertebrate.

Introduction

An important aspect in studies on ecology and evolution is the scale that is being considered, because the processes that originate patterns may operate at different scales (Levin, 1992). Exploring multiple scales can provide inferences that connect complex interactions of evolutionary, behavioral, ecological and stochastic processes (Balkenhol *et al*. 2009; Anderson *et al*. 2010), especially because inferences on patterns and processes beyond or below the extent of a study cannot be made (Wiens, 1989). In this sense, gene flow has a strong role in determining spatial scale over which genetic differentiation happens. Thus, conducting population genetic analysis including multiple spatial scales of sampling can reveal relevant processes affecting genetic variation, for example, micro-differentiation in spatially closer individuals (Epperson, 1993; Andrade & Solferini, 2007).

Besides that, natural selection may be responsible for adaptive phenotypic divergence when there is low gene flow among variable habitats, and, on the other hand, there is reduced trait divergence when there is high gene flow (Garant *et al*., 2007). Therefore, the balance of natural selection-gene flow will determine if an ecologically important trait in a given population will diverge or homogenize in relation to other locations (Slatkin, 1987; Schluter, 2000).

Marine environments, in contrast to terrestrial, were once considered as open systems, where organisms would show very low geographical differentiation especially because of the lack of physical barriers to gene flow (Palumbi, 1992). However, factors as marine currents, water temperature and salinity, habitat discontinuity, mobility of species, anthropogenic activities and, notably, the diversity of life history of marine organisms can affect subdivision of populations and adaptive divergence (Palumbi 1992; Edmands & Potts 1997; Collin 2001; Nielsen & Kenchington 2001; Luttikhuizen *et al*. 2003; Baus *et al*. 2005; Kenchington *et al*. 2006; Crispo & Champman, 2008). Dispersal potential has been considered an important biological aspect of species to predict geographical differentiation. Larval phases are commonly associated with long-distance dispersal organisms, which maintain genetic

homogeneity of interconnected local populations (Ayre *et al*, 1997); contrastingly, lack of larval phases (i.e. direct developer species) are associated with increased genetic subdivision and inbreeding (e.g., Ellstrand & Ellam 1993; Knowlton & Jackson 1993; Thornhill 1993; Frankham 1995). However, not all species fit this theoretical implications, and understanding how dispersal occurs determines the scale at which species are interacting with their habitat, responding to disturbance and evolving (Roughgarden *et al*. 1988, Hanski 1999).

In this context, studies about plant-herbivore interactions in marine environments are an interesting model for testing geographical differentiation in multiple scales, because host-plant may play an important role on the evolution of small herbivores (Arrontes, 1999). Plants and macroalgae, in local scales, represents not only food for these animals, but refuge from predators as well (Price *et al*. 1980, Hay *et al*. 1987). Besides, these interactions may be influenced by chemical and morphological traits that plants and macroalgae may present in fine scales, and by dispersal capabilities of different taxa and occurrence of host-plant in macro scales (Hay, 1996; Singer & Stireman III et. al., 2003; Pereira & Gama, 2008; Paul et. al., 2011). However, these are questions that are not so explored for marine taxa.

It is described for terrestrial environments, more frequently, that the host plant specificity can be the cause of genetic structuring of insects, as aphids and butterfly populations (Peccoud *et al*., 2009; Nice & Shapiro, 2001), influencing evolution of these small herbivores (Futuyma & Moreno, 1988). In these examples, these animals may become adapted to the plants because conditions provided by hosts are a strong selection force considering that the choice of habitat and food source is connected with mate encounter (Via, 2001). Host-plant association may cause morphological differences in insects as well, especially because diet, which is connected with habitat choice, may influence the morphology of a given structure (Jorge *et al*., 2011) or because hosts are not structurally equal , which may cause natural selection to act differently (Nosil & Crespi, 2004) . In marine realm, amphipods are a very common small herbivores in rocky shore environments (Leite *et al*, 2007), being considered ‘insect-like’ organisms because these animals are small in comparison to their host macroalgae, they occur in high densities and have a strict relationship with their host (Hay *et al*., 1987; Poore *et al*., 2008).

These small herbivores use species-specific pheromones in order to find sexual partners to reproduce (Borowsky, 1984; Borowsky, 1985; Borowsky & Borowsky, 1987). Pheromones may be modified depending on the host substrate that amphipods inhabit

promoting different rates of mate encounter in a local population (Stanhope *et al*., 1992a). The estuarine amphipod *Eogammarus confervicolus* that lives in the macroalgae *Fucus*, for example, is more pheromon attracted by the ones that inhabits and eats *Fucus* in comparison to other individuals that inhabit others substrates (Stanhope *et al*., 1992a). The authors argued that food source was able to modify the molecular structure of pheromones during their physiological production and these modifications were being recognized by individuals that inhabit that specific substrate (i.e. macroalgae *Fucus*), connecting host substrate with mate encounter.

Additionally, it is common that herbivorous amphipods construct tubes using algae and inhabit it, being considered sedentary organisms (Appadoo & Myers, 2003), and they usually feed on their host macroalgae (Taylor & Steinberg, 2005), which are different in chemical traits and composition (Hay & Fenical, 1988). This strict relation between the amphipod and its host macroalgae is able to cause local adaptation in some populations, especially because of differences in chemical traits and composition (Hay & Fenical, 1988; Sotka & Hay, 2002; Sotka *et al*, 2003). Once that amphipod-macroalgae and insect-plant interactions might be similar ecological systems, we might expect similar responses related to genetic and morphological variation among host-plant in local scales.

Variation in macro scales, on the other hand, may be influenced by dispersion of amphipods. This group has direct development, brooding juveniles in a ventral pouch called marsupium (Havermans *et al*. 2007). It is described that direct developers species, commonly, are highly genetic subdivided or present a pattern of isolation by distance (Kyle & Bounding, 2000; Sherman *et al*., 2008), and studies on genetic structure of amphipods usually confirm this prediction (Kane *et al*., 1992; Duan *et al*., 2000; Baird *et al*., 2011).

In the present study, we aimed to investigate the role of hosts on genetic and morphological diversity of small herbivores in marine environment. We asked here if host macroalgae influence on genetic structure and in shape variation of the herbivorous amphipod *Cymadusa filosa* Savigny,1816, investigating this influence on local scale (i.e. individuals from the same rocky shore) and on regional scale (i.e. individuals from different rocky shores). Host macroalgae play an important role in mate encounter for these small herbivorous because it may be causing non-random reproduction and are chemically and structurally different, so we expect genetic and morphological signatures of population subdivision in local scales. Additionally, dispersion in these animals may be limiting gene

flow in regional scales. Here, we tested the hypothesis that: 1) in local scales, the identity of host macroalgae is related to genetic subdivision (i.e. each host plant specie are inhabited by a different population) and phenotypic divergence among hosts; 2) in regional scales, *Cymadusa filosa* from different locations are genetically structured because of poor dispersal capability, as predicted for direct developer species.

Material and Methods

Sampling design

In order to explore local and regional scale structure, we performed a hierarchical sampling design. The sampling area is located in Southeastern Brazil, Ubatuba region (Figure 1). We collected individuals of *Cymadusa filosa* located in five different rocky shores: Fortaleza (23º52'S, 45º16'W), Domingas Dias (23º49'S, 45º16'W), Enseada (23º49'S, 45º09'W), Lamberto (23º50'S, 45º11'W) and Itaguá (23º45'S, 45º05'W). Fortaleza and Itaguá shores (south and north limits) are separated by approximately 30 km. In each location we sampled *C. filosa* inhabiting different host-macroalgae: *Sargassum* spp., *Galaxaura stupocaulon* and *Padina gymnospora*. *Sargassum* spp. are brown algae that are very abundant, and provide a highly complex habitat for the associated fauna; *Padina gymnospora* is a brown algae as well, but provide a less complex habitat in comparision with *Sargassum* spp.; *Galaxaura stupocaulon* is a red algae that provide a highly heterogenous environment as well (Joly, 1965). Every location presented all macroalgae species occurring in patches on the sublittoral zone. This method enables us to explore aspects of populations within rocky shores (local scale) and aspects among different locations (regional scale).

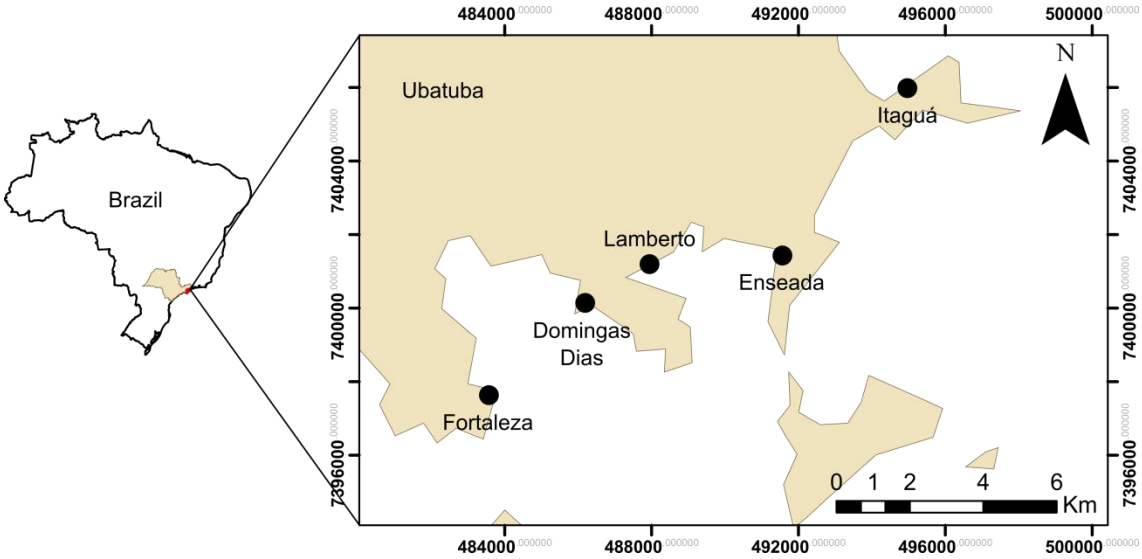

Figure 1. Map of study sites in Ubatuba, southeastern Brazil. Datum of map is WGS 1984 UTM Zone 23S.

We were not able to collect the individuals of *C. filosa* directly in the field, so we sampled 40 fronds of each macroalgae species in each shore. Each individual plant was detached from the rock with a knife and gently enclosed in a plastic bag, both procedures occurred underwater. The minimum distance among fronds was 1m, and the maximum was 50 m. On each shore we assigned the exact place in which macroalgae were collected. This was done by stipulating a horizontal transect along all the extension of the rocky shore where seaweeds occurred, which allowed us to attribute the exact position where hosts were collected by using its location on the horizontal transect and the perpendicular distance that macroalgae were from the transect. Therefore, we could represent *C. filosa* geographic position within the rocky shore as a (x,y) coordinate. We performed this in all the locations. In the laboratory, each sample was transferred to a bucket with water and vigorously shaken, so *C. filosa* individuals could be separated. Each individual had an indication of its host-macroalgae, which was its position in the rocky shore, and from which location it was sampled. We preserved all individuals in 100% ethanol and stored at -20ºC. Although we attempted to sample the same number of individuals for each host-macroalgae, sampling was constrained by the scarcity of individuals species depending on the rocky shore.

DNA collection

Amphipod juveniles may recruit to the immediate vicinity of their parents (Thiel, 1999). Thus, in order to avoid genetically related individuals in the analysis, when more than

one individual was present in a unique frond, we selected the bigger one to perform genetic procedures. In total, 279 individuals of *C. filosa* were included in genetic analysis, (Table 1). Before DNA extraction, the females were examined in stereomicroscope to guarantee that they were not carrying eggs or juveniles in the marsupium, what could led to amplification of extra DNA samples and misinterpretation of results. Genomic DNA was extracted from the entire individuals following a modified salt-extraction protocol according to Aljanabi & Martinez (1997).

Table 1. Number of individuals of *Cymadusa filosa* used for genetic and geometric morphometric analysis

Location	**Host macroalgae**	**Nº individuals for genetic analysis**	**Nº individuals for geometric morphometric analysis**
Domingas Dias (23º49'S, 45º16'W)	*Galaxaura*	21	10
	Padina	23	10
	Sargassum	28	10
	Total	72	30
Enseada (23º49'S, 45º09'W)	*Galaxaura*	15	5
	Padina	10	2
	Sargassum	14	10
	Total	39	17
Fortaleza (23º52'S, 45º16'W)	*Galaxaura*	12	10
	Padina	15	9
	Sargassum	19	8
	Total	46	27
Itaguá (23º45'S, 45º05'W)	*Galaxaura*	23	10
	Padina	25	10
	Sargassum	29	9
	Total	77	29
Lamberto (23º50'S, 45º11'W)	*Galaxaura*	20	10
	Padina	16	9
	Sargassum	9	2
	Total	45	21

Microsatellite analysis

All amphipods were genotyped at the same 10 polymorphic microsatellite loci developed by Peres *et al*. (submitted results). Polymerase chain reaction (PCR) amplifications were performed in a final volume of 10uL or 15 uL, depending on the locus, as described in Peres *et al*. Each PCR contained 1.5 ng of DNA template; 1X PCR buffer; 3mM magnesium chloride; 4ug BSA; 0.2 uM of each dNTP; 0.1 uM of each primer; 0.1 uM of 700 or 800 nm infrared dyes (Li-Cor Biosciences) and 1 U*Taq* DNA polymerase. All loci were amplified using touchdown PCR, according to the following thermocycling conditions: 94ºC for 4 min; 10 X [94ºC for 45s, 65 or 57 or 52ºC (-0,5ºC/cycle) for 1 min and 72ºC for 1 min 15s]; 25 X [94ºC for 45s, 50ºC for 1 min and 72ºC for 1min 15s]; and 72ºC for 10 min. To each forward primer we added a M13 tail (5'- CACGACGTTGTAAAACGAC -3') at its 5' end, which enabled the fragments to be scored on 6.5% polyacrylamide gels on Li-Cor 4300 DNA Analyser (Li-Cor Biosciences). Allele were determined based on its length.

For statistical analysis, we organized our data in fifteen groups that represent the three different hosts in each rocky shore (i.e. one group per host-macroalgae) in five different locations (i.e. five rocky shores where individuals were sampled). To evaluate genetic differentiation within and among groups, we calculated the fixation index F_{IS} as an estimator of inbreeding using GENETIX 4.05 (Belkhir *et al.*, 2004) employing 10000 bootstrap iterations. Pairwise F_{ST} was used as an estimator of subdivision of populations (Weir & Cockerham, 1984), and was performed in FSTAT ver 2.9.3.2 (Goudet, 1995). The significance levels of 0.05 were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989).

We performed an analysis of molecular variance (AMOVA) with 1000 permutations to explore the partitioning of genetic variation among groups (Excoffier *et al*.,1992) using Arlequin 3.5 (Excoffier & Lischer, 2010). Levels tested were: variation among individuals inhabiting a same host-macroalgae species; variation among host-macroalgae nested within rocky shore; variation among locations (AMOVA I). Additionally, based on geometric morphometrics analysis (see Results), we also performed a second test (AMOVA II) using groups that were separated based on morphological similarity of the locations, which enable us to evaluate if this morphological clusters are also separated genetic clusters.

The patterns of population structure were further investigated using a Bayesian approach implemented in STRUCTURE 2.3.3 (Pritchard *et al*., 2000) without a priori

assumption of subdivisions in the populations. Under an admixture model and correlated allele frequencies, 30 independent Markov Chain Monte Carlo (MCMC) runs were carried out with 5.0 x 10^5 iterations following a burn-in-period of 5.0 x 10^5 for each value of the number of clusters (K) ranging from 1 to 20. We determined the most likely number of clusters (K) using the comparisons of Ln Pr (X|K) (Pritchard *et al*., 2000) and the *ad hoc* ΔK method (Evanno *et al*. 2005). We also conducted a principal coordinates analysis (PCoA) to evaluate population structure, which considers *a priori* assumption of populations, performed using GeneAlEx 6.5 (Peakall & Smouse, 2006; Peakall & Smouse, 2012).

In order to examine the effect of geographic distance on genetic differentiation (isolation by distance - IBD) in both local and regional scales, a Mantel test (Mantel, 1967) was carried out between pairwise log transformation of geographic distances and pairwise $F_{ST}/(1-F_{ST})$ estimates with 1000 permutations using GENEPOP'007 (Rousset, 1997; Rousset, 2000 Rousset, 2008). This analysis were performed within each of the five rocky shore using comparisons between individuals and their specific geographic position (local scale), and among all locations (regional scale). Therefore, we were able to evaluate effects of IBD among individuals in each shore, and among the groups that represented the five locations.

Geometric Morphometric analysis

To test the hypothesis that morphology varies in local scale (among different host algae from the same rocky shore) and regional scale (among different locations), we used a geometric morphometric approach (Rohlf, 1990; Zelditch *et al*., 2004). Only adult males were used to avoid misinterpretation of results due to sexual dimorphism (Conlon, 1991). We choose the outer surface of the right gnathopod II propodus because it is a rigid structure that is not susceptible to deformation caused by sample procedures (Zelditch *et al*., 2004), and because it is a structure associated with biological aspects of this species (e.g. tube construction, hold onto host-macroalgae)(Appadoo & Myers, 2003). In total, we analyzed 125 individuals from *Sargassum*, *Galaxaura* and *Padina* from five locations (for details, see Sampling). We made an effort to guarantee 10 individual per host macroalgae in each rocky shore, however, in some cases this was not possible because males were absent (Table 1).

Images were acquired using a Zeiss AxioCam camera and AxioVision software version 4.8 (Carl Zeiss, Inc., Thornwood, New York). Landmarks were digitized using the software tpsDig 2.14 (Rohlf, 2009). Six landmarks were defined along the right gnathopod II margin (Figure 2).

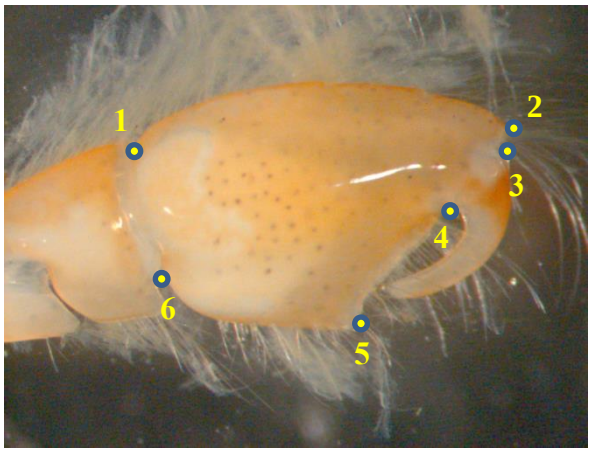

Figure 2. Landmarks used in geometric morphometric analysis of *Cymadusa filosa* gnathopod II. 1: proximal carpus-propodus articulation point of dorsal region; 2: distal corner of dorsal region; 3 – 4: propodus-dactilus articulation point; 5: distal corner of ventral region; 6: proximal carpus-propodus articulation point of ventral region.

After digitizing, we performed a Generalized Procrustes Analysis (GPA), which is an optimally superimposition of homologous landmarks that uses a least-squares algorithm to align all landmarks, removing any shape unrelated information, such as position, orientation and scale (Rohlf & Slice, 1990). The thin-plate spline (TPS) analysis was used to visualize changes in morphology. This procedure generates deformation grids by dividing shape change into uniform components (UC), describing global changes, and non-uniform components, which describes local variation in specific regions (Zelditch et. al., 2004). Finally, a relative warps analysis (RW), analogous to principal component analysis (PCA), was undertaken for the non-uniform componente generating new shape variables (relative warps scores - RWs) (Rohlf, 1993), providing coefficients that can be used in descriptive and inferential statistical tests (Rohlf, 1999). All this routine was performed in tpsRelw 1.46 (Rohlf, 2008).

Then, the values of UC and RW scores were compared separately using a 2-way multivariate analysis of variance (MANOVA), using ‘host-macroalgae’ and ‘location’ as factors. Centroid size (CS), defined as the square-root of the summed squared distances between all landmarks, was used as a proxy for propodus size (Bookstein, 1991; Zelditch *et al*., 2004) and compared using a 2-way analysis of variance (ANOVA), using the same factors described above. Propodus shape variation along relative warps, or uniform axes, was

described by deformation grids obtained in TPS analysis. When necessary, Student-Newman-Keuls (SNK) post hoc test was performed.

Results

Genetic analysis

Measures of fixation index were low for groups inhabiting different host-macroalgae within each rocky shore, and when considering locations, F_{IS} values were low too (Table 2). Pairwise $F_{ST,}$ which was used to represent genetic differentiation among groups, were also low for every scale considered (Table 3 and 4). Even though our test after 1000 permutations presented some significant p values, average F_{ST} suggests low population genetic structure (Wright, 1978).

Table 2. Genetic diversity of *Cymadusa filosa* for 10 polymorphic microsatellite loci. Values represent the mean for the 10 loci ± standard error. N_A : number of alleles; NE: number of effective alleles; I: information index; H_O: observed heterozygosity; H_E: expected heterozygosity; F_{IS}: fixation index with confidence interval (95%).

		N_A	N_E	I	H_O	H_E	F_{IS}
Domingas Dias	*Galaxaura*	3.50 ± 1.54	2.42 ± 1.11	0.51 ± 0.26	0.25 ± 0.11	0.23 ± 0.10	-0.044 (-0.204 - 0.069)
	Padina	3.60 ± 1.73	2.68 ± 1.32	0.55 ± 0.27	0.28 ± 0.10	0.25 ± 0.10	-0.074 (-0.208 - 0.004)
	Sargassum	3.70 ± 1.63	2.50 ± 1.19	0.51 ± 0.27	0.20 ± 0.08	0.22 ± 0.10	0.079 (-0.061 - 0.185)
	Total	5.1 ± 2.79	3.02 ± 1.67	0.58 ± 0.30	0.24 ± 0.10	0.24 ± 010	0.001 (-0.073 - 0.064)
Enseada	*Galaxaura*	3.40 ± 1.21	2.48 ± 1.07	0.59 ± 0.24	0.30 ± 0.11	0.27 ± 0.10	-0.065 (-0.239 - 0.012)
	Padina	2.80 ± 0.94	2.21 ± 0.85	0.53 ± 0.22	0.29 ± 010	0.26 ± 0.09	-0.049 (-0.320 - 0.062)
	Sargassum	2.80 ± 1.15	2.15 ± 0.91	0.45 ± 0.24	0.20 ± 0.09	0.21 ± 0.09	0.088 (-0.131 - 0.223)
	Total	4.1 ± 1.9	2.66 ± 1.33	0.58 ± 0.27	0.26 ± 0.10	0.25 ± 0.09	-0.019 (-0.122 - 0.055)
Fortaleza	*Galaxaura*	3.20 ± 1.55	2.48 ± 1.22	0.49 ± 0.27	0.23 ± 0.10	0.21 ± 0.10	-0.029 (-0.222 - 0.075)
	Padina	3 ± 1.35	2.37 ± 1.13	0.47 ± 0.25	0.20 ± 0.08	0.21 ± 0.09	0.084 (-0.086 - 0.172)
	Sargassum	4.40 ± 2.08	2.62 ± 1.32	0.55 ± 0.28	0.23 ± 0.10	0.23 ± 0.09	-0.002 (-0.152 - 0.104)
	Total	5.20 ± 2.88	3.26 ± 1.99	0.57 ± 0.31	0.22 ± 0.09	0.22 ± 0.10	0.017 (-0.049 - 0.097)
Itaguá	*Galaxaura*	3.70 ± 1.83	2.66 ± 1.37	0.50 ± 0.28	0.21 ± 0.10	0.21 ± 0.10	0.085 (-0.097 - 0.214)
	Padina	4.10 ± 2.23	3.08 ± 1.83	0.50 ± 0.30	0.21 ± 0.10	0.20 ± 0.10	-0.042 (0.151 - 0.026)
	Sargassum	4 ± 2.12	2.57 ± 1.31	0.51 ± 0.28	0.20 ± 0.09	0.21 ± 0.10	0.054 (-0.093 - 0.175)
	Total	5.2 ± 2.99	3.18 ± 1.91	0.54 ± 0.31	0.21 ± 0.10	0.21 ± 0.10	0.028 (-0.049 - 0.097)
Lamberto	*Galaxaura*	3.7 ± 1.49	2.31 ± 1.03	0.54 ± 0.25	0.21 ± 0.08	0.24 ± 0.09	0.124 (-0.021 - 0.190)
	Padina	2.5 ± 0.87	1.71 ± 0.54	0.38 ± 0.21	0.16 ± 0.09	0.17 ± 0.09	0.088 (-0.120 - 0.214)
	Sargassum	2.6 ± 0.85	1.86 ± 0.60	0.45 ± 0.21	0.24 ± 0.09	0.24 ± 0.09	-0.003 (-0.267 - 0.050)
	Total	4.5 ± 2.18	2.30 ± 1.06	0.53 ± 0.27	0.20 ± 0.08	0.22 ± 0.09	0.085 (-0.005 - 0.149)

Table 3. Pairwise F_{ST} among groups of *Cymadusa filosa* from different locations. Values in bold represent significant values after Bonferroni correction.

	Domingas Dias	Enseada	Fortaleza	Itaguá	Lamberto
Domingas Dias	0				
Enseada	**0.0257**	0			
Fortaleza	0.0173	**0.0352**	0		
Itaguá	**0.0261**	**0.0196**	**0.0262**	0	
Lamberto	**0.0306**	**0.0141**	**0.034**	0.0054	0

Tabela 4. Pairwise F_{ST} among groups of *Cymadusa filosa* from different host macroalgae in each rocky shore. None of the values were significant.

Domingas Dias			
	Galaxaura	*Padina*	*Sargassum*
Galaxaura	0		
Padina	0.012	0	
Sargassum	0.0156	0.0214	0

Enseada			
	Galaxaura	*Padina*	*Sargassum*
Galaxaura	0		
Padina	-0.0226	0	
Sargassum	0.0001	0.0115	0

Fortaleza			
	Galaxaura	*Padina*	*Sargassum*
Galaxaura	0		
Padina	-0.0144	0	
Sargassum	0.0116	0.0004	0

Itaguá			
	Galaxaura	*Padina*	*Sargassum*
Galaxaura	0		
Padina	-0.0081	0	
Sargassum	-0.006	0.0032	0

Lamberto			
	Galaxaura	*Padina*	*Sargassum*
Galaxaura	0		
Padina	-0.0005	0	
Sargassum	-0.004	0.0035	0

When considering the hierarchical partitioning of genetic variation for AMOVA I, almost all genetic variance was a result of differences within groups (97.5%), showing that they were not forming a structured population (Table 5).

Table 5. Results for analysis of molecular variance (AMOVA) testing for differences among locations and host macroalgae (AMOVA I) and considering morphological clusters (AMOVA II). Groups are individuals from the same location inhabiting the same macroalgae specie. Values in bold represent $p<0.05$.

Source of variation	Variance components	Fixation Index	% variation
AMOVA I			
Among locations	0.02710	**0.02481**	2.26909
Among groups within rocky shore	0.00253	0.00217	0.21227
Within groups	1.16492	**0.02269**	97.51864
AMOVA II			
Among morphological clusters	0.0109	**0.0212**	0.91178
Among groups within morphological clusters	0.01911	0.01613	1.59874
Among individuals within groups	0.02471	**0.00912**	2.06668
Within individuals	1.1407	0.04577	95.4228

Regarding the STRUCTURE analysis, the most likely number of groups is K = 2 for both methods of estimating clusters (Pritchard *et al.*, 2000; Evanno *et al.*, 2005). However, the probability of assignment of our individuals were all intermediate (Figure 3), which we may consider as that there is no genetic structure among populations, once the methods proposed to estimate K cannot find the best K if K = 1 (Evanno *et al*., 2005).

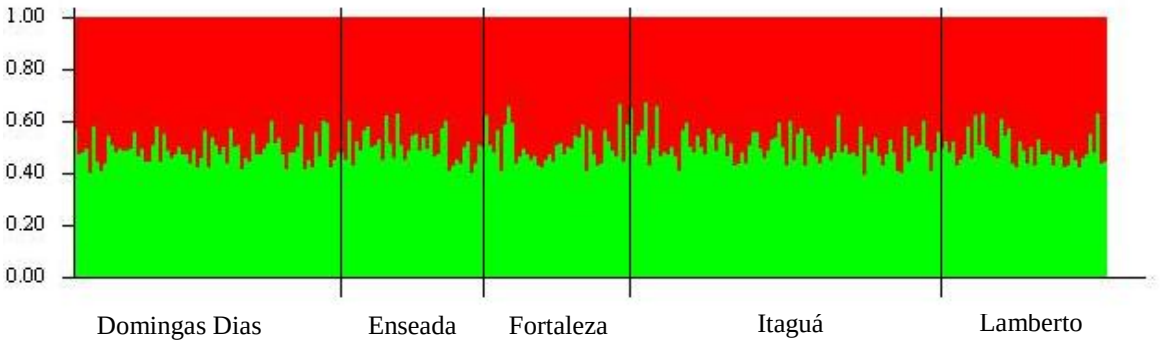

Figura 3. STRUCTURE analysis considering K = 2. Each vertical bar represent na individual and each colour the probability of that individual be assigned for each genetic cluster. Individuals location is indicated.

Similarly, the PCoA retained 11.25% and 10.28% of the total variance in the first and the second axes, respectively, indicating that individuals from populations are not genetically different (Figure 4).

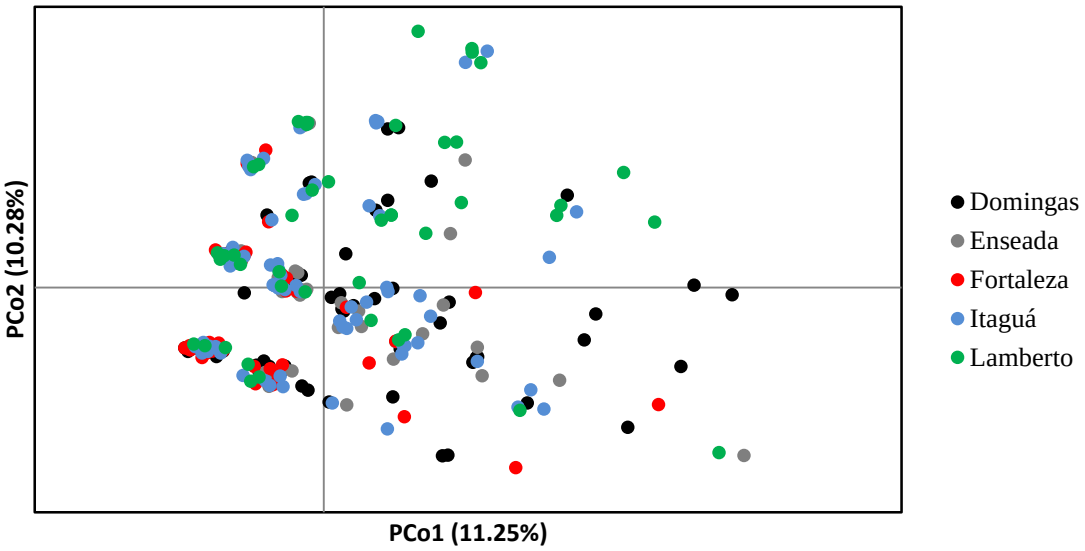

Figure 4. Principal Coordinate Analysis (PCoA) scatterplot considering the two principal coordinates.

Mantel test did not reveal a pattern of IBD among locations, indicating that genetic distances were not correlated with geographic distances. When testing for this pattern within rocky shores, our results also reported that individuals spatially closer were not related in four of the shores. However, we found an IBD pattern among individuals in one rocky shore (Domingas Dias).

Geometric Morphometric analysis

Morphometric analysis on propodus shape of C. filosa resulted in eight relative warps (RWs) and two uniform components (UCs). The first relative warps (RW1) accounted for 37.68% of variation, and second relative warps (RW2) for 22.25%. Variation in distal corner of dorsal region and in propodus-dactilus articulation point were the ones that most counted for distinct morphologies. Results from the MANOVA showed that there were no significant differences in local scale morphology (i.e. among individuals inhabiting different host-macroalgae in the same rocky shore), but revealed the existence of two different clusters formed by a Domingas Dias–Enseada (D-E) group and a Itaguá–Lamberto–Fortaleza (I-L-F)

group at both the relative warps and the uniform components (Table 7)**.** The propodus dorsal margin is longer than the ventral, distal corner of dorsal region were more pronounced, and the palm is acute in relation to the dactilus in D-E group; on the other hand, I-L-F group did not present these traits, having a dorsal and ventral margin of propodus more equal, with a palm not so acute. In Figure 5, the thin plane spline grids allow the visualization of changes in geometric terms, for RW1 and RW2. The size of propodus (CS), however, was not significant distinct among populations, neither in local nor regional scales comparisons.

Table 7. Results of analysis of variance (ANOVA) for centroid size (CS) and multivariate analysis of variance (MANOVA) for relative warps (RW) and uniform components (UC) for shape variables of *C. filosa.* H-m: host macroalgae; L: location.

	ANOVA for CS			MANOVA for RWs				MANOVA for Ucs			
	df	F	P	Pillai's Trace	*df*	F	P	Pillai's Trace	*df*	F	P
H-m	2	2.367	ns	0.126	16	0.871	ns	0.059	4	1.683	Ns
L	4	1.749	ns	0.581	32	2.253	**	0.181	8	2.739	*
H-m*L	8	1.354	ns	0.644	64	1.204	ns	0.125	16	0.913	Ns
Error	55										

ns: not significant; * P<0.05; **P<0.001

When testing for genetic differentiation in morphologically distinct D-E and I-L-F groups (AMOVA II), we also found that genetic variance was a result of differences within populations (95.4%), and morphological clusters are not genetically different. These results show that variance in propodus shape are not related with connectivity among populations.

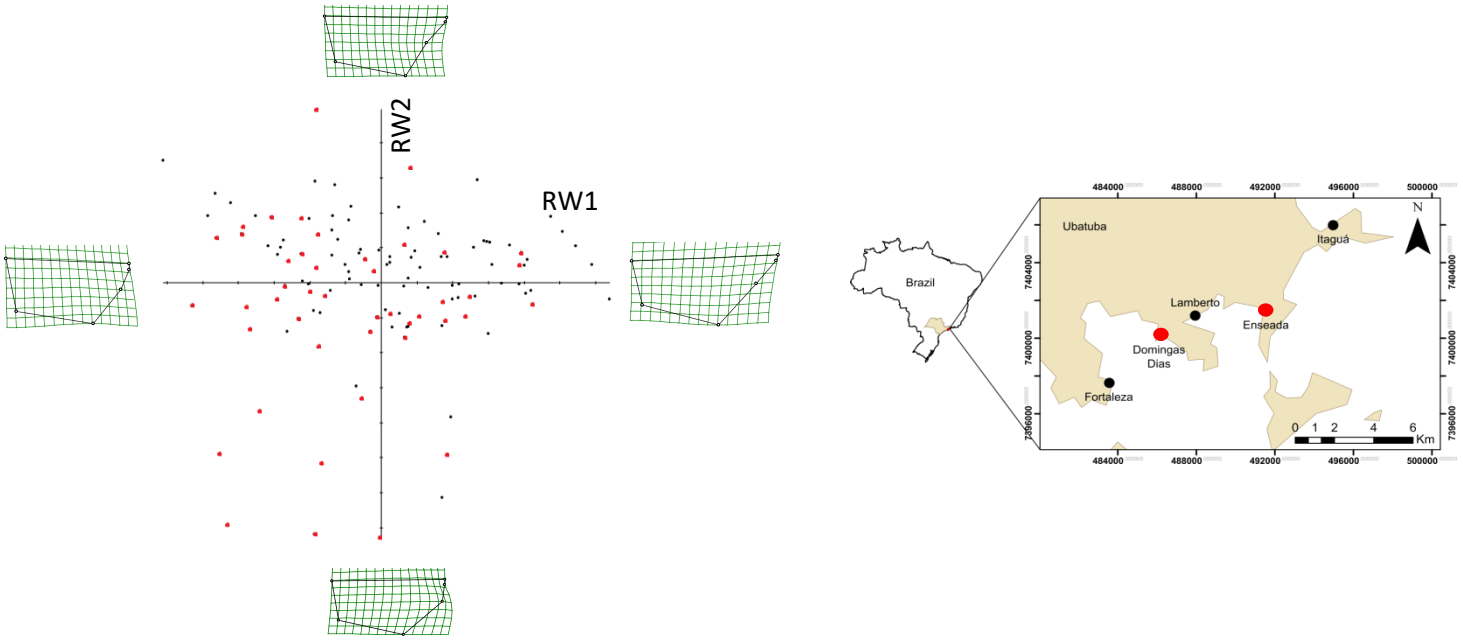

Figure 5. Geometric morphometric results for differences in propodus shape for *Cymadusa filosa*. Axes are relative warps 1 (RW1) and relative warps 2 (RW2), which explained 59,93% of total shape variation. Deformation grids represent propodus shape at extreme values along relative warp axes. Colors represent the two morphological statistically different groups (red: Domingas Dias and Enseada; black: Fortaleza, Itaguá and Lamberto). The map indicate the location of the two morphological clusters.

Discussion

This study represents the first one that compares marine and terrestrial host-herbivore interaction using a genetic and morphological perspective. Our results indicates that, even though exists a potential for ecological divergence among amphipods that are inhabiting different host macroalgae or that are from different rocky shores, there were no genetic structure. *Cymadusa filosa* was not structured in local scales and host macroalgae did not predict different subpopulations, and, in regional scales, rocky shores did not represent different populations as well. Besides that, we did not find genetic support to affirm that juveniles are recruiting next to their parents, so as that *C. filosa* dispersion is explained by distance among locations. On the other hand, we find two different morphological clusters, which were associated with different locations. Our results suggest that this species is organized in a metapopulation structure with phenotypic divergence (Hansky, 1998).

Association of amphipods and its host macroalgae is considered strict because it represents refuge and food for these small herbivorous (Hay *et al*., 1987; Poore *et al*., 2008). This interaction has already shown to be able to generate local adaptation, for example, in the amphipod specie *Ampithoe longimana* (Sotka & Hay, 2002; Sotka *et al*, 2003). Authors observed that individuals from distinct geographical regions from USA coast presented different habitat/food choice and performance depending if their original habitat had or had not the occurrence of the brown algae *Dictyota*, which presents secondary compounds (Sotka & Hay, 2002; Sotka *et al*, 2003). On the other hand, the species *Perampithoe parmerong* had potential for local adaptation according to their host macroalgae in laboratory experiments, however, this potential was not realized in field populations (Poore & Steinberg, 2001). This last study reported that generations of *P. parmerong* raised in aquarium with only one macroalgae species available presented host choice as a heritable trait, but this was not in accord to what was found in natural conditions. Colonization experiments performed in field using multiple potential hosts macroalgae revealed that animals did not show different rates of habitat choose (Poore & Steinberg, 2001).

Macroalgae community in rocky shores is not constant over time, occurring a turnover of species in this environment (Underwood, 1981). This means that a specific macroalgal species cannot be used as food and shelter constantly by small herbivorous. So, considering that there is a turnover of species and variation in abundance of algae, a generalist strategy would be naturally selected instead of a specialist one. It is often reported that these animals habit and feed on many host species (Jacobucci & Leite, 2014), even though a different host choice may induce changes in fecundity and survivorship rates according to which host is used (Cruz-Rivera & Hay, 2000).

Once amphipods did not present a dispersal larval phase (i.e. these animals present direct development), a low potential to disperse over great distances is expected. Besides that, it is considered that amphipods distribution is aggregated, especially because juveniles recruit next to their parents (Thiel & Vásquez, 2000). Additionally, herbivorous amphipods are described as organisms of low mobility, given the fact that some of them are tubicolous (Appadoo & Myers, 2003), which explains the strict interaction with their host macroalgae. In our study, we hypothesize that these biological aspects would result in ecological specialization and/or genetic subdivision of populations in local and regional scales. However, this was not confirmed. Even though there is theoretical support to affirm that the reproductive system and habits of amphipods would make these organisms locally limited,

there is also experimental studies that did not support that (Tanaka & Leite, 2004; Poore & Steinberg, 2001), so as the present work. Field studies exploring local dispersion of amphipods and colonization of macroalgae in rocky shores reported high rates of colonization and high abundances of animals in small periods of time, regarding the distance that algae were attached from the shore (Tanaka & Leite, 2004). Macroalgae species and its traits (e.g. nutritional values) were not related to colonization in local scales (Poore & Steinberg, 2001). Therefore, herbivorous amphipods, despite their natural history, are capable move themselves over distances in the rocky shore and inhabiting a great diversity of host macroalgae.

We did not find genetic differentiation among individuals that inhabit different host species nor that there is a pattern of isolation by distance among them. We expected that because of their pattern of distribution, local recruitment and parental care as a result from direct development, *C. filosa* that were spatially closer would be genetically related (Thiel & Vásquez, 2000). This pattern of isolation by distance in local scales was just explicit in one location (Domingas Dias). A possible explanation of our results is that adults are capable to move along the whole extension of the shore, besides their tubicolous habit (Appadoo & Myers, 2003). Thus, there would not occur a genetic neighborhood, which is the preference of breeding with spatially closer individuals (Rousset, 2000). We may hypothesize that *C. filosa* is actually a highly mobile species when adult, and is capable to find mates along great distances. Another possible explanation for our results is that dispersion for this species occurs during juvenil stages, in other words, right after juveniles hatch from their eggs. For the species *Bemlos unicornis* is already reported that dispersion occurs in this life stage (Munguia *et al.*, 2007), and for *Paracorophium* spp. it is supposed that dispersion occurs by young females (Stevens *et al.*, 2006). Juvenile based dispersion may be a way to avoid inbreeding, given the fact that natural history characteristics of amphipods would favor this situation. A dispersion based on juveniles would cause that mate encounter could occur among individuals that were not geographically close at the same shore, given the fact that they are considered tubicolous and not so mobile organisms, avoiding the existence of clusters of genetically similar animals,. We may hypothesize that a juvenile based dispersion is favored by natural selection because it results in more genetic variability for populations, considering that adults are not so active. However, our data shows that in Domingas Dias rocky shore *C. filosa* had a pattern of IBD. This may be explained by the fact that dispersion and the distance in which it occurs may vary because of local conditions, as wave activity and

predation intensity, which are able to limit movements of juveniles and adults modifying patterns of genetic diversity as a consequence.

The dispersion of amphipods in macro scales (i.e. among locations) have not been elucidated because it is not clear how adults are able to move from one place to another, given the fact that there is not a larval phase. In this sense, it is considered that they are transported by rafting, that is, they are passively drifted by ocean currents using floating substrates (Thiel & Gutow, 2005). For example, kelp-boring species of the isopod genus *Limnoria* excavate extensive burrows in the holdfasts and haptera of large kelps (Thiel & Vasquez, 2000) and have an extensive geographical distribution. Molecular analysis of this brooding species along the coast of Chile indicates that they are able to disperse along great distances because kelps are transported by ocean currents and these animals survive inside the burrows (Haye *et al.*, 2012). On the other hand, the amphipod *Orchestia montagui* has presented genetic structure among populations of Mediterranean Sea that were separated for over 600km and analyzed using microsatellites and mitochondrial DNA as molecular markers (Pavesi et al., 2012). The authors reported the occurrence of two distinct genetic clusters representing populations from Adriatic Sea and other location from Mediterranean Sea, respectively. The genetic structure was revealed in broad scales, however, two populations that were less than 100 km of distance were not genetically distinct, which agrees with the scale that our study was done. This may indicate that rafting can be a powerful dispersal force for animals that not present a larval phase, but distances in which dispersal occurs are modified according to location, local ocean currents and floating substrate. Regardless how amphipods are dispersing, this transport can occur in stepping-stone way (Baird et al., 2012) or through long distance dispersal (Pavesi et al., 2012), which may generate or not a pattern of isolation by distance, respectively. In this study, the distance among of locations studied apparently is not enough to interfere on gene flow, and our locations are continuously changing migrants. Amphipods, besides their natural history, seem to be well adapted to disperse along geographical distances.

Although our results show that there is no genetic structure, which could mean that there is an intense gene flow among locations, we identified two different morphological clusters regarding the shape of gnathopod II. Gnathopods is an important adaptive structure in amphipods because it is used for reproduction, male mating success and feeding (Conlan, 1991; Arndt et al., 2005; Wellborn & Bartholf, 2005), and changes in this body part may have impacts for the individual. Structure divergences that represent adaptive changes under

different environmental conditions can be interpreted as natural selection acting distinctly among population that are under different selective forces or that dispersing individuals are able to phenotypically adapt to new conditions through a habitat induced response (Crispo, 2008). Sultan & Spencer (2002) proposed through modelling that in a metapopulation with migration, plasticity is favored over adaptive divergence. So, in our potential high gene flow scenario with no genetic structure, we can interpret *C. filosa* morphological variance probably as a phenotypic plasticity, which should be explored in future studies. Phenotypic characteristics and patterns of genetic diversity are in some way independent (Brian et al., 2006) and factors as habitat, wave action, predation, food supply, salinity may play an important role on shape variation. For example, amphipods from the genus *Hyalella* present different gnathopods morphology depending on environmental stressors, as predation and food resources (Cothran & Jeyasingh, 2010). Changes in gnathopods may occur via labile plasticity (i.e. when is induced by environment during any life stage and is reversible) or via developmental plasticity (i.e. when is induced by environment during development and is not reversible) (Crispo, 2008), but this can only be affirmed depending on the stage at which selection and or dispersal takes place. We may hypothesize that ocean conditions is one of the causes of different gnathopods shapes because of similar geographical location of Domingas Dias and Enseada, and predation intensity and diet may also have an important role. These topics should be explored in future studies.

In conclusion, the species *C. filosa* is dispersing through all scales that were analyzed, that is, among host macroalgae and rocky shores, which resulted in low genetic differences and in populations that are, actually, multiple subpopulations interconnected as a metapopulation. We suggest two possible scenarios: these amphipods are highly active during adults or during juveniles stages. Both of them could cause admixture of individuals in local and regional scales resulting in a panmitic population. Amphipod-macroalgae interactions are probably more related to factors as food and shelter than with reproduction preferences related to distance or induction of specific pheromones, but algae still provides a way to these animals to disperse long distance via rafting. Even though there is potential for high gene flow among shores, there are distinct morphologies that are associated with locations, probably because of local conditions. Our work gives support to affirm that natural history and reproduction should not be the only way to forecast and describe how species are dispersing, and this topic has to be more explored, especially in marine environments.

Considerações Finais

Este trabalho é o primeiro a utilizar marcadores moleculares em estudos ecológicos com anfípodes para a costa do Brasil, assim como explorando a diversidade genética em pequenas escalas, o que trouxe resultados inéditos sobre aspectos ecológicos e evolutivos desses organismos. Demonstramos que não devemos utilizar apenas informações sobre história natural e reprodução das espécies como base para conclusões sobre dispersão dos indivíduos e interações ecológicas. Nossos resultados demonstram que a espécie *C. filosa* está se dispersando continuamente entre suas algas hospedeiras e entre costões rochosos próximos, gerando uma baixa diferenciação genética entre as localidades, podendo as populações amostradas serem consideradas, na verdade, uma metapopulação (Hanski, 1998), ao contrário do que era previsto baseando-se na literatura. Ou seja, as localidades que foram estudadas formam subpopulações que não se diferenciam geneticamente, provavelmente devido a constante fluxo de migrantes entre os costões rochosos. Além disso, quando analisamos as populações localmente (e.g. mesmo costão rochoso), podemos explicar o cenário encontrado tanto considerando o fato de os indivíduos serem altamente móveis originarem uma população panmítica como também uma dispersão pelos indivíduos jovens. Dessa forma, a interação entre os anfípodes herbívoros e suas macroalgas aparenta estar atrelada mais ao balanço abrigo-alimentação que a fatores como reprodução preferencial de indivíduos de mesma alga hospedeira. Sendo assim, a hospedeira não é um fator que influencia a estrutura genética desses animais, mas que pode servir como veículo para uma dispersão passiva a maiores distâncias. Por outro lado, esse trabalho representa um caso em encontramos grupos morfologicamente distintos apesar do provável fluxo gênico alto. Tal resultado provavelmente indica plasticidade fenotípica gerada por fatores ambientais aos quais não puderam ser totalmente compreendidos. Assim, são necessários estudos complementares para aprofundarmos as discussões aqui propostas. Dentre os tópicos os quais esse trabalho direciona para serem explorados estão a estrutura genética de anfípodes em diferentes escalas, a capacidade de dispersão de espécies com história de vida diferente (e.g. sem hábito tubícola) também explorando as relações de indivíduos próximos, experimentos que visem explicar os fatores capazes de modificar as estruturas desses animais e como a diferenciação morfológica ocorre, assim como estudos de genética de populações de anfípodes para o litoral brasileiro.

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***Interações entre anfípodes herbívoros e algas: a identidade da alga hospedeira influencia na diversidade genética e morfológica de populações de herbívoros?***", desenvolvida no Programa de Pós-Graduação em Ecologia do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Data: 08 de setembro de 2016

Profa. Dra. Rachel Meneguello
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Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Interação entre anfípodes herbívoros e algas: a identidade da alga hospedeira influencia na diversidade genética e morfológica de populações de herbívoros?**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 08 de setembro de 2016

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