Universidade Estadual de Campinas

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Padrões de distribuição genotípica em litorinídeos (Mollusca: Gastropoda) da costa Brasileira

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Genética e Biologia Molecular na área de Genética Animal e Evolução.

Orientadora: Profa. Dra. Vera Nisaka Solferini

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E valeria a pena, afinal,
Teria valido a pena,
Após os poentes, as ruas e os quintais polvilhados de rocio,
Após as novelas, as chávenas de chá, após
O arrastar das saias no assoalho
— Tudo isso, e tanto mais ainda? —
Impossível exprimir exatamente o que penso!
Mas se uma lanterna mágica projetasse
Na tela os nervos em retalhos
Teria valido a pena,
Se alguém, ao colocar um travesseiro ou ao tirar seu xale às pressas,
E ao voltar em direção à janela, dissesse:
"Não é absolutamente isso,
Não é isso o que quis dizer, em absoluto."

- **"A canção de Amor de J. Alfred Prufrock"**, T. S. Elliot
("Prufrock e outras observações", 1917)

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RESUMO

Uma das questões particularmente interessantes em Biologia é compreender o vínculo entre a ecologia e evolução das espécies. Avaliar a relação entre a capacidade de dispersão e a escala espacial na qual as populações diferem geneticamente é uma das formas de entender como esse vínculo é estabelecido. A variação espacial das freqüências alélicas em populações naturais pode ser resultado de isolamento por distância, história populacional ou seleção diversificadora. Análises populacionais em grande e pequena escala são relevantes para avaliar como essas freqüências podem variar espacial e temporalmente. O objetivo desse estudo foi investigar a distribuição da variabilidade em litorinídeos utilizando isozimas como marcador molecular. No primeiro capítulo, o padrão de desvio das proporções de Hardy-Weinberg foi analisado em três espécies de litorinídeos (*Echinolittorina lineolata*, *Littoraria flava* e *L. angulifera*) em uma escala macrogeográfica ao longo da costa Brasileira (cerca de 4.000 km). Um teste de homogeneidade dentro das amostras mostrou que os valores dos F_{IS} são, em sua maioria, heterogêneos. Este resultado exclui endogamia e efeito Wahlund como principais causas do excesso de homozigotos. Em todas as espécies, pelo menos um loco do sistema PGM apresentou valores homogêneos de desvio de Hardy-Weinberg em todas as amostras, sugerindo que essa enzima pode estar sob efeito de seleção natural ou em desequilíbrio de ligação com um loco sob seleção.

No segundo capítulo, avaliamos a subdivisão em escala local em *Littoraria flava* a fim de testar se os desvios de Hardy-Weinberg podem ser explicados por estruturação genética em pequena escala, apesar de essa espécie possuir fase larval planctotrófica. As

amostras foram coletadas em transectos horizontais no costão rochoso em três praias, três vezes em um período de cerca de um ano. Foi realizada uma análise hierárquica de 15 locos polimórficos comparando a estruturação de uma escala de 200 km em relação a uma escala de dezenas a poucas centenas de metros. *Littoraria flava* apresentou maior estruturação dentro dos transectos e entre as diferentes coletas temporais do que entre as praias. Cerca de 18% dos testes de neutralidade de Ewens-Watterson apresentaram desvio significativo de neutralidade. Esses resultados sugerem um equilíbrio entre colonizações recorrentes e coeficientes seletivos variando no tempo e espaço sobre diferentes locos.

No terceiro capítulo estão apresentados os resultados de uma avaliação do efeito do ambiente sobre a forma da rádula de *L. flava* e *L. angulifera*. Além da caracterização da variação morfológica da rádula, foi realizado um experimento de transferência recíproca dos indivíduos entre o mangue e o costão. Nas duas espécies, foi observada menor variação na forma da rádula nos indivíduos coletados no mangue, indicando que cada ambiente tem um efeito diferente sobre esse caráter. No experimento de transferência, as rádulas de *L. flava* apresentaram mudança de forma 40 dias após o início do experimento, apesar do tamanho da fita radular ser fortemente influenciado pelo substrato original ($F_{6,22}$=17,13, p<0,001). Foram observadas mudanças na forma em diferentes intensidades, sugerindo plasticidade fenotípica da forma da rádula.

ABSTRACT

One of the main questions in biology is the link between a species ecology and its evolution. Evaluating the relationship between the geographical scale over which populations differ genetically and the species dispersal ability is a way to understand how this link is established. Spatial variation in allellic frequencies of natural populations may be explained by isolation by distance, population history or diversifying selection. Populational analyses at different scales are appropriate to evaluate how gene frequencies vary in time and space. The main goal of this study was to analyse the distribution of the genetic variability in littorinids using allozymes as molecular marker. In the first chapter, the pattern of heterozygote deficiency was evaluated in three littorinid species (*Echinolittorina lineolata*, *Littoraria flava* and *L. angulifera*) at a macrogeographic scale - along the Brazilian coast (4,000 Km). A homogeneity test among loci showed heterogeneous F_{IS} values in most populations. This result ruled out inbreeding and Wahlund effect as the main causes of departure of Hardy-Weinberg expectations. In the three littorinid species, at least one Pgm locus had homogeneous F_{IS} values along all sampled populations, which suggests that this enzyme may have an important role in the fitness or may be linked to a locus under selection.

In the second chapter, local-scale subdivision in *Littoraria flava* was investigated in order to test if Hardy-Weinberg deviations could be explained by micro-structuring, despite the planktotrophic larval phase. Samples were collected along horizontal transects in rocky shores of three different beaches, three times over a year. With this sampling design, and using 15 polymorphic allozymic loci, we searched for indications of any micro-scale or short-temporal subdivision in contrast with macrogeographic (200 Km) structuring.

Littoraria flava samples presented significantly more structure within transects and along the temporal scale than at large-scale. Eighteen percent of the Ewens-Watterson neutrality test showed significant deviation of neutrality expectation. This suggested that there could be a balance among several recurrent colonizations by cohorts with different allelic frequencies, followed by a directional selection on different loci at different times and localities.

In the last chapter, we assessed if environmental heterogeneity could affect radular form in *L. flava* and *L. angulifera*. We also made a reciprocal transfer experiment in natural conditions between mangrove and rocky shore locations, apart nearby 100 m. Individuals of both species from mangrove showed less variation in the shape of radula than those from rocky shores, implying in a different environmental effect in each species. In the natural transfer experiment, radulae morphology of *L. flava* individuals changed within 40 days, but the length of the radulae were strongly influenced by the original substrate ($F_{6,22}$=17.13, $p<0.001$). Changes in the shape had different intensities, suggesting that this trait could be subject to phenotypic plasticity.

INTRODUÇÃO GERAL

Uma das questões particularmente interessantes em Biologia é compreender o vínculo entre a ecologia e a evolução das espécies. Avaliar a relação entre a capacidade de dispersão e a escala espacial na qual as populações diferem geneticamente é uma das formas de entender como esse vínculo é estabelecido. Estudos sobre estruturação populacional são particularmente relevantes, já que permitem inferências de como as forças evolutivas interagem ao longo da história evolutiva de uma espécie. A estruturação populacional pode refletir o efeito da seleção natural, da história da separação das populações assim como da intensidade do fluxo gênico entre elas.

A estruturação populacional em invertebrados marinhos é mais comum em espécies com desenvolvimento direto devido à falta de conexão entre habitats e à menor possibilidade de colonização de locais distantes (Johannesson, 1988). De forma geral, invertebrados marinhos com altas taxas de dispersão possuem grandes tamanhos populacionais, fluxo gênico constante e, conseqüentemente, grande amplitude de distribuição geográfica; mesmo em ambientes disjuntos, a dispersão planctônica reduziria potencialmente a divergência genética entre as populações (Palumbi, 1992, 1994; Bohonak, 1999). Nessas espécies espera-se também que a escala de estruturação populacional seja da ordem de centenas a milhares de quilômetros de extensão (Palumbi, 1992; Bohonak, 1999).

Hohenloe (2004) realizou simulações usando como modelo duas espécies de gastrópodes litorinídeos com fase larval planctônica e concluiu que: (1) correntes marinhas podem criar uma eficiente barreira ao fluxo gênico, porém temporalmente efêmera; (2) variações temporais longas em fatores abióticos podem ter mais efeito sobre o fluxo gênico do que mudanças temporalmente mais curtas; (3) a duração da fase planctônica tem um

efeito pequeno sobre a taxa de migração, ou seja, há um limite mínimo no qual o tempo de duração larval é suficiente para que haja fluxo gênico a média e longa distância; (4) uma estação longa de desova pode suplantar supostas barreiras ao fluxo gênico geradas por correntes marinhas que variam sazonalmente.

Estudos de populações de diferentes espécies em larga e pequena escala espacial são interessantes para entender as condições necessárias à diferenciação em organismos marinhos com vida adulta bentônica. Mesmo em espécies que possuem fase larval planctotrófica (na qual a desova é lançada na água, e, após a eclosão, as larvas podem ter longa fase pelágica, que dura entre poucas semanas a alguns meses), há vários fatores que podem limitar a dispersão, criando oportunidades para diferenciação genética. Fatores abióticos, tais como gradientes de salinidade, temperatura e correntes marinhas, e alguns processos, tais como isolamento por distância, fatores comportamentais e seleção podem reduzir a migração entre locais relativamente próximos (Palumbi, 1994).

De forma inesperada, alguns invertebrados marinhos com desenvolvimento planctotrófico apresentam subdivisão populacional em uma escala entre dezenas de metros a poucos quilômetros (Koehn *et al.*, 1973; Levinton e Suchanek, 1978; Johnson e Black, 1982, 1984; Watts *et al.*, 1990; Tatarenkov, 1995; Hilbish, 1996; Johnson e Black, 2000; Sotka *et al.*, 2004). Em alguns casos, as espécies apresentaram homogeneidade genética ao longo de grandes distâncias, mas significativa estruturação em uma menor escala (Johnson e Black, 1982, 1984; Watts *et al.*, 1990; Ayvazian *et al.*, 1994; Tatarenkov, 1995; Parsons, 1996; Kyle e Boulding, 2000; Johnson *et al.*, 2001). Essa subdivisão local foi atribuída a fatores seletivos relacionados à heterogeneidade ambiental (Tatarenkov, 1995; Johnson *et al.*, 2001), a condições ambientais (ou biogeográficas) que impediriam a migração e favoreceriam recrutamento local, tais como arquipélagos e estuários (Parsons, 1996;

Ayvazian *et al.*, 1994), ao comportamento da larva em relação a correntes marinhas ou salinidade (Tatarenkov, 1995; Kyle e Boulding, 2000) e também ao recrutamento não sincronizado entre coortes de larvas (Johnson e Black, 1982, 1984; Watts *et al.*, 1990).

Heterogeneidade ambiental e efeitos sobre a variabilidade genética

A formação de populações subdivididas, ocupando ambientes diferentes quanto a características biológicas ou fatores abióticos, seria suficiente para manter a variabilidade genética. Nesse caso, diferentes alelos são selecionados, ou simplesmente fixados, nos diferentes ambientes (Levene, 1953), resultando em excesso de homozigotos especialmente em casos em que há um número limitado de indivíduos fundadores de uma nova população (Santos, 1994; Fernández Iriarte *et al.*, 2002).

Em algumas espécies, a quantidade de variabilidade e polimorfismo genético são apontados como reflexo da heterogeneidade ambiental. Populações que vivem em ambientes mais diversificados ou têm um nicho mais largo apresentariam maior variabilidade em relação àquelas que habitam ambientes mais simples ou possuem um nicho estreito (Powell, 1971; Levinton e Suchanek, 1978; Nevo, 1978; Ward e Warwick, 1980; Janson, 1985; Noy *et al.*, 1987; Mitchell-Olds, 1992; Schmidt e Rand, 1999).

O efeito do ambiente sobre a variabilidade genética, principalmente quando se trata de variação isozímica, é bastante questionado (Gooch e Schopf, 1972; Hedrick *et al.*, 1976; Hedrick, 1986; Solé-Cava e Thorpe, 1991; Prout e Savolainen, 1996; Johannesson 2003). Na maioria dos trabalhos, a associação ambiente-variação genética foi baseada em correlações mas não foi confirmada por abordagens experimentais. Além disso, quando se avalia o efeito da variação espacial sobre as freqüências genotípicas de populações naturais deve-se também levar em conta o possível efeito da variação temporal, que pode acentuar o

efeito da heterogeneidade ambiental (Bryant, 1976). Outro importante fator é o tamanho efetivo populacional, quando se comparam diferentes espécies quanto à variabilidade genética. O alto tamanho efetivo pode ser o principal fator na manutenção da variabilidade (Ward, 1990).

Ambientes bentônicos e especialmente costões rochosos estão entre os ambientes mais heterogêneos conhecidos (Johannesson, 2003). Em bivalves do gênero *Mytilus*, que possuem estágio larval planctotrófico, as freqüências dos locos da enzima aminopeptidase da leucina (LAP) parecem estar intimamente relacionadas com gradientes de salinidade, formando "clines" ao longo da costa (Hillbish e Koehn, 1985; Mitton, 1997). Schimdt e Rand (1999) observaram um padrão de freqüência genotípica de um loco da manose 6-fosfato da isomerase (MPI) dependente de temperatura em *Semibalanus balanoides*, uma espécie de craca de ampla distribuição, com desenvovimento planctotrófico.

A família Littorinidae (Gastropoda: Mollusca) tem sido foco de vários estudos ecológicos e genéticos devido à diversidade de suas características bionômicas, sua ampla distribuição e sua importância nas comunidades litorâneas, pois são encontrados em substratos rochosos de regiões entremarés e em mangues (Reid, 1989). Esses moluscos são dióicos, possuem fertilização interna e o desenvolvimento larval pode ser planctônico ou não (Rios, 1994). Vários estudos têm sido realizados na família, a fim de descrever, em diversas escalas, a diferenciação genética em locos enzimáticos em costões rochosos (Behrens Yamada, 1989; Johannesson *et al.*, 1995; Rolán-Alvarez *et al.*, 1995; Johannesson e Tatarenkov, 1997; Johnson e Black, 1998; Panova and Johannesson, 2004).

Em Littorinidae, a maioria dos estudos que investigam o efeito da heterogeneidade ambiental sobre a variabilidade e estruturação genética em costões rochosos tem sido realizada principalmente em espécies com desenvolvimento direto. Johannesson e

colaboradores têm abordado de forma sistemática o efeito da variação do ambiente na distribuição de genótipos e fenótipos de *Littorina saxatilis* e *L. fabalis*, utilizando principalmente a análise isozímica (e.g Johannesson e Johannesson, 1989; Johannesson *et al.*, 1993; Johannesson *et al.*, 1995; Tatarenkov e Johannesson, 1999; Johannesson *et al.*, 2004; Johannesson e Mikhailova, 2004; Panova and Johannesson, 2004). Johannesson *et al.* (1993) observaram, em *L. saxatilis*, variação na forma e na coloração da concha associada à distribuição das populações no costão. A divergência genética entre amostras do mesmo morfo foi menor em relação à encontrada entre amostras de morfos distintos. Nesta mesma espécie também foi encontrada associação entre freqüências dos genótipos do sistema enzimático AAT (aspartato aminotransferase) e diferentes ambientes do costão (Johannesson e Johannesson, 1989; Johannesson *et al.*, 1995; Johannesson e Tatarenkov, 1997; Panova e Johannesson, 2004). Um resultado similar foi observado em *L. fabalis*, para um loco do sistema quinase da arginina (ARK), que apresentou variação nas freqüências genotípicas entre amostras coletadas em áreas protegidas e expostas do costão (Tatarenkov e Johannesson, 1999). Quando as amostras foram analisadas em conjunto, observou-se desvio das proporções de Hardy-Weinberg no sentido de deficiência de heterozigotos, o que pôde ser inteiramente explicado alta estruturação genética.

Recentemente, Johannesson e Mikhailova (2004) obtiveram evidências de que os locos responsáveis pela expressão da enzima ARK e pela forma da concha em *L.* fabalis estão em desequilíbrio de ligação e situados dentro de uma inversão cromossômica paracêntrica. A presença dessa inversão está altamente correlacionada com dois micro-habitats no costão ocupados pela espécie, resultando em uma estruturação genética local e sugerindo especialização das populações a micro-ambientes situados a poucos metros de distância.

Poucos são os estudos que avaliaram o efeito da heterogenidade ambiental sobre a variabilidade genética em litorinídeos com desenvolvimento planctônico. Tatarenkov (1995) observou que populações de *Littorina brevicula* (que tem um período larval de cerca de 10 dias) apresentaram grande heterogeneidade nas freqüências alélicas em três locos enzimáticos a dezenas de metros de distância. As explicações apresentadas para esse padrão foram seleção diferencial e variação nas freqüências gênicas de diferentes coortes de recrutas.

Heterogeneidade ambiental e efeitos sobre a variabilidade morfológica

O efeito da heterogeneidade ambiental em costões rochosos sobre a variação morfológica intra-específica também tem sido bastante estudado. A variação morfológica pode ser uma resposta à complexidade ambiental, surgindo de várias maneiras: (1) indivíduos se tornam adaptados a partes diferentes do ambiente; (2) ou apresentam um fenótipo plástico; (3) ou evoluem para um único fenótipo "fixo", com um valor adaptativo médio nos vários ambientes (Scheneir e Callahan, 1999). A compreensão da base, genética ou ecofenotípica, da variação morfológica em diferentes ambientes é importante para a elaboração de teorias e hipóteses sobre mecanismos adaptativos, especiação e variação biogeográfica (Trussell, 2000). Gastrópodes da região entremarés comumente mostram variação morfológica em grandes e pequenas escalas espaciais (Trussell, 1997, 2000). Embora a plasticidade fenotípica seja uma das formas mais estudadas de adaptação no indivíduo, seu mecanismo ainda é pouco conhecido (Marchinko, 2003).

Em gastrópodes, a variação morfológica mais estudada refere-se, principalmente, a alguns caracteres relacionados à concha. A forma, a coloração e a espessura dessa estrutura em litorinídeos apresentam variação, geralmente relacionada com fatores abióticos, como

radiação solar (tolerância à temperatura), taxas de dessecação e exposição à ação das ondas (Grahame e Mill, 1989; Trussell, 1997; De Wolf *et al.*, 1998; Lee e Williams, 2002).

Outros caracteres, tais como o tamanho do pé e a rádula também têm revelado uma clara relação com a heterogeneidade ambiental (Rolán-Alvarez *et al.*, 1996; Trussell, 1997; Padilla, 1998; Reid e Mak, 1999; Trussell, 2000; Ito *et al.*, 2002). A rádula é um órgão característico do filo Mollusca; é uma fita constituída por fileiras de dentes quitinosos, utilizada por moluscos herbívoros para raspar o substrato e obter alimento. Em litorinídeos, a rádula é do tipo tenioglossa, em que cada fileira é composta por quatro dentes marginais, 2 laterais e 1 raquidiano ou central, sendo constantemente produzida, devido ao seu desgaste (Reid, 1986).

Algumas espécies da família Littorinidae apresentam substituição completa da rádula a cada 2 a 4 semanas (Padilla *et al.*, 1996; Padilla, 1998). Em espécies do gênero *Lacuna* foi observada variação intra-específica no formato dos dentes da rádula de acordo com o tipo de alga que revestia o substrato (Padilla, 1998). Reid e Mak (1999) observaram em 36 espécies do gênero *Littoraria* um claro padrão de variação na forma dos dentes marginais e laterais entre espécimes coletados no mangue e no costão rochoso. Os autores observaram que indivíduos que ocupam o costão apresentam dentes com as cúspides principais mais arredondadas enquanto que os do mangue apresentam cúspides longas e afiadas. Essa variação pode ser atribuída à plasticidade ecofenotípica, ou seja, a variação da forma é induzida pelo substrato ocupado e/ou dieta e mostra uma clara convergência na forma dos dentes, altamente dependente do tipo de ambiente ocupado pelas diferentes espécies.

Comportamento larval e recrutamento não sincronizado

Os fatores que limitam o fluxo gênico podendo levar à estruturação local incluem: predação (especialmente pelos adultos sobre as larvas: Burton e Feldman, 1981; Naylor e McShane, 2001), diferentes histórias de recrutamento (Johnson e Black, 1982; Watts *et al.*, 1990; Parsons, 1996; Hoarau *et al.*, 2002; Sotka *et al.*, 2004) e variação no sucesso da capacidade reprodutiva (Li e Hedgecock, 1998; Dawson, 2001; Planes e Lenfant, 2002). Migração vertical, ou seja, na coluna d'água, é comum em larvas de crustáceos e limitaria o fluxo gênico em espécies onde se esperariam amplas taxas de dispersão (Palumbi, 1994).

Mudanças temporais e espaciais na abundância relativa e absoluta das larvas de diferentes genótipos durante recrutamento podem ser responsáveis pela estruturação microgeográfica em lapas (Johnson e Black, 1982, 1984). Uma comparação entre locais com diferentes histórias de recrutamento indicou que a diferenciação genética em populações do ouriço *Echinometra mathaei* é resultante de eventos que ocorreram logo antes e durante o processo de recrutamento das larvas na superfície de recifes de corais (Watts *et al.*, 1990). Parsons (1996) observou uma aparente ausência de fluxo gênico entre populações de poças adjacentes do gastrópode *Austrocochlea constricta*, que possue larva planctônica. As explicações apresentadas foram recrutamento local e/ou mortalidade seletiva na migração das larvas entre as poças durante a maré alta. Diversos autores observaram que alguns ambientes parecem favorecer a estruturação genética local, tais como arquipélagos, baías e estuários (Johnson e Black, 1991; Ayvazian *et al.*, 1994; Tatarenkov, 1995; Johnson *et al.*, 2001; Pampoulie *et al.*, 2004).

Alguns autores consideram que o principal fator determinante da variação das freqüências alélicas seria a mortalidade seletiva durante o estágio larval e a fase de recrutamento (Koehn e Gaffney, 1984; Zouros e Foltz, 1984; Fairbrother e Beaumont,

1993). Essa variação também poderia ser reflexo da variância do sucesso reprodutivo (Li e Hedgecock, 1998; Dawson, 2001; Planes e Lenfant, 2002). Quanto maior a variância, menor seria o tamanho efetivo das populações, já que poucos indivíduos estariam contribuindo com gametas para a próxima geração e haveria uma maior chance de um par de alelos ser idêntico por descendência. Parece contraditório que espécies com larva planctônica e ampla distribuição apresentem esse tipo de padrão, no entanto a variância no sucesso reprodutivo, no entanto, assim como a não sincronização durante o recrutamento, podem promover desvio das proporções esperadas pelo equilíbrio de Hardy-Weinberg (Johnson e Black, 1982, 1984; Watts *et al.*, 1990; Li e Hedgecock,1998). Além disso, seleção durante o recrutamento também pode gerar desvio das proporções esperadas pelo equilíbrio de Hardy-Weinberg (Koehn e Gaffney, 1984; Burton, 1986; Gilg e Hillbish, 2000), de forma que é difícil isolar os mecanismos seletivos do efeitos da colonização por diferentes coortes com freqüências alélicas variando no tempo e espaço.

Desvio das proporções esperadas pelo equilíbrio de Hardy-Weinberg

Devido à existência de estágio larval planctônico e ao fato de possuírem populações grandes, espera-se que invertebrados marinhos apresentem panmixia e que suas freqüências genotípicas se encontrem dentro das proporções esperadas pelo equilíbrio de Hardy-Weinberg. No entanto, vários trabalhos mostram desvios, geralmente tendendo para o excesso de homozigotos (e.g. Nevo *et al.*, 1984; Singh e Green, 1984; Zouros e Foltz, 1984; Janson, 1985; Noy *et al.*, 1987; Gaffney *et al.*, 1990; Ward, 1990; McQuaid, 1996; Rio-Portilla e Beaumont, 2000; Johnson *et al.*, 2001).

Diferentes hipóteses foram propostas para explicar cada caso, tais como: efeito Wahlund (Berger, 1983; Johnson e Black, 1984; Janson, 1985; Noy *et al.*, 1987; Gaffney *et*

al., 1990), seleção natural (Zouros e Foltz, 1984; Janson, 1985; Gaffney *et al.*, 1990), alelos nulos (Janson, 1985), entre outras. Alguns estudos (Zouros *et al.*, 1983; Zouros e Foltz, 1984) mostram que a heterozigosidade poderia estar relacionada a maiores taxas de crescimento e a uma maior eficiência metabólica em algumas espécies de bivalves. Alguns bivalves aparentemente apresentariam uma associação entre idade e heterozigosidade (Singh e Green, 1984; Zouro e Foltz, 1984; Fairbrother e Beaumont, 1993; Toro e Vergara, 1995). Segundo a hipótese de seleção bifásica, em alguns locos a homozigosidade seria favorável durante a fase larval, após a qual os indivíduos heterozigotos seriam favorecidos (Singh e Green, 1984; Zouro e Foltz, 1984). A relação entre crescimento e heterozigosidade não foi encontrada quando testada em *Littorina littorea* (Foltz *et al.*, 1993).

A subdivisão em uma escala local foi uma teoria proposta por Tracey *et al.* (1975) para explicar a deficiência de heterozigostos em *Mytilus californianus*. Os chamados "grupos de acasalamento", ou seja, conjuntos de indivíduos com freqüências genotípicas diferentes, às vezes a poucos metros de distância uns dos outros, seriam responsáveis pela deficiência de heterozigotos nessa espécie de bivalve. Essas diferenças nas freqüências alélicas podem ser causadas por uma variação temporal na composição e distribuição dos recrutas resultante, por exemplo, de uma diferenciação genotípica para época reprodutiva ou, ainda, por uma variação espacial na distribuição desses genótipos. Nesse último caso, a heterogeneidade do costão, com seus microambientes distintos, poderia estar causando ou acentuando essa estruturação populacional. Embora essa hipótese se baseie na estruturação espacial, assim como o efeito Wahlund, nesse caso a deficiência de heterozigotos não dependeria da forma como a população foi amostrada. Os "grupos de acasalamento" violariam o pressuposto de panmixia do modelo de Hardy-Weinberg e, portanto, mesmo a população de larvas no plâncton deveria apresentar deficiência de heterozigotos.

Diversos autores apresentam essa hipótese para explicar a deficiência de heterozigotos em moluscos (Levinton e Suchanek, 1978 e Gaffney *et al.*, 1990, com bivalves; Johnson e Black, 1982, 1984, estudando o pulmonado *Siphonaria sp.*). No entanto, Gaffney *et al.* (1990) propõem que vários fatores (e.g. seleção, alelos nulos, os "grupos de acasalamento", entre outros) estariam atuando em conjunto sobre as freqüências genotípicas dos invertebrados com estágio larval planctônico, uma vez que a deficiência de heterozigotos foi observada em vários locos de uma mesma espécie. Essa subdivisão seria mantida por seleção, recrutamento em diferentes períodos, diferenciação entre os genótipos quanto à época reprodutiva (acasalamento não aleatório) ou, simplesmente, amostras compostas por mais de uma população (gerando efeito Wahlund em uma pequena escala).

A alta freqüência do desvio das proporções esperadas pelo equilíbrio de Hardy-Weinberg em invertebrados marinhos ainda não foi completamente esclarecida. O fato de esses organismos apresentarem padrões que contrariam o esperado em espécies panmíticas é um indício de que o investimento em seu estudo poderá trazer contribuições interessantes para compreender a distribuição e organização dos genótipos em populações naturais.

Família Littorinidae na costa brasileira

A costa brasileira tem cerca de 8.000 km de extensão, abrangendo áreas de costão abrigadas ou expostas, praias e manguezais. Apesar da ampla extensão, existem poucas espécies da família Littorinidae, sendo documentados dois gêneros na costa continental do Brasil: *Nodilittorina* Martens 1897 e *Littoraria* Griffith e Pidgeon 1834 (Rios, 1994; Magalhães, 1998). O gênero *Nodilittorina* pode ser encontrado em quase todos os continentes, sendo freqüente nas regiões tropicais e subtropicais (Bandel e Kadolsky, 1982; McQuaid, 1996). Recentemente, baseando-se em caracteres morfológicos e dados

moleculares, Reid (2002) e Williams *et al.* (2003), chegaram à conclusão de que o gênero *Nodilittorina* é polifilético. *Nodilittorina* foi dividido em quatro gêneros (*Echinolittorina*, *Austrolittorina*, *Afrolittorina* e *Nodilittorina*.), sendo que a espécie amplamente distribuída no Brasil, *N. lineolata* D'Orbigny 1840, foi incluída, juntamente com mais 49 espécies, no gênero *Echinolittorina* Habe, 1956.

Echinolittorina lineolata (originária do Caribe) pertence a um complexo de espécies denominado *ziczac*, podendo ser encontrada em toda a costa da América do Sul (Williams e Reid, 2004). No costão rochoso, *E. lineolata* ocorre desde a faixa de cirripédios e bivalves, no mediolitoral superior (em algumas pedras, estes gastrópodes chegam a ficar submersos durante a maré alta) até o supralitoral (Magalhães, 1998).

Dentro do gênero *Littoraria* são encontradas, no Brasil, as espécies *L. flava* (King e Broderip 1832) e *L. angulifera* Lamarck 1822. Esse gênero é restrito às áreas tropicais e suas espécies estão geralmente associadas à vegetação do mangue e a costões próximos a ambientes de água doce ou salobra (Reid, 1986). *Littoraria angulifera* é encontrada principalmente dentro do mangue e, em menor densidade, no costão. Com *L. flava* ocorre o contrário: alguns poucos indivíduos são observados no mangue e a maioria dos espécimes parece estar concentrada nos costões, em áreas próximas a estuários ou filetes de água doce (Gallagher e Reid, 1974; Reid, 1986; observações pessoais).

As espécies de litorinídeos e do Brasil são dióicas, com fecundação interna e apresentam desenvolvimento larval completo ou planctotrófico (Gallagher e Reid, 1974; Mileikovsky, 1975; Bandel e Kadolsky, 1982; Reid, 1999). *Echinolittorina lineolata* apresenta uma estágio larval de cerca de quatro semanas e estima-se que indivíduos pertencentes a esse gênero apresentem uma capacidade de dispersão de até 1.400 km (Williams e Reid, 2004). Todas as espécies brasileiras são ovíparas, com exceção de

Littoraria angulifera, que apresenta ovoviviparidade incompleta: os ovos, depois de fecundados, permanecem em uma cavidade do manto da fêmea por cerca de quatro dias, até a eclosão, quando as larvas e alguns ovos imaturos são liberados na água. Estima-se que a duração da fase pelágica de *L. angulifera* seja de 8 a 10 semanas (Bandel, 1974; Gallagher e Reid, 1974; Reid, 1986).

Andrade *et al.* (2003; Apêndice 1) amostraram populações de *E. lineolata*, *L. angulifera* e *L. flava* desde o Estado do Ceará até Santa Catarina em 22 locais (entre praias e mangues). Para estas espécies foi encontrada alta diversidade gênica e alta porcentagem de locos polimórficos (médias H_e = 0,17 e P = 45%). As amostras de *E. lineolata* apresentaram baixa estruturação genética (F_{st}=0,028), as de *L. flava*, baixa a moderada (F_{st}=0,054) e de *L. angulifera*, alta estruturação (F_{st}=0,185), valores esperados considerando as características bionômicas dessas espécies.

No presente trabalho, foram estudadas as três espécies da família Littorinidae que ocupam a costa continental brasileira. Como a eletroforese de isozimas tem sido muito utilizada em vários estudos de genética dessa família, os resultados obtidos podem ser comparados aos encontrados em outras espécies de litorinídeos. Além disso, como resultados prévios (Andrade *et al.*, 2003) mostraram que os locos enzimáticos apresentaram alta variabilidade (com uma média de 9-10 alelos por loco), e como forma de dar continuidade ao trabalho já iniciado no mestrado, além de tentar explicar os padrões encontrados, optamos por manter isozimas como marcador molecular. O trabalho é composto por três capítulos, todos na forma de manuscritos.

O estudo apresentado no primeiro capítulo é baseado nos dados coletados durante o mestrado. O objetivo principal foi verificar a existência de padrões de desvio das proporções de Hardy-Weinberg, ao longo da costa brasileira, nas três espécies de

litorinídeos, *E. lineolata, Littoraria flava* e *L. angulifera*. Foi usada uma abordagem baseada na análise de homogeneidade de coeficientes de correlação (Crow e Kimura, 1970; Sokal e Rohlf, 1995). Esse artigo está publicado no periódico *Journal of Molluscan Studies*.

No segundo capítulo, estão descritos os padrões de variabilidade genética da espécie *Littoraria flava* em uma escala local. A fim de averiguar se a deficiência de heterozigotos observada é gerada por variação microgeográfica das freqüências alélicas, foram coletadas amostras em transectos horizontais (que variaram de 64 a 300 m) em três praias do litoral norte do Estado de São Paulo. A coleta foi repetida duas vezes nos mesmos pontos (previamente marcados) para investigar se há estruturação temporal entre os mesmos. Esses dados foram analisados de forma hierárquica para testar se há maior estruturação local (dentro do costão) do que entre praias (distantes cerca de 200 km).

O terceiro capítulo descreve a variação da forma dos dentes da rádula de *L. flava* e *L. angulifera*. Indivíduos de diversos tamanhos e que ocupam diferentes ambientes (mangue e costão rochoso) foram comparados. Além disso, é apresentado o resultado de um experimento de transferência com marcação e recaptura em condições naturais de espécimes de *L. flava*. O objetivo desse experimento foi testar a hipótese de plasticidade ecofenotípica apresentada por Reid e Mak (1999). Com base nos indivíduos controle e nos padrões de forma de dente encontrados na literatura, investigamos se as rádulas dos espécimes transplantados apresentaram alterações relacionadas a cada ambiente. Esse manuscrito está submetido ao periódico *Journal of Molluscan Studies*.

No apêndice 1 está apresentado o trabalho publicado com os resultados obtidos durante meu mestrado, onde podem ser observados alguns do dados apresentados nessa introdução para as mesmas amostras analisadas no Capítulo 1.

Referências Bibliográficas

Andrade S.C.S., Magalhães C.A. e Solferini V.N. 2003. Patterns of genetic variability in Brazilian littorinids (Mollusca): a macrogeographic approach. Journal of Zoological Systematics and Evolutionary Research, 41: 249-255.

Ayvazian S.G., Johnson M.S. e McGlashan D.J. 1994. High levels of genetic subdivision of marine and estuarine populations of the estuarine catfish *Cnidoglanis macrocephalus* (Plotosidae) in southwestern Australia. Marine Biology, 118: 25-31.

Bandel, K. 1974. Studies on Littorinidae from the Atlantic. Veliger, 17: 92-114.

Bandel K. e Kadolsky D. 1982. Western Atlantic species of *Nodilittorina* (Gastropoda: Prosobranchia): comparative morphology and its functional, ecological, phylogenetic and taxonomic implications. The Veliger, 25: 1-42.

Behrens-Yamada S. 1989. Are direct developers more locally adapted than planktonic developers? Marine Biology, 103: 403-411.

Berger E.M. 1983. Chap. 13: Population genetics of marine gastropods and bivalves *in* The Mollusca, Vol. 6. Ecology. Academic Press, New York, NY.

Bohonak A.J. 1999. Dispersal, gene flow, and population structure. Quarterly Review of Biology, 74: 21-45.

Bryant E.H.. 1976. A comment on the role of environmental variation in maintaining polymorphisms in natural populations. Evolution, 30: 188-190

Burton R.S. 1986. Evolutionary consequences of restricted gene flow among natural-populations of the copepod, *Tigriopus californicus*. Bulletin of Marine Science, 39: 526-535.

Burton R.S. e Feldman M.W. 1981. Population-genetics of *Tigriopus californicus*.2. Differentiation among neighboring populations. Evolution, 35: 1192-1205.

Crow J. F. e Kimura M. 1970. An Introduction to Population Genetics Theory. Harper & Row, New York.

Dawson M.N. 2001. Phylogeography in coastal marine animals: a solution from California? Journal of Biogeography, 28: 723-736.

De Wolf H., Backeljau T. e Verhagen R. 1998. Spatio-temporal genetic structure and gene flow between two distinct shell morphs of the planktonic periwinkle *Littorina striata* (Mollusca: Prosobranchia). Marine Ecology Progress Series, 163: 155-163.

Fairbrother J.E. e Beaumont A.R. 1993. Heterozygote deficiencies in a cohort of newly settled *Mytilus edulis* spat. *Journal* of Marine Biology Association of United Kingdom, 73: 647-653.

Fernández Iriarte P.J., Rodriguez C. e Hasson E. 2002. Inversion and allozyme polymorphism show contrasting patterns of microgeographical population structure in a natural population of *Drosophila buzzatii* from Argentina. Journal of Evolutionary Biology, 15: 226-234.

Foltz D.W., Shumway S.E. e Crisp D. 1993. Genetic structure and heterozygosity-related fitness effects in the marine snail *Littorina littorea*. American Malacological Bulletin, 10: 55-60.

Gaffney P.M., Scott T.M., Koehn, R.K. e Diehl, W.J.. 1990. Interrelationships of heterozygosity, growth rate and heterozygote deficiency in the coot clam, *Mulinia lateralis*. Genetics, 124: 687-699.

Gallagher S.B. e Reid G.K. 1974. Reproductive behavior and early development in *Littorina scabra angulifera* and *Littorina irrorata* (Gastropoda: Prosobranchia) in the Tampa Bay region of Florida. Malacological Review, 7: 105-125.

Gilg M.R. e Hilbish T.J. 2000. The relationship between allele frequency and tidal height in a mussel hybrid zone: a test of the differential settlement hypothesis. Marine Biology, 137: 371-378.

Gooch J.L. e Schopf T.J.M. 1972. Genetic variability in deep-sea - relation to environmental variability. Evolution, 26: 545-552.

Grahame J. e Mill P.J. 1989. Shell shape variation in *Littorina saxatilis* and *Littorina arcana*, a case of character displacement. Journal of the Marine Biological Association of the United Kingdom, 69: 837-855.

Hedrick P.W., Ginevan, M.E. e Ewing E.P. 1976. Genetic polymorphism in heterogeneous environments. Annual Review of Ecology and Systematics, 7: 1-32.

Hedrick P.H. 1986. Genetic polymorphism in heterogeneous environments: a decade later. Annual Review of Ecology and Systematics, 17: 535-566.

Hillbish T.J. 1996. Population genetics of marine species: the interaction of natural selection and historically differentiated populations. Journal of Experimental Marine Biology and Ecology, 200: 67-83.

Hillbish T.J. e Koehn R.K. 1985. Dominance in physiological phenotypes and fitness at an enzyme locus. Science, 229: 52-54

Hohenlohe P.A. 2004. Limits to gene flow in marine animals with planktonic larvae: models of Littorina species around Point Conception, California. Biological Journal of the Linnean Society, 82: 169–187.

Hoarau G., Rijnsdorp A.D., Van der Veer H.W., Stam W.T. e Olsen J.L. 2002. Population structure of plaice (*Pleuronectes platessa* L.) in northern Europe: microsatellites revealed large-scale spatial and temporal homogeneity. Molecular Ecology, 11: 1165-1176.

Ito A., Ilano A.S. e Nakao S. 2002. Seasonal and tidal height variations in body weight and radular length in *Nodilittorina radiata* (Eydoux and Souleyet, 1852). Journal of Molluscan Studies 68: 197-203.

Janson K. 1985. Genetic variation in three species of Caribbean periwinkles, *Littorina angustior*, *L. lineolata* and *L. ziczac* (Gastropoda: Prosobranchia). Bulletin of Marine Science, 37: 871-879.

Johannesson K. 1988. The paradox of rockall - why is a brooding gastropod (*Littorina saxatilis*) more widespread than one having a planktonic larval dispersal stage (*Littorina littorea*). Marine Biology, 99: 507-513.

Johannesson K. 2003. Evolution in *Littorina*: ecology matters. Journal of Sea Research, 49: 107-117.

Johannesson K. e Johannesson B. 1989. Differences in allele frequencies of AAT between high-rocky and mid-rocky shore populations of *Littorina saxatilis* (Olivi) suggest selection in this enzyme locus.Genetical Research, 54: 7-11.

Johannesson K., Johannesson B. e Rolán-Alvarez E. 1993. Morphological differentiation and genetic cohesiveness over a microenvironmental gradient in the marine snail *Littorina saxatilis*. Evolution, 47: 1770–1787.

Johannesson K. , Johannesson B. e Lundgren U. 1995. Strong natural selection causes microscale allozyme variation in a marine snail. Procedings of National Academy of Sciences of U.S.A., 92: 2602–2606.

Johannesson K. e Tatarenkov, A. 1997. Allozyme variation in a snail (*Littorina saxatilis*)-deconfounding the effects of microhabitat and gene flow. Evolution, 51: 402-409.

Johannesson K., Lundberg J., André C. e Nilsson P.G. 2004. Island isolation and habitat heterogeneity correlate with DNA variation in a marine snail (*Littorina saxatilis*). Biological Journal of the Linnean Society, 82: 377–384.

Johannesson K. e Mikhailova N. 2004. Habitat-related genetic substructuring in a marine snail (*Littorina fabalis*) involving a tight link between allozyme and a DNA locus. Biological Journal of the Linnean Society, 81: 301–306.

Johnson M.S. e Black R. 1982. Chaotic genetic patchiness in an intertidal limpet, *Siphonaria* sp. Marine Biology, 70: 157-164.

Johnson M.S. e Black R. 1984. Pattern beneath the chaos: the effect of recruitment on genetic patchiness in an intertidal limpet. Evolution, 38: 1371–1383.

Johnson M.S. e Black R. 1991. Genetic subdivision of the intertidal snail *Bembicium vittatum* (Gastropoda, Littorinidae) varies with habitat in the Houtman Abrolhos Islands, Western-Australia. Heredity, 67: 205-213

Johnson M.S. e Black R. 1998. Increased genetic divergence and reduced geneic variation in populations of the snail *Bembicium vittatum* in isolated tidal ponds. Heredity, 80: 163-172.

Johnson M.S. e Black R. 2000. Associations with habitat versus geographic cohesiveness: size and shape of *Bembicium vittatum* Philippi (Gastropoda: Littorinidae) in the Houtman Abrolhos Islands. Biological Journal of the Linnean Society, 71: 563-580.

Johnson M.S., Bentley S.L., Ford S.S., Ladyman M.T. e Lambert G.J. 2001. Effects of a complex archipelago on genetic subdivision of the intertidal limpet *Siphonaria kurracheensis*. Marine Biology, 139: 1087-1094.

Koehn R.K., Turano F.J. e Mitton J.B. 1973. Population genetics of marine pelecypods. II. Genetic differences in microhabitats of Modiolus demissus. Evolution, 27:100–105.

Koehn R.K. e Gaffney P.M. 1984. Genetic heterozygosity and growth-rate in *Mytilus edulis*. Marine Biology, 82: 1-7.

Kyle C.J. e Boulding E.G. 2000. Comparative population genetic structure of marine gastropods (*Littorina spp.*) with and without pelagic larval dispersal. Marine Biology, 137: 835-845.

Lee O.H.K. e Williams G.A. 2002.Spatial distribution patterns of *Littoraria* species in Hong Kong mangroves. Hydrobiologia, 481: 137-145.

Levene H. 1953. Genetic equilibrium when more than one ecological niche is available. The American Naturalist, 87: 331-333

Levinton J.S. e Suchanek, T.H.. 1978. Geographic variation, niche breadth and genetic differentiation at different geographic scales in the mussels *Mytilus californianus* and *M. edulis*. Marine Biology, 49: 363-375.

Li G. e Hedgecock D. 1998. Genetic heterogeneity, detected by PCR-SSCP, among samples of larval Pacific oysters (*Crassostrea gigas*) supports the hypothesis of large variance in reproductive success. Canadian Journal of Fisheries and Aquatic Sciences, 55: 1025-1033.

Magalhães C.A. 1998. Density and shell-size variation of *Nodilittorina lineolata* (Orbigny, 1840) in the intertidal region in southeastern Brazil. Hydrobiologia, 378: 143-148.

Marchinko K.B. 2003. Dramatic phenotypic plasticity in barnacle legs (*Balanus glandula* Darwin): magnitude, age dependence, and speed of response. Evolution, 57: 1281-1290.

McQuaid C.D. 1996. Biology of the gastropod family Littorinidae. I. Evolutionary aspects. Oceanography and Marine Biology, 34: 233-262.

Mileikovsky S.A. 1975. Types of larval development in Littorinidae (Gastropoda: Prosobranchia) of the world ocean, and ecological patterns of their distribution. Marine Biology, 30: 129-135.

Mitchell-Olds T. 1992. Does environmental variation maintain genetic variation? A question of scale. Trends in Ecology and Evolution, 7: 397-398.

Mitton J.B. 1997. Selection in Natural Populations. Oxford University Press , New York.

Naylor J.R. e McShane P.E. 2001. Mortality of post-settlement abalone *Haliotis iris* caused by conspecific adults and wave exposure. New Zealand Journal of Marine and Freshwater Research, 35: 363-369.

Nevo E. 1978. Genetic variation in natural populations: patterns and theory. Theoretical Population Biology, 13: 127-177.

Nevo E., Ben-Shlomo E. e Lavie B. 1984. Mercury selection of allozymes in marine organisms: predictionand verification in nature. Proceedings of the National Academy of Sciences USA, 81: 1258-1259.

Noy R., Lavie, B. e Nevo, E.1987. The niche-width variation hypothesis revisited: genetic diversity in the marine gastropods *Littorina punctata* (Gmelin) and *L. neritoides* (L.). Journal of Experimental Marine Biology and Ecology, 109: 109-135.

Padilla D.K., Dittman D.E., Franz J. e Sladek R. 1996. Radular production rates in two species of *Lacuna* Turton (Gastropoda: Littorinidae). Journal of Molluscan Studies 62: 275-280.

Padilla D.K. 1998. Inducible phenotypic plasticity of the radula in *Lacuna* (Gastropoda: Littorinidae). The Veliger, 41: 201-204.

Palumbi S.R. 1992. Marine speciation on a small planet. Trends in Ecology and Evolution, 7:114-118.

Palumbi S.R. 1994. Genetic divergence, reproductive isolation, and marine speciation. Annual Review of Ecology and Systematics, 25: 547–572.

Pampoulie C., Gysels E.S., Maes G.E., Hellemans B., Leentjes V., Jones A.G. e Volckaert F.A.M. 2004. Evidence for fine-scale genetic structure and estuarine colonisation in a potential high gene flow marine goby (*Pomatoschistus minutus*) . Heredity, 95: 434-445.

Panova M. e Johannesson K. 2004. Microscale variation in Aat (aspartate aminotransferase) is supported by activity differences between upper and lower shore allozymes of *Littorina saxatilis*. Marine Biology, 144: 1154-1167.

Parsons K.E. 1996. The genetic effects of larval dispersal depend on spatial scale and habitat characteristics. Marine Biology, 126: 403-414

Planes S. e Lenfant P. 2002. Temporal change in the genetic structure between and within cohorts of a marine fish, *Diplodus sargus*, induced by a large variance in individual reproductive success. Molecular Ecology, 11: 1515-1524.

Powell J.R. 1971. Genetic polymorphisms in varied environments. Science, 174: 1035.

Prout T. e Savolainen O. 1996. Genotype-by-environment interaction is not sufficient to maintain variation: Levene and the leafhopper. American Naturalist, 148: 930-936.

Reid D.G. 1986. The Littorinid Molluscs of Mangrove Forests in the Indo-Pacific Region. The Genus *Littoraria*. British Museum (Natural History).

Reid D.G. 1989. The comparative morphology, phylogeny and evolution of the gastropod family Littorinidae. Philosophical Transactions of the Royal Society of London, B324: 1-110.

Reid D.G. 1999. The genus *Littoraria* Griffith and Pidgeon, 1834 (Gastropoda: Littorinidae) in tropical eastern Pacific. The Veliger, 42: 21-53.

Reid D.G. 2002. Morphological review and phylogenetics analysis of *Nodilittorina* (Gastropoda: Littorinidae). Journal of the Molluscan Studies, 68: 259-281.

Reid D.G. e Mak Y.M. 1999. Indirect evidence for ecophenotypic plasticity in radular dentition of *Littoraria* species (Gastropoda: Littorinidae). Journal of the Molluscan Studies, 65: 355-370.

Rio-Portilla M.A. e Beaumont A.R. 2000. Larval growth, juvenile size and heterozygosity in laboratory reared mussels, *Mytilus edulis*. Journal of Experimental Marine Biology and Ecology, 254: 1-17.

Rios E.C. 1994.Seashells of Brazil. Ed. UFRG, Rio Grande do Sul.

Rolán-Alvarez E., Zapata C. and Alvarez G. 1995. Distinct genetic subdivision in sympatric and sibling of the genus *Littorina* (Gastropoda: Littorinidae). Heredity, 74: 1-9.

Rolán-Alvarez E., Rolán E. e Johannesson K. 1996. Differentiation in radular and embryonic characters, and further comments on gene flow, between two sympatric morphs of *Littorina saxatilis* (Olivi). Ophelia 45: 1-15.

Santos M. 1994. Heterozygote deficiencies under Levene's population subdivision structure. Evolution 48: 912–920.

Scheneir S.M. e Callahan H.S. 1999. Measuring natural selection on phenotypic plasticity. Evolution, 53: 1704-1713.

Schimdt P.S. e Rand D.M. 1999. Intertidal microhabitat and selection at Mpi: Interlocus contrasts in the northern acorn barnacle, *Semibalanus balanoides*. Evolution, 53: 135-146.

Singh S.M. e Green R.H. 1984. Excess of allozyme homozygosity in marine molluscs and its possible biological significance. Malacologia, 25: 569-581.

Sokal R.R. e Rohlf F.J. 1995. Biometry: The Principles and Practice of Statistics in Biological Research. 3ª ed. WH Freemam Company, NY.

Solé-Cava A.M. e Thorpe J.P. 1991. High levels of genetic variation in natural populations of marine lower invertebrates. Biological Journal of the Linnean Society. 44: 65-80.

Sotka E.E., Wares J.P., Barth J.A., Grosberg R.K. e Palumbi S.R. 2004. Strong genetic clines and geographical variation in gene flow in the rocky intertidal barnacle *Balanus glandula*. Molecular Ecology, 13: 2143–2156

Tatarenkov A. N. 1995. Genetic heterogeneity in populations of *Littorina brevicula* (Philippi) (Mollusca: Gastropoda) in the northern part of Peter the Great Bay (Sea of Japan). Veliger, 38: 85–91.

Tatarenkov A. e Johannesson K. 1999. Micro- and macrogeogeographic allozyme variation in *Littorina fabalis*; do sheltered and exposed forms hybridize? Biological Journal of the Linnean Society, 67: 199-212.

Toro J.E. and Vergara A.M. 1995. Evidence for selection against heterozygotes - postsettlement excess of allozyme homozygosity in a cohort of the chilean oyster, *Ostrea chilensis* Philippi, 1845. Biological Bulletin, 188: 117-119.

Tracey, M.L., Bellet, N.F. e Gravem, C.D. 1975. Excess allozyme homozygosity and breeding population structure in the mussel *Mytilus californianus*. Marine Biology, 32: 303-311.

Trussell G.C. 1997. Phenotypic plasticity in the foot size of an intertidal snail. Ecology 78: 1033-1048.

Trussell G.C. 2000. Phenotypic clines, plasticity, and morphological trade-offs in an intertidal snail. Evolution 54: 151-166.

Ward R.D. 1990. Biochemical genetic variation in the genus *Littorina* (Prosobranchia: Mollusca). Hydrobiologia, 193: 53-69.

Ward R.D. e Warwick T. 1980. Genetic differentiation in the molluscan species *Littorina rudis* and *Littorina arcana* (Prosobranchia-Littorinidae). Biological Journal of the Linnean Society ,14: 417-428

Watts R.J., Johnson M.S. e Black R. 1990. Effects of recruitment on genetic patchiness in the urchin *Echinometra mathaei* in western Australia. Marine Biology, 105:145–151.

Williams S.T., Reid D.G. e Littlewood D.T.J. 2003. A molecular phylogeny of Littorininae (Gastropoda: Littorinidae): unequal evolutionary rates, morphological parallelism, and biogeography of Southern Ocean. Molecular Phylogenetics and Evolution, 28: 60-86.

Williams S.T. e Reid D.G. 2004. Speciation and diversity on tropical rocky shores: a global phylogeny of snails of the genus *Echinolittorina*. Evolution, 58: 2227-2251.

Zouros E., Singh S.M., Foltz D.W. e Mallet A.L. 1983. Post-settlement viability in the american oyster (*Crassostrea virginica*) - an overdominant phenotype. Genetical Research, 41: 259-270.

Zouros E. e Foltz D.W. 1984. Possible explanations of heterozygote deficiency in bivalve molluscs. Malacologia, 25: 583-591.

Capítulo 1

HOMOGENEITY TEST OF HARDY-WEINBERG DEVIATIONS IN BRAZILIAN LITTORINIDS: EVIDENCE FOR SELECTION?

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Running Head: HARDY-WEINBERG DEVIATIONS IN BRAZILIAN LITTORINIDS

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ABSTRACT

Echinolittorina lineolata, *Littoraria flava* and *L. angulifera* (Gastropoda: Littorinidae) have a wide distribution along the Brazilian coast. The aim of this study was to evaluate the occurrence of Hardy-Weinberg disequilibrium in these species and to verify if there is a pattern in deviations. Isozyme analyses were done on 20 populations of *E. lineolata*, nine of *L. flava* and 10 of *L. angulifera*, collected along 4,000 km of the Brazilian coast. Sixteen polymorphic loci were analysed in *E. lineolata*, 15 in *L. angulifera* and 17 in *L. flava*. Most populations showed heterozygote deficiency in several loci. A homogeneity test among loci showed heterogeneous F_{IS} values in most populations. This result ruled out inbreeding and the Wahlund effect as the main causes of departure in these populations. In all littorinid species, at least one PGM locus had homogeneous F_{IS} values. This finding may suggest that this enzyme could have an important role in the fitness of these periwinkles.

INTRODUCTION

Due to their planktonic larval stage and large populations, marine invertebrates are expected to have genotypic frequencies in Hardy-Weinberg equilibrium. However, genetic studies of marine mollusc populations have reported heterozygote deficiencies in several enzymatic loci, in many bivalve and gastropod species. Several different explanations have been proposed, including Wahlund effect (Johnson & Black, 1984; Noy, Lavie & Nevo, 1987; Gaffney *et al.*, 1990), natural selection (Nevo, Ben-Shlomo & Lavie, 1984; Zouros & Foltz, 1984; Gaffney *et al.*, 1990), null alleles (Skibinski, Beardmore & Cross, 1983; Gaffney *et al.*, 1990; Gardner, 1992) and small breeding groups (Tracey, Bellet & Gravem, 1975). Some studies have related heterozygosity to growth rates and metabolic efficiency in certain bivalves (Zouros & Foltz, 1984), and this has been suggested but not confirmed in *Mytilus edulis* (Rio-Portilla & Beaumont, 2000) and *Littorina littorea* (Foltz, Shumway & Crisp, 1993).

In this work, we analysed deviations from Hardy-Weinberg equilibrium in three littorinid species found along the Brazilian coast, *Nodilittorina lineolata* (D'Orbigny, 1840), *Littoraria flava* (King & Broderip, 1832) and *Littoraria angulifera* (Lamarck, 1822), all of which have internal fertilization and planktotrophic larvae, but with different distribution patterns on the shore: *N. lineolata* occupies rocky shores from upper barnacle zone to supralittoral (Bandel & Kadolsky, 1982; Magalhães, 1998); *L. flava* is restricted to rocky shores near freshwater, but is sometimes found on mangroves (Reid & Mak, 1999; personal observation); *L. angulifera* occurs on mangroves and rocky shores near them (Gallagher & Reid, 1974; personal observation). Based on molecular data (Williams, Reid & Littlewood, 2003), the species *N. lineolata* belongs to the genus *Echinolittorina* Habe, 1956. So, from this point we follow these authors in using *Echinolittorina lineolata* instead of *Nodilittorina lineolata*.

We tested the homogeneity of heterozygote deficiencies across loci in order to check for inbreeding or the Wahlund effect. The heterozygote deficiencies among populations for each locus were also examined to determine if they could be ascribed to a common factor that could act in a directional manner.

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MATERIAL AND METHODS

Sampling

Twenty-one localities, covering about 4,000 km of the Brazilian coast, were sampled between May 1996 and January 2000. We collected 20 populations of *Echinolittorina lineolata*, nine of *Littoraria flava* and 10 of *L. angulifera*; some sites were sampled more than once (Table 1). The molluscs were transported alive to the laboratory where they were frozen in liquid nitrogen. The number of individuals scored for each locus and sample is presented in Figiure 2. In this study we use the term 'population' to refer to each sample of individuals, even though we cannot define the true limits of each biological population.

Electrophoresis

A total of 1109 individuals of *E. lineolata*, 239 of *L. flava* and 271 *of L. angulifera* were analysed. The entire soft body was squashed in extraction buffer (0.1 M Tris pH 8.0, 0.5% β-mercaptoethanol) and the extracts were loaded on to 8.5% starch gels (hydrolysed potato starch, Sigma). The electrophoretic conditions were described by Andrade, Magalhães & Solferini (2003). The electrophoretic analyses were done with 14 enzyme systems for *E. lineolata*, and with 12 for *L. flava* and *L. angulifera*: leucine aminopeptidase (EC 3.4.11.1; LAP), L-leucyl-L-glycylglycine peptidase (EC 3.4.11.-; PLGG), L-leucyl-L-alanine peptidase (EC 3.4.13.18; PLA), pyrophosphatase (EC 3.6.1.1; PP), aspartate aminotransaminase (EC 2.6.1.1; AAT), lactate dehydrogenase (EC 1.1.1.27; LDH), mannose phosphate isomerase (EC 5.3.1.8; MPI), sorbitol dehydrogenase (EC 1.1.1.14; SDH), malic enzyme (EC 1.1.1.40; ME), phosphoglucose isomerase (EC 5.3.1.9; GPI), phosphoglucomutase (EC 5.4.2.2; PGM), malate dehydrogenase (EC 1.1.1.37; MDH), isocitric dehydrogenase (EC 1.1.1.42; IDH), adenylate kinase (EC 2.7.4.3; AK), and hexokinase (EC 2.7.1.1; HK). The staining procedures were adapted from Shaw & Prasad (1970), Soltis & Soltis (1989) and Alfenas (1991), with modifications (recipes can be obtained from authors on request).

Data analysis

Alleles were identified by their mobility relative to the most common allele of each genus. Within the genus *Littoraria*, alleles

Table 1. Localities sampled for *Echinolittorina lineolata*, *Littoraria flava* and *L. angulifera*.

Species	Abbreviation of locality	Locality	Collection date	Lat/long
E. lineolata	El Fo	Fortaleza	Jul 1998	3°43′ S/38°30′E
	El Ti96	Tibau	Aug 1996	4°48′ S/37°15′ E
	El Ti97		Feb 1997	
	El Na	Natal	Apr 1997	5°48′ S/35°10′ E
	El Po	Porto (Barreiro)	Jan 1997	8°49′ S/35°07′ E
	El SJ97	S. José da Coroa Grande	Jan 1997	8°54′ S/35°08′ E
	El Pe	Pepino	Jan 1998	22°56′ S/43°16′ E
	El Fr	Frade	Aug 1997	23°00′ S/44°16′ E
	El IP	Porcos Pequena Is (Ubatuba)	Feb 1999	23°23′ S/44°53′ E
	El Pre96	Preta (S. Sebastião)	Nov 1996	23°48′ S/45°23′ E
	El Pre97		Jun 1997	
	El Pre98		Jun 1998	
	El Ba96	Barequeçaba (S. Sebastião)	Nov 1996	23°50′ S/45°26′ E
	El Ba98		Jun 1998	
	El Gu96	Guaecá (S. Sebastião)	May 1996	23°50′ S/45°27′ E
	El Gu3/97		Mar 1997	
	El Gu6/97		Jun 1997	
	El PG97	Praia Grande (S. Sebastião, Ilhabela)	Feb 1997	23°52′ S/45°25′ E
	El PG98		Jun 1998	
	El Bo	Bombas	Oct 1998	27°08′ S/48°29′ E
L. flava	Fl Ti	Tibau	Jul 1999	4°48′ S/37°15′ E
	Fl Na	Natal	Nov 1999	5°48′ S/35°10′ E
	Fl Su	Suape	Oct 1999	8°28′ S/34°57′ E
	Fl SJ97	S. José da Coroa Grande	Jan 1997	8°54′ S/35°08′ E
	Fl SJ00		Jan 2000	
	Fl Ar	Aracaju	Jul 1997	10°55′ S/37°02′ E
	Fl Ma	Mangaratiba	Sept 1998	22°56′ S/44°04′ E
	Fl PG98	Praia Grande (Ilhabela)	Jun 1998	23°52′ S/45°25′ E
	Fl Bo	Bombas	Oct 1998	27°08′ S/48°29′ E
L. angulifera	An MB	Morro Branco	Sept 1997	4°09′ S/38°07′ E
	An Ge	Genipabu	Feb 1998	5°41′ S/35°12′ E
	An Na	Natal	Nov 1999	5°48′ S/35°10′ E
	An JP	João Pessoa	Aug 1997	7°08′ S/34°57′ E
	An SA	Cabo de Sto Agostinho	May 1997	8°21′ S/34°57′ E
	An SJ97	S José da Coroa Grande	Jan 1997	8°54′ S/35°08′ E
	An SJ00		Jan 2000	
	An Mg	Maragoji	Nov 1999	9°01′ S/35°13′ E
	An SB	Abrolhos (Sta Bárbara Is)	Oct 1998	18°21′ S/38°36′ E
	An Ma	Mangaratiba	Sept 1998	22°56′ S/44°04′ E

and loci were named according to those of *L. flava* population Fl Ma. Sixteen of 18 loci scored were polymorphic in *Echinolittorina lineolata*. Of the 19 loci scored for *Littoraria*, 17 and 15 were polymorphic in *L. flava* and *L. angulifera*, respectively.

The Biosys-1 program (Swofford & Selander, 1981) was used to obtain the gene diversity (H_e) per population (unbiased estimate, Nei, 1978) and the observed heterozygosity (H_o) estimates. Departures from Hardy-Weinberg expectations were calculated using the TFPGA program (Miller, 1997): an exact test for each locus was done for all populations using a conventional Monte Carlo method (adapted from Guo & Thompson, 1992), with 10 batches of 1,000 permutations (10,000 permutations). The sequential Bonferroni procedure was used to correct for type 1 errors introduced by the use of multiple tests (Rice, 1989).

The F_{IS} fixation index was estimated for each species, populations and single locus using Weir & Cockerham's (1984) *f*. This index measures the reduction of heterozygosity within populations based on a correlation of alleles within individuals (Crow & Kimura, 1970; Weir & Cockerham, 1984). For each species, 95% confidence intervals were determined for F_{IS} values by bootstrapping 10,000 times across loci using the GDA program (Lewis & Zaykin, 1999).

The program Genetix v. 4.02 (Belkhir, 2001) was used to calculate the F_{IS} coefficient per population and locus. The F_{IS} values obtained in this analysis were used in the homogeneity test. We also tested the significance of this estimate for each population by making random permutations in the matrix of individuals *vs* genotypes in order to obtain the expected distribution of F_{IS} under the null hypothesis ($F_{IS} = 0$). The probability of occurrence of a value larger than or equal to that estimated was calculated as $P = (n + 1)/(N + 1)$, where n is the number of pseudo-values (1,000, obtained by permutations of genotypes in the total population) larger than or equal to the estimate, and N is the number of random permutations (Sokal & Rohlf, 1995). The sequential Bonferroni procedure was also used in this case.

The homogeneity of heterozygote deficiencies among loci within each sample was tested following Gaffney *et al.* (1990).

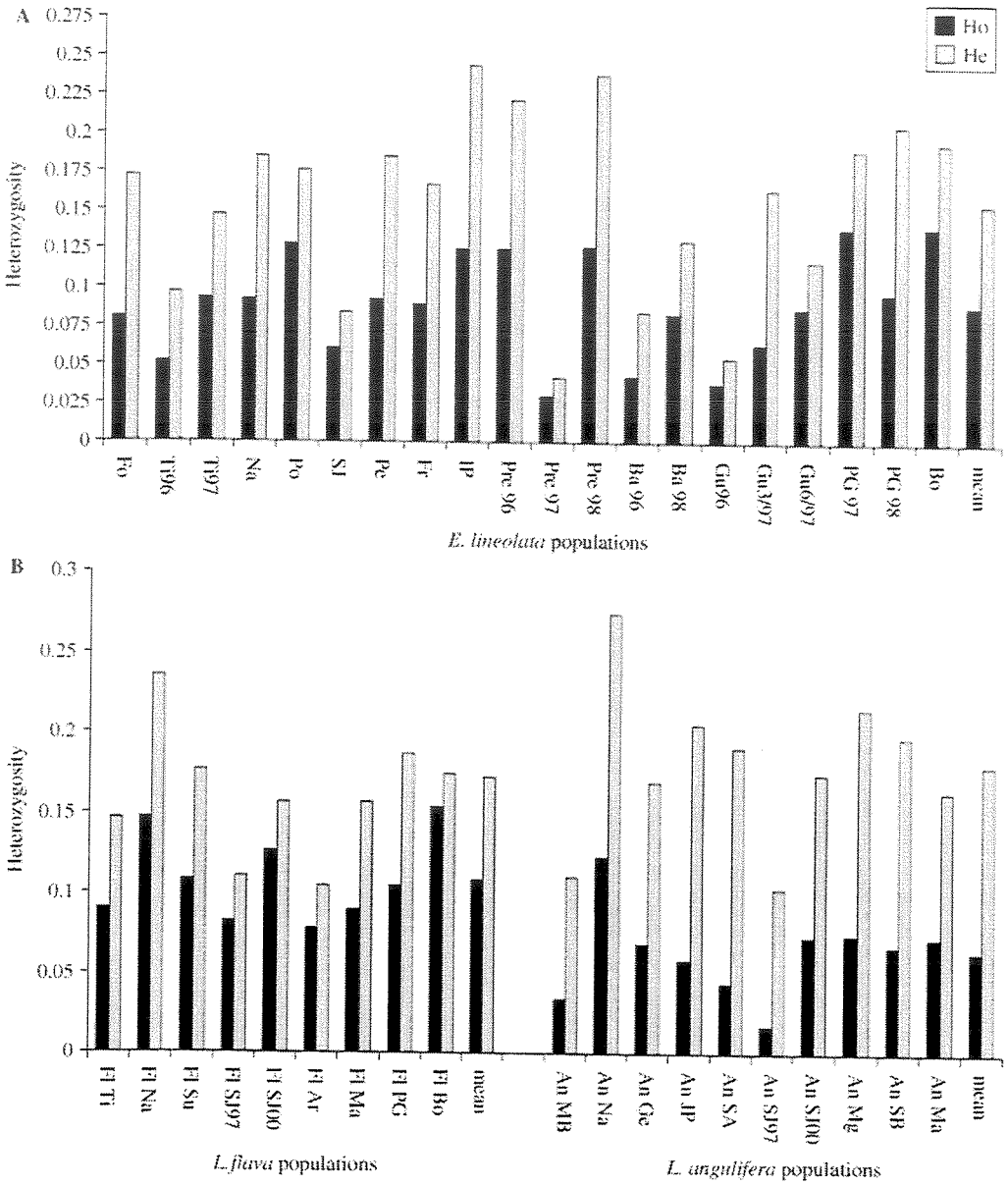

Figure 1. Populations and their respective values of gene diversity (H_e) and observed heterozygosity (H_o) over all loci. **A.** *Echinolittorina lineolata*, **B.** *Littoraria* species (on the left, *L. flava*; on the right, *L. angulifera*). Abbreviation of populations as in Table 1.

According to Crow & Kimura (1970) the F_{IS} can be considered a correlation coefficient and its homogeneity can be tested as described by Sokal & Rohlf (1995). In this test, correlation coefficients are transformed to a function z, which is approximately normally distributed. If the variation of z-values is randomly distributed in a sample, the weighted sum of squares of these values has an expected χ^2 distribution ($k-1$ degrees of freedom), which provides a probability of the true null hypothesis. The alternative hypothesis is that the variation of F_{IS} taken from different loci in the same sample is not due to chance, i.e. the values are heterogeneous among loci in the sample.

The same procedure was applied to compare the F_{IS} values obtained for each locus in all populations of each species. With this procedure, we tried to assess if each locus exhibited the same value along the populations. The F_{IS} of each locus was z-transformed and treated as described above. The null hypothesis was similar to the previous analysis, in which we assessed whether populations had similar F_{IS} values for each locus. For homogeneity analyses, populations smaller than six were not used (as suggested by Crow & Kimura, 1970).

A weighted average F_{IS} was obtained for each population and locus for the three species by calculating the corresponding F_{IS} value for the mean z value, using the z-test formula: $z = 0.5 \ln(1 + r)/(1 - r)$ where r is the correlation coefficient, F_{IS}.

A test for linkage disequilibrium using the GENEPOP program (Raymond & Rousset, 1995a, b) was made for *Littoraria* species. The default program was used: 100 batches of 1,000 iterations per batch with 1,000 dememorization steps. The sequential Bonferroni procedure was also applied. This analysis had already been made for *E. lineolata* (Andrade *et al.*, 2003).

RESULTS

The comparison among values of H_e and H_o of the three species is presented in Figure 1. The number of alleles in *Echinolittorina lineolata* ranged from four in MDH-1 to 10 in PGM-1 and LAP-1; in *Littoraria flava* populations there were two alleles in MDH-1

A

	AK1	AK2	HK1	HK2	IDH	LAP	LDH	MDH	ME	MPI	PLA	PLGG	GPI	PGM1	PGM2	PP	SDH1	SDH2	F_{IS}
Fo	54		44	42	56		31	57	51	55	**54**			**54**	34		**37**	**32**	0.530
Ti96	**65**	16	27	23	92	35	34	77	56	30	38	8	85	**65**	68	7	9	8	0.467
Ti97			24	20	49		*	51	34	40	14	15	65	**40**	28		*	*	0.377
Na	28	7	17	26	30		24	30	28	30	23	10	30	28	30	20	*	6	0.410
Po	57	39	42	24	57		18	64	47	47	31	10	57	**57**	58	9	6	*	0.275
SJ	29	13	12	7	48		8	38	9	26	*	*	50	**29**	16	*	*	*	0.283
Pe	45	40	38	38	47	**18**	21	48	48	50	43	20	50	**45**	45		**34**	**21**	0.507
Fr	63	27	28	32	66		29	56	49	67	45	8	67		55	**44**	18		0.474
IP		40	49	38	51		42	51	51	51	48	32	51	**47**	43	**36**	**37**	**29**	0.491
Pre96	79		33	40	63		29	82	66	63	42	*	74	**79**	71	**30**		*	0.440
Pre97	14	*	*	*	32	32	8	30	8	39	*	*	15	**14**	23	*	*	*	0.297
Pre98	23	18	**23**	20	30	**17**	17	31	30	23	20	12	31	**23**	14	**15**	**14**	**16**	0.473
Ba96	54		24	14	65	**43**	14	75	30	47	10	*	67		44	*	*	*	0.500
Ba98		12	10	18	17	17	13	19	19	19	15	*	18	**17**	17		6	*	0.381
Gu96	17	8	17	*	18	22	*	31	19	42	*	*	32	17	*	*	*	*	0.310
Gu3/97	45	20	24		57	32		52	29	33	28	16	58		31		12	**14**	0.615
Gu6/97	32	*	*	*	33	18	*	26	20	32	*	*	43		20	*	*	*	0.295
PG97	33	22	32	30	45	30	31	42	43	35	28	13	45		44	**14**	11	12	0.271
PG98	36	24	27	25	35	36	22	35	21	33	27	15	36	**36**	29	**24**			0.537
Bo	56	28		36	58	42		59	52	60	50	12	60				22	**17**	0.283
F_{IS}	**0.38**	**0.28**	**0.19**	**0.33**	**0.28**	0.31	**0.35**	0.00	**0.95**	**0.64**	**0.80**	**0.66**	0.02	0.38	**0.11**	**0.89**	**0.93**	**0.83**	

B

	AK1	AK2	AK3	IDH1	LAP1	LAP2	MDH2	ME	MPI	PLA	GPI	PGM1	PGM2	PGM3	PP	SDH1	AAT1	AAT2	F_{IS}
Ti	34	34	32	**34**	22		34	30		34	32	**31**	33	27	34		15	23	0.386
Na	19						21	20	21	21	19		20			21	21		0.377
Su		39	34	**39**	40	**39**	40		40	40		**39**		38			29	40	0.385
SJ97	16	17	14	16	11		17	17	17	17	11	7	7	7	16	17	10	10	0.271
SJ00	23	24		23	20	21	24	20	24	24	20		24	24	24	24	23	24	0.197
Ar	22	25		27	*	35	35	18	35	*					32	34	*	35	35	0.252
Ma		41		42	35	**44**	48			**41**		**35**			36	46	48	40	45	0.434
PG	9	7	8	8	*		10	*	10	**9**	9	8	*	*	9	10	10	10	0.454
Bo	10	10	9	10	10	10	10	10	10	10	10		10	10	10	9	9	10	0.125
F_{IS}	0.14	0.22	**0.60**	0.28	**0.59**	**0.58**	–0.01	0.22	**0.61**	**0.86**	0.22	**0.72**	0.31	0.21	**0.38**	**0.67**	**0.42**	**0.29**	

C

	AK1	AK3	IDH2	LAP2	ME	MPI	PLA	GPI	PGM2	PGM3	PP	SDH1	SDH2	AAT1	AAT2	F_{IS}
MB	16	12	15	17	16	17		**15**	**12**	15	15	17	14		17	0.697
Ge	**12**	10	11	**10**		12	**11**	**11**		12	12	12	10	9	12	0.606
Na		24	24	22		24	21			18	**24**			24		0.568
JP	**23**	22		**31**	20	31	**23**	**30**	**23**		32	32	27	33	33	0.719
SA		13	10	**13**	**13**	13	8	**10**	*	*		13	11		13	0.775
SJ97	**20**	18	16	18		18	**19**		*	*	19	20	12	**20**	20	0.841
SJ00	**23**	23	23	23	**23**	25	25		**24**		22	23	17	20	25	0.583
Mg	38	39		**35**	40	41	**41**	**37**	**41**	**41**	40	**33**			**41**	0.661
SB	41	40	40	**29**	**38**	**41**	**34**	**35**	**22**			40		35	36	0.664
Ma	41	41	43	41	45	40	37	39	28	28	43	45	43	45	45	0.563
F_{IS}	**0.65**	0.04	**0.91**	**0.84**	**0.57**	**0.34**	0.99	**0.76**	0.46	**0.37**	**0.90**	**0.59**	**0.87**	0.99	**0.92**	

loci with no heterozygote deficiency

loci with heterozygote deficiency using the Monte Carlo method

loci with heterozygote deficiency when the Bonferroni procedure was applied

* samples with less than six individuals (not tested)

Figure 2. Diagram of the Monte Carlo and homogeneity analyses for *Echinolittorina lineolata* (**A**), *Littoraria flava* (**B**) and *L. angulifera* (**C**). Populations are on the left and loci are above. The mean F_{IS} values are given to the right of and below the populations and loci, respectively. The values in bold are those significant from the homogeneity test ($P < 0.05$). Sample sizes are indicated for each locus and sample inside the diagram. The loci and populations with df = 0 are not shown. Abbreviation of populations as in Table 1.

Table 2. Test for homogeneity of Hardy-Weinberg deviations.

Among loci within populations			Among populations for each locus			
Populations	df	χ^2	Locus	sps	df	χ^2
El Fo	6	28.85***	AK1	El	13	285.35***
El Ti96	13	552.13***		Fl	7	11.11
El Ti97	8	226.72***		An	8	203.53***
El Na	11	276.94***	AK2	El	16	94.93***
El Po	9	100.26***		Fl	1	2.96
El SJ	12	111.85***	AK3	Fl	6	107.31***
El Pe	4	98.40***		An	6	4.17
El Fr	12	288.76***	HK1	El	13	34.51**
El IP	5	3.90	HK2	El	9	111.58***
El Pre96	12	409.99***	IDH 1	El	2	22.29***
El Pre97	3	3.57		Fl	8	13.03
El Pre98	13	224.84***	IDH2	An	2	151.42***
El Ba96	6	121.61***	LAP1	El	18	14.37
El Ba98	8	88.57***		Fl	2	78.35***
El Gu96	13	71.81***	LAP2	Fl	8	48.84***
El Gu3/97	12	228.01***		An	7	79.41***
El Gu6/97	13	469.69***	LDH	El	10	148.07***
El PG97	14	268.74***	MDH1	El	3	0.01
El PG98	12	318.03***	MDH2	Fl	1	0.01
El Bo	16	99.33***	ME	El	4	322.57***
Fl Ti	12	302.90***		Fl	6	11.65
Fl Na	15	167.65***		An	8	145.23***
Fl Su	15	48.54***	MPI	El	7	356.95***
Fl SJ97	7	36.74***		Fl	5	226.39***
Fl SJ00	11	208.71***		An	9	49.28***
Fl Ar	11	19.58	PLA	El	16	465.98***
Fl Ma	14	448.42***		Fl	3	158.19***
Fl PG	10	88.80***		An	7	0.01
Fl Bo	9	48.22***	PLGG	El	12	109.99***
An MB	6	129.97***	GPI	El	19	12.20
An Ge	14	77.02***		Fl	8	2.63
An Na	10	248.52***		An	9	52.25***
An JP	13	347.62***	PGM1	El	19	23.02
An SA	7	74.13***		Fl	8	64.93***
An SJ97	5	97.82***	PGM2	El	12	61.87***
An SJ00	7	34.99***		Fl	8	6.73
An Mg	14	634.15***		An	7	2.01
An SB	11	184.48***	PGM3	Fl	8	2.04
An Ma	11	517.97***		An	7	17.43*
			PP	El	13	295.19***
				Fl	2	13.54**
				An	4	138.26***
			SDH1	El	8	149.13***
				Fl	5	133.24***
				An	4	155.48***
			SDH2	El	10	81.69***
				An	5	191.12***
			AAT1	Fl	3	105.12***
				An	4	0.01
			AAT2	Fl	5	111.67***
				An	2	128.26***

Abbreviations of populations as in Table 1.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

and 13 in PGM-2; and the range of allelic number in *L. angulifera* was 2 in MDH-1, MDH-2 and IDH-2 loci and 16 in PGM-2.

The mean F_{IS} was high for the three species: $F_{IS} = 0.461$ (95% CI = 0.333–0.666) for *E. lineolata*; $F_{IS} = 0.346$ (95% CI = 0.250–0.465), for *L. flava*; and $F_{IS} = 0.645$ (95% CI = 0.533–0.775), for *L. angulifera*.

Most populations showed departures from Hardy-Weinberg expectations in several loci and all deviations were explained by deficit of heterozygotes (Figs 1, 2). All population F_{IS} estimates were significantly different from zero. Using the Monte Carlo method followed by sequential Bonferroni correction, 43 out of 228 tests were significant for *E. lineolata*, nine out of 113 for *L. flava*, and 38 out of 109 tests for *L. angulifera* (Fig. 2).

Tests for homogeneity of deviations from Hardy-Weinberg equilibrium showed that 36 out of 39 populations showed some heterogeneity among loci (Table 2, Fig. 2).

In the analysis for each locus among populations, most loci (35 out of 51, for all species) showed significant heterogeneity in heterozygote deficiencies (Table 2, Fig. 2). The PGM loci showed heterozygote deficiency in 12 of the 20 populations of *E. lineolata*, in three of the nine of *L. flava*, and in five of the 10 populations of *L. angulifera*. The loci PGM-1 in *E. lineolata*, PGM-2 in *L. angulifera* and PGM-2 and PGM-3 in *L. flava* presented a strong pattern of high and homogeneous F_{IS} values among populations (Table 2, Fig. 2).

None of the 653 linkage disequilibrium tests in *Littoraria flava* populations was significant. Seven out of 509 tests for *L. angulifera* showed a significant deviation from a random distribution of genotypes. The loci associations that showed disequilibrium were: PLGG and LAP-2 (An Ge), AK-1 and PLGG (An JP), GPI and LAP-2 (An JP and An Mg), AK-1 and AAT-1 (An SJ97), ME and AK-1 (An SJ00), LAP-2 and AAT-2 (An Mg).

DISCUSSION

Many reports have demonstrated heterozygote deficiency in natural populations of marine invertebrates. In the present work, we found significant high F_{IS} values in a large number of loci in three littorinid species, an indication that deviations from Hardy-Weinberg equilibrium are common in these gastropods (Figs 1, 2).

Pooling of genetically differentiated specimens would produce heterozygote deficiencies, but would also result in gametic disequilibrium or non-random association among genes in a population (Hartl & Clark, 1997). We have not detected significant deviations from random associations between loci in *Littoraria flava* populations, nor have they been observed in previous studies on *E. lineolata* populations (Andrade *et al.*, 2003); however, a few significant associations were found in *L. angulifera*, but this is probably a consequence of genetic subdivision, as observed by Andrade *et al.* (2003).

Several explanations have been proposed to account for Hardy-Weinberg deviations in marine molluscs. Aneuploidy was suggested as a probable cause in some species (Dixon, 1982; Thiriot-Quiévreux, 1986) and could explain deficiencies when a single tissue type (e.g. gill) is analysed (David, 1998). Null alleles could be responsible for such heterozygote paucity if the deficiencies were borderline at one or two loci (Gardner, 1992; Rio-Portilla & Beaumont, 2000). Otherwise, it is necessary for mutation rates to be high or for strong selection to exist that favours null heterozygotes. In our populations, all individuals produced active enzyme patterns at all loci and there was no indication of null homozygotes. Therefore, null alleles and aneuploidy may have some minor effect, but certainly cannot explain the deviations for all loci and populations.

Other possible explanations for the excess of homozygotes include molecular imprinting and mis-scoring. However, imprinting is a transient effect and is generally not repeated

over generations (Chakraborty, 1989). Mis-scoring was unlikely since no allele migrated with an electrophoretic mobility close enough to another to cause misinterpretation.

The Wahlund effect consists of pooling populations that differ in allele frequencies, resulting in a deficiency of heterozygotes in the combined sample (Gaffney *et al.*, 1990). A simple example of this would occur in the extreme case of pooling populations fixed for different alleles, where homozygotes for two alleles would be found, but no heterozygotes.

Genetic divergence among populations reflects interactions between natural selection and breeding systems. Whereas natural selection may act differently on each locus, non-random mating and population subdivision should affect all loci in a similar way. Inbreeding would generate uniform heterozygote deficiencies within populations regardless of gene frequencies. Most of our populations (36 out of 39) had heterogeneous F_{IS} across loci, and this has also been observed in other molluscs (Gaffney *et al.*, 1990; Pecon Slattery, Vrijenhoek & Lutz, 1991). Since *Echinolittorina lineolata* and *L. flava* have planktotrophic larval development and there is only weak structure among populations suggesting high gene flow, the effects of any local inbreeding would be lost in the next generation by mixing of the larvae (Andrade *et al.*, 2003). The Wahlund effect alone could explain these results only if each population had many breeding groups, but these could hardly be maintained given the planktonic development of these species. Besides, it would require a larger variance of allelic frequencies among the populations to generate the high values of F_{IS}. Thus, although inbreeding and the Wahlund effect cannot be discounted, our data do not support them as major factors responsible for the deviations observed here.

In the analysis of homogeneity of F_{IS} value at each locus, most of the loci showed heterogeneous F_{IS} across populations (Table 2, Fig. 2). This result was expected, since the intensity of the factors above may be different on each population. These factors could have a minor role but when summed up lead to the observed deviations. Another aspect that could account for these results is the association between size and heterozygosity, reported in many bivalves (Singh & Green, 1984; Zouros & Foltz, 1984; Gaffney *et al.*, 1990; Fairbrother & Beaumont, 1993; Toro & Vergara, 1995). However, in this study only the adults were analysed, ruling out large variation in size, although individuals were possibly derived from different cohorts. Furthermore, some loci showed high homogeneous heterozygote deficiencies among populations (Fig. 2); so, the deviations showed evidence of being locus-specific and often consistent across populations.

Littoraria angulifera showed the highest F_{IS} values (Fig. 2), in particular for PLA and AAT-1 loci, where we rarely found heterozygotes (even with relatively good sampling in some populations, like An Mg; Fig. 2). This species has incomplete ovoviviparity: the female retains the eggs until the swimming veliger stage (Bandel, 1974; Gallagher & Reid, 1974). Although *L. angulifera* has planktotrophic development, their populations show high genetic structure ($\theta = 0.185$, Andrade *et al.*, 2003). The populations of this species are therefore not a single breeding unit, which may explain the small amount of linkage disequilibrium we observed and part of the heterozygote deficiency. This explanation does not seem sufficient to explain completely the deviations, for the few significant associations between loci were not found in all populations. Moreover, even with the high structuring, there is evidence of gene flow among populations where we could observe shared rare alleles (Andrade *et al.*, 2003).

The homogeneity analysis over populations highlights the PGM system: all three species had homogeneous positive F_{IS} values in at least one PGM locus across populations (Table 2, Fig. 2). This result may suggest a pattern that could be explained by a directional factor acting on this enzyme or on a linked gene over different localities. However, very high coefficients of selection on these loci would be necessary to explain the F_{IS} values observed.

Hardy-Weinberg deviations caused by natural selection have been reported in marine molluscs (Johannesson, Johannesson & Lundgren, 1995; Lemaire *et al.*, 2000). Several studies have shown that different genotypes of PGM strongly affect survival of many marine invertebrates (Wilkins, Fulino & Sasaki, 1980; Nevo *et al.*, 1984; Moraga, Jollivet & Denis, 1994; Tanguy *et al.*, 1999). A relationship between some PGM allelic combinations and temperature tolerance has also been observed in littorinids (Wilkins, O'Regan & Moynihan, 1978 cited in Moraga *et al.*, 1994). We did not measure a possible relation between temperature and sampling site, so we cannot discount the effect of temperature on PGM allelic frequencies in Brazilian populations.

In marine organisms, the possibility that selection may act on some loci through local adaptation has been considered (Johannesson *et al.*, 1995; Lemaire *et al.*, 2000). Some authors have also mentioned selective mortality in the larval stage and during early settlement as the major determinant of allele frequencies (Johnson & Black, 1984; Zouros & Foltz, 1984; Fairbrother & Beaumont, 1993). The subdivision of a population could result from differences in the genetic composition of settling recruits, or from site-specific genotypic selection during benthic existence. As adults have low mobility and a high probability of mating with neighbours, genetic sub-structuring would result in heterozygote deficiency in the larval pool and, in consequence, in the entire population. A possible effect of habitat heterogeneity could be considered as these individuals are distributed in a highly heterogeneous environment.

Assortative mating could lead to deficiency of heterozygotes; however, that in the subsequent generations could only be maintained under continuing selection pressure, otherwise, due to the planktonic development phase, they would return to Hardy-Weinberg equilibrium. Balancing selection tends to homogenize gene frequencies, which can lead to the incorrect interpretation of high gene flow. The effect of balancing selection together with assortative mating on PGM loci could provide an explanation for the observed deviation. The coefficients of selection necessary to overcome the dispersal ability to explain the F_{IS} values observed would be high, as well as the genetic load on these populations. Hence, natural selection cannot account for all cases of heterozygote deficiency in our populations, but it may be acting together with other factors. The homogeneity tests give some idea of how the deviations are organized in our populations. The high F_{IS} in PGM loci maintained along a large spatial and temporal scale (4,000 km and 3 years), cannot be entirely explained by a stochastic mechanism. It seems likely that this enzyme may have an important role in the fitness of the species studied here, although the factors responsible for natural selection are not known.

The causes of Hardy-Weinberg deviations in many marine invertebrates remain bewildering after more than three decades of investigation. The most difficult cases to explain are those involving species with a planktonic larval phase, as studied here, which makes them interesting models. Our data have not determined the causes for heterozygote deficiency, and indeed raise more questions about the unexpected homogeneity at PGM loci for the three species of Brazilian littorinids. The uniqueness of these data may be a clue to their Hardy-Weinberg deviations. This study suggests the need to study the occurrence of micro-spatial differentiation in this system, as well as the possible explanations for it. It is also necessary to test the homogeneity of F_{IS} values in other marine invertebrates that have heterozygote deficiencies at so many loci.

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REFERENCES

ALFENAS, A.C. 1991. *Eletroforese de isozimas e proteínas afins*. Editora Universidade Federal de Viçosa, MG.

ANDRADE, S.C.S. & MAGALHÃES, C.A. 2003. Solferini, V.N. Patterns of genetic variability in Brazilian littorinids (Mollusca): a macrogeographic approach. *Journal of Zoological Systematics and Evolutionary Research*, **41**: 249–255.

BANDEL, K. 1974. Studies on Littorinidae from the Atlantic. *Veliger*, **17**: 92–114.

BANDEL, K. & KADOLSKY, D. 1982. Western Atlantic species of *Nodilittorina* (Gastropoda: Prosobranchia): comparative morphology and its functional, ecological, phylogenetic and taxonomic implications. *Veliger*, **25**: 1–42.

BELKHIR, K. 2001. *GENETIX, logiciel sous WindowsTM pour la génétique des populations Laboratoire Génome et Populations, CNRS UPR 9060*. Université de Montpellier II, Montpellier, France.

CHAKRABORTY, R. 1989. Can molecular imprinting explain heterozygote deficiency and hybrid vigor? *Genetics*, **122**: 713–717.

CROW, J. F. & KIMURA, M. 1970. *An introduction to population genetics theory*. Harper & Row, New York.

DAVID, P. 1998. Heterozygote-fitness correlations: new perspectives on old problems. *Heredity*, **80**: 531–537.

DIXON, D.R. 1982. Aneuploidy in mussel embryos (*Mytilus edulis*) originating from a polluted dock. *Marine Biology Letters*, **3**: 155–161.

FAIRBROTHER, J.E. & BEAUMONT, A.R. 1993. Heterozygote deficiencies in a cohort of newly settled *Mytilus edulis* spat. *Journal of the Marine Biological Association of the United Kingdom*, **73**: 647–653.

FOLTZ, D.W., SHUMWAY, S.E. & CRISP, D. 1993. Genetic structure and heterozygosity-related fitness effects in the marine snail *Littorina littorea*. *American Malacological Bulletin*, **10**: 55–60.

GAFFNEY, P.M., SCOTT, T.M., KOEHN, R.K. & DIEHL, W.J. 1990. Interrelationships of heterozygosity, growth rate and heterozygote deficiency in the coot clam, *Mulinia lateralis*. *Genetics*, **124**: 687–699.

GALLAGHER, S.B. & REID, G.K. 1974. Reproductive behavior and early development in *Littorina scabra angulifera* and *Littorina irrorata* (Gastropoda: Prosobranchia) in the Tampa Bay region of Florida. *Malacological Review*, **7**: 105–125.

GARDNER, J.P.A. 1992. Null alleles and heterozygote deficiencies among mussels (*Mytilus edulis* and *M. galloprovincialis*) of two sympatric populations. *Malacologia*, **34**: 99–106.

GUO, S.W. & THOMPSON, E.A. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, **48**: 361–372.

HARTL, D.H. & CLARK, A.G. 1997. *Principles of population genetics*. Edn 3. Sinauer Associates, Massachusetts.

JOHANNESSON, K., JOHANNESSON, B. & LUNDGREN, U. 1995. Strong natural selection causes micro-escale allozyme variation in a marine snail. *Proceedings of the National Academy of Sciences of the USA*, **92**: 2602–2606.

JOHNSON, M.S. & BLACK, R. 1984. The Wahlund effect and the geographical scale of variation in the intertidal limpet *Siphonaria* sp. *Marine Biology*, **79**: 295–302.

LEMAIRE, C., ALLEGRUCCI, G., NACIRI, M., BAHRI-SFAR, L., KARA, H., and BONHOMME, F. (2000) Do discrepancies between microsatellite and allozyme variation reveal differential selection between sea and lagoon in the sea bass (*Dicentrarchus labrax*)? *Molecular Ecology*, **9**: 457–467.

LEWIS, P.O., ZAYKIN, D. 1999. *Genetic Data Analysis: computer program for the analysis of allelic data* Version 10 (d12). Available from http://lewis.eeb.uconn.edu/lewishome/software.html.

MAGALHÃES, C.A. 1998. Density and shell-size variation of *Nodilittorina lineolata* (Orbigny, 1840) in the intertidal region in southeastern Brazil. *Hydrobiologia*, **378**: 143–148.

MILLER, M.P. 1997. Tools for population genetic analyses (TFPGA) 13: a Windows program for the analysis of allozyme and molecular population genetic data. Available from http://www.marksgenetic software.net

MORAGA, D., JOLLIVET, D. & DENIS, F. 1994. Genetic differentiation across the western Pacific populations of the hydrothermal vent bivalve *Bathymodiolus* spp and the eastern Pacific (13°N) population of *Bathymodiolus thermophilus*. *Deep-Sea Research I*, **41**: 1551–1567.

NEI, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**: 583–590.

NEVO, E., BEN-SHLOMO, E. & LAVIE, B. 1984. Mercury selection of allozymes in marine organisms: prediction and verification in nature. *Proceedings of the National Academy of Sciences of the USA*, **81**: 1258–1259.

NOY, R., LAVIE, B. & NEVO, E. 1987. The niche-width variation hypothesis revisited: genetic diversity in the marine gastropods *Littorina punctata* (Gmelin) and *L. neritoides* (L). *Journal of Experimental Marine Biology and Ecology*, **109**: 109–135.

PECON SLATTERY, J., VRIJENHOEK, R.C. & LUTZ, R.A. 1991. Heterozygosity, growth, and survival of the hard clam, *Mercenaria mercenaria*, in seagrass vs sandflat habitats. *Marine Biology*, **111**: 335–342.

RAYMOND, M. & ROUSSET, F. 1995a. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**: 248–249.

RAYMOND, M. & ROUSSET, F. 1995b. An exact test for population differentiation. *Evolution*, **49**: 1280–1283.

REID, D.G. & MAK, Y.M. 1999. Indirect evidence for ecophenotypic plasticity in radular dentition of *Littoraria* species (Gastropoda: Littorinidae). *Journal of Molluscan Studies*, **65**: 355–370.

RICE, W.R. 1989. Analyzing tables of statistical tests. *Evolution*, **43**: 223–225.

RIO-PORTILLA, M.A. & BEAUMONT, A.R. 2000. Larval growth, juvenile size and heterozygosity in laboratory reared mussels, *Mytilus edulis*. *Journal of Experimental Marine Biology and Ecology*, **254**: 1–17.

SHAW, C.R. & PRASAD, R. 1970. Starch gel electrophoresis of enzymes—a compilation of recipes. *Biochemical Genetics*, **4**: 297–320.

SINGH, S.M. & GREEN, R.H. 1984. Excess of allozyme homozygosity in marine molluscs and its possible biological significance. *Malacologia*, **25**: 569–581.

SKIBINSKI, D.O.F., BEARDMORE, J.A. & CROSS, T.F. 1983. Aspects of the population genetics of *Mytilus* (Mytilidae; Mollusca) in the British Isles. *Biological Journal of the Linnean Society*, **19**: 137–183.

SOKAL, R.R. & ROHLF, F.J. 1995. *Biometry: the principles and practice of statistics in biological research*. Edn 3. WH Freeman Company, NY.

SOLTIS, D.E. & SOLTIS, P.S. 1989. *Advances in plant sciences series. Isozymes in plant biology*. **Vol. 4**. Dioscorides Press.

SWOFFORD, D.L. & SELANDER, R.B. 1981. BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. *Journal of Heredity*, **72**: 281–283.

TANGUY, A., FERNANDEZ CASTRO, F., MARCHIC, A. & MORAGA, D. 1999. Effects of an organic pollutant (tributyltin) on genetic structure in the Pacific oyster *Crassostrea gigas*. *Marine Pollution Bulletin*, **38**: 550–559.

THIRIOT-QUIÉVREUX, C. 1986. Study of aneuploidy in spats of Ostreidae (Bivalvia). *Genetica*, **70**: 225–231.

TORO, J.E. & VERGARA, A.M. 1995. Evidence for selection against heterozygotes: post-settlement excess of allozyme homozygosity in a cohort of the Chilean Oyster, *Ostrea chilensis* Philippi, 1845. *Biological Bulletin*, **188**: 117–119.

TRACEY, M.L., BELLET, N.F. & GRAVEM, C.D. 1975. Excess allozyme homozygosity and breeding population structure in the mussel *Mytilus californianus*. *Marine Biology*, **32**: 303–311.

WEIR, B.S. & COCKERHAM, C.C. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution*, **38**: 1358–1370.

WILKINS, N.P., O'REGAN, D. & MOYNIHAN, E. 1978. Electrophoretic variability and temperature sensitivity of phosphoglucose isomerase and phosphoglucomutase in littorinids and other marine molluscs. In: *Marine organisms genetics, ecology, and evolution* (B. Battaglia, ed.). Plenum Press, Chicago, NATO Conference Series.

WILKINS, N.P., FULINO, K. & SASAKI, K. 1980. Genetic studies on the Pacific abalone-IV Thermostability difference among phosphoglucomutase variants. *Bulletin of the Japanese Society of Scientific Fisheries*, **46**: 549–553.

WILLIAMS, S.T., REID, D.G. & LITTLEWOOD, D.T.J. 2003. A molecular phylogeny of Littorininae (Gastropoda: Littorinidae): unequal evolutionary rates, morphological parallelism, and biogeography of the Southern Ocean. *Molecular Phylogenetics and Evolution*, **28**: 60–86.

ZOUROS, E. & FOLTZ, D.W. 1984. Possible explanations of heterozygote deficiency in bivalve molluscs. *Malacologia*, **25**: 583–591.

Capítulo 2

Fine-scale genetic structure and heterozygote deficiency in *Littoraria flava* (Mollusca: Gastropoda): demographic events or selection?

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ABSTRACT

Littoraria flava, a Brazilian littorinid species with planktotrophic larval phase, occupies a continuous habitat on rocky shores close to brackish and freshwater sources. Previous studies of this species have shown a moderate genetic structure over a broad geographic scale, with high deviations from Hardy-Weinberg expectations in many allozymic loci, always towards heterozygote deficiency. Local-scale subdivision in marine species with a long dispersal phase is unexpected, but occasionally found. Using a horizontal transect at three locations, we examined whether micro-scale and short-term subdivision also occurred in *L. flava* populations and, if so, whether this could explain the Hardy-Weinberg deviations. *Littoraria flava* showed even more structuring on a microgeographic scale (4-300 m) than on a large-scale (>200 km). The Ewens-Watterson neutrality test showed that 18% of the tests deviated significantly from the neutrality model, suggesting that there are more rare alleles than predicted. A homogeneity test for each locus across samples within transects showed homogeneous and high F_{IS} values in about half of tests. A multivariate analysis of environmental characteristics indicated that there was more heterogeneity in species composition along a transect than among them, in accordance with the genetic data. These results and the apparent genetic patchiness within transects suggest that there could be a balance between recurrent colonizations through intense dispersal and selection acting on different loci at different times and localities.

Key words: environmental effect; Hardy-Weinberg deviation; hierarchical analysis; *Littoraria flava*; local-scale subdivision.

INTRODUCTION

The study of microgeographic structure provides a way to understand the maintenance and distribution of genetic variability in marine invertebrates. Populational subdivision in non-planktonic species is common as a result of low motility of adults and juveniles. Genetic divergence among populations results from the interaction of selection and breeding system, thereby subdivision and genetic drift. In marine species with high dispersal rates, but relatively little geographic interpopulational variation, many cases of divergence have been reported at a scale from less than a hundred meters to a few kilometers (Johnson & Black, 1984; Watts, Johnson & Black, 1990; Tatarenkov, 1995; Johnson & Black, 2000; Sotka *et al.*, 2004). Some species showed uniformity over large distances, but high levels of local genetic structuring (Johnson & Black, 1984; Watts *et al.*, 1990; Parsons, 1996). As observed by Parsons (1996), most cases of enhanced subdivision have been observed in estuaries, embayments and islands, places that favor genetic divergence (e.g., Johnson & Black, 1991; Ayvazian, Johnson & McGlashan, 1994; Tatarenkov, 1995; Johnson *et al.*, 2001).

Rocky shores are among the most extreme environments in terms of physical heterogeneity, mainly because of marked abiotic gradients such as temperature, salinity, wave action, and incidence of irradiation. Associated with these gradients are sharp transitions in the distribution of organisms, with grazing, predation and species interactions contributing to this heterogeneous scenario. In littorinids, several allozyme studies investigated the effects of genetic drift and selection by describing population differentiation and structuring on 'horizontal' or 'vertical', micro or macrogeographic scales on rocky shores, most of them dealing with direct-development species (e.g., Johannesson, Johannesson & Rolán-Alvarez, 1993; Johannesson, Johannesson & Lundgren, 1995;

Johannesson & Tatarenkov, 1997; Tatarenkov & Johannesson, 1999; Johannesson *et al.*, 2004; Panova & Johannesson, 2004).

Littoraria flava (King & Broderip, 1832) is commonly found on rocky-shores near freshwater sources slightly above the mean water level, but also occurs in mangroves. This snail is dioceous, oviparous and, like most marine species, has a life-cycle that alternates between a relatively sedentary stage and an ecologically distinct larval phase capable of great dispersal (Reid, 1986, 1999). In Brazil, *L. flava* is common in beaches with estuaries or near mangroves, with a continuous distribution along rocky shore.

In common with littorinids that have a planktonic development (e.g, Janson, 1987; Ward, 1990; Parsons, 1996; De Wolf, Verhagen & Backeljau, 2000), *L. flava* shows substantial levels of isozyme variability (Andrade, Magalhães & Solferini, 2003). On a macrogeographic scale (4,000 km of Brazilian coast), *L. flava* shows moderate structure (all loci: θ=0.054) and high deviation from Hardy-Weinberg expectations (F_{IS}=0.346), always towards deficiency of heterozygotes, as also reported for many marine mollusks (Johnson & Black, 1984; Zouros & Foltz, 1984; Gaffney *et al.*, 1990; Johnson *et al.*, 2001; Andrade, Medeiros & Solferini, 2005). These results are difficult to interpret because the planktotrophic development should promote random matings and panmitic populations.

Populational subdivision in a microgeographic or temporal scale could be an explanation. To test whether *L. flava* populations are locally subdivided, we sampled three localities (two estuaries and a boulder shore); at each site, samples were taken along a horizontal transect. This procedure was applied three times at each locality, with an interval of 5-6 months between each sampling. The aim was to determine whether there was any microgeographic structuring among *L. flava* samples. The variability within transects were

compared to the variation within localities (apart up to 220 km). Hierarchical analyses were performed to test two different hypotheses:

(1) there is more structuring among sites along transects than among localities. If so, are the samples collected on rocky shores along estuaries more structured than those collected elsewhere?;

(2) there is genetic structuring in a temporal scale among the different times of collection over a year period. If so, is this structuring greater than among samples located a few meters apart along a transect?

A possible relationship between genetic differentiation and geographic distance along the transects was evaluated to detect any indication of isolation-by-distance pattern.

Material and Methods

Sites

Samples of *L. flava* were collected on three beaches, Juquehy, Dura and Itamambuca, located on north coast of São Paulo state in three periods (collections): November 2001, May 2002 and October 2002, providing a total of 63 samples (with 10-49 individuals per sample, Table 1). Juquehy is the most southerly of the three beaches and is a sheltered locality characterized by a boulder rocky shore, unlike Dura and Itamambuca. Dura is about 165 km apart from Juquehy and about 60 km from Itamambuca, which has the most northerly location. Itamambuca and Dura are on the estuaries of the Itamambuca and Escuro rivers, respectively. Itamambuca is more exposed than Dura and its river is about 10 m wide while the Escuro River is about 20 m wide. There are mangroves close to both locations, but only at Dura were specimens of *L. flava* easily found.

Sampling design

On each shore, we collected samples along a horizontal transect that began close to the beach, where the first specimens of *L. flava* were found, in direction to the sea. The distances among sampling sites were established using the formulae $D_n = 2^{n-1}$, where *D* is the distance in meters and *n* is the number of the site. At the first collection, we marked the rock at each sampled site with Tubolit® polyethylene to ensure that the next samplings were done at the same point, or very close. The periwinkles were collected within 1 m^2 around the marked point at sites 0, 4, 8, 16, 32, 64, 128 and 256 m along the transect (sites 1 and 2 were excluded, due to the large area of collect, 1 m^2). This sampling design was an attempt to include many several possible scales of structuring. The length of the transects varied among shores depending on the species distribution: at Itamambuca it was 64 m long, at Juquehy, 128 m and at Dura, 232 m (the last sites where *L. flava* was seen). The last sample of Itamambuca and the last two samples of Dura were collected on the coast (not inside the estuary). At Dura, an additional sampling site (identified as Dm) located in mangrove 100 m away from the shore, was included, where the individuals were found in fields of *Spartina* sp., a very common grass in Brazilian mangroves. Some sites at Itamambuca were not re-sampled because of lack of individuals.

Environmental data

For environmental characterization, we collected samples of invertebrates (only colonizers of primary substratum) and algae on the 1 m^2 area at each site along the transects. The specimens were stored in 70% ethanol (invertebrates) and 7.5% formalin (algae) for subsequent identification to the lowest taxon possible. The sites were characterized by the absence/presence of the following species: the bivalves *Brachidontes*

darwinianus, *B. solisianus* and *Crassostrea rizhophorae*, the gastropods *Mellampus coffeus*, *Collisella subrugosa*, *Echinolittorina lineolata* and *Littoraria angulifera*, the barnacle *Chthamalus bisinuatus*; and one species of isopod. The algae included the green *Ulva lactuca*, *U. fasciata*, *Enteromorpha compressa*, *Cladophora coelothrix*, *Rhizoclonium riparium*, and the red algae *Porphyra atropurpurea*, *Bostrychia radicans*, *B. calliptera*, *B. tenella*, *B. montagnei*, *Murrayella periclados*. Composite indices for the variables were obtained from non-centered principal component analysis, calculated using the FITOPAC package (Shepherd, 1995).

Electrophoresis of L. flava

Specimens were collected and frozen in liquid nitrogen until electrophoresis. One thousand eight hundred and fifty-one individuals were analyzed. The entire soft body was squashed in extraction buffer (0.1 M Tris pH 8.0, 0.5% β-mercaptoethanol) and the extracts were loaded on to 8.5% starch gels (hydrolyzed potato starch, Sigma). The electrophoretic conditions were described by Andrade *et al.* (2003).

Of the 12 enzymes used in that study, only seven were scored here. The electrophoretic analyses were done with 15 polymorphic loci: malic enzyme (EC 1.1.1.40; ME, buffer II), isocitric dehydrogenase (EC 1.1.1.42; IDH, buffer I), adenylate kinase (EC 2.7.4.3; AK, buffer I), L-leucyl-L-glycylglycine peptidase (EC 3.4.11. -; PLGG, buffer II), leucine aminopeptidase (EC 3.4.11.1; LAP, buffer I), phosphoglucose isomerase (EC 5.3.1.9; GPI, buffer I), and phosphoglucomutase (EC 5.4.2.2; PGM, buffer I). Two buffer systems were used: (I) electrode: 0.04 M citric acid and N (3 aminopropyl) morpholine adjusted to pH 6.1, and gel: electrode solution diluted 1:20 (Clayton & Tetriak, 1972); (II)

electrode: 0.01 lithium hydroxide, 0.095 boric acid and 0.003 EDTA, pH 8.0, and gel: electrode solution diluted 1:40. For LAP, in this study only Lap-1 was scored; Me-2, Idh-2, Pep-1, Pep-3 were scored here but not in the samples of Andrade *et al.* (2003, 2005). The staining procedures were adapted from Shaw & Prasad (1970) and Alfenas (1991), with modifications (recipes can be obtained on request).

Analysis of genetic data

Intrapopulational estimates

Alleles were identified by their mobility relative to the most common allele. Estimates of genetic variability were calculated per sample and included the percentage of polymorphic loci (P, 95% criterion), the gene diversity (H_e, unbiased estimate; Nei, 1978), the observed heterozygosity (H_o) and the fixation index F_{IS} (f; Weir & Cockerham, 1984). Departures from Hardy-Weinberg expectations were calculated using the TFPGA program (Miller, 1997). An exact test for each locus was done for all samples using a conventional Monte Carlo method (adapted from Guo & Thompson, 1992), with 10 batches of 1,000 permutations. The sequential Bonferroni procedure was used to correct type I errors introduced by the use of multiple tests (Rice, 1989).

The program Genetix v. 4.02 (Belkhir, 2001) was used to calculate the F_{IS} coefficient per sample and loci. The F_{IS} values obtained in this analysis were used in the homogeneity test (as described in Andrade *et al.*, 2005). According to Crow & Kimura (1970), the F_{IS} can be considered a correlation coefficient and its homogeneity can be tested as described by Sokal & Rohlf (1995). The homogeneity of heterozygote deficiencies across loci within each sample was tested according to Gaffney *et al.* (1990). The same procedure was applied to compare the F_{IS} values of each locus across all samples from each

transect. With this procedure, we tried to assess whether each locus had homogeneity of deviation within transects. The alternative hypothesis was that the values of the F_{IS} for different loci on the same sample were heterogeneous. Samples with less than six individuals were not used for the analyses of homogeneity (Crow & Kimura 1970). Along each transect, any locus that was scored by less than four samples was not included in this analysis.

The Ewens-Watterson neutrality test (Ewens, 1972; Watterson, 1978; Slatkin, 1994 implementation) was applied to allelic frequencies distributions using the program PyPop (Lancaster *et al.*, 2003). This test compares the sum of the squared allele frequencies (F or homozygosity) in a sample with the expected distribution of F under neutral theory equilibrium. The p-value is the probability that the observed homozygosity was obtained in a neutral sample of equal size and k distinct alleles, equal to the samples. The observed F is compared with the simulated F from 1,000 samples obtained by the Markov-chain Monte Carlo method (with 2,000 dememorization steps). If F lies within the top or bottom 2.5% of the theoretical distribution, then the assumption of equilibrium neutrality can be rejected. A method for combining probabilities for each locus in each transect was applied, as developed by Fisher (1954, in Sokal & Rohlf 1995). In this analysis p-values from all tests within each transect were transformed and combined in one p-value.

Genotypic data were tested for linkage disequilibrium within samples and transects using the GENEPOP program (Raymond & Rousset, 1995a, b). Default program settings were used: 200 batches of 5,000 iterations per batch with 1,000 dememorization steps. Again, the sequential Bonferroni procedure was applied.

Interpopulational variability estimates

The heterogeneity of the allelic frequencies in each locus was estimated using Fisher's R x C test (Raymound & Rousset, 1995b; Sokal & Rohlf, 1995), with 10 batches of 10,000 permutations and 1,000 dememorization steps.

The F_{ST} coefficients were calculated using the Weir & Cockerham (1984) parameter (θ). Ninety-five percent confidence intervals were determined for the total F_{ST} value by bootstrapping 10,000 times across the loci using the GDA program (Lewis & Zaykin, 1999). To test whether there was microspatial structuring, we calculated the F_{ST} among samples in each transect. A four-level hierarchical analysis was done to test whether there was more structuring between localities than among sites within transects (Figure 1). The four hierarchical levels for the test of our first hypothesis were: (1) individuals within samples, (2) samples within each transect, (3) between the nine transects, and (4) between the three localities (Juquehy, Dura and Itamambuca). A hierarchical analysis of the short temporal effect on the samples was also done to test the second hypothesis. In this case, the samples of each transect were grouped and also the transects were grouped according to the time of collection [(J_1, D_1 and I_1) x (J_2, D_2 and I_2) x (J_3, D_3 and I_3)]. A three-level hierarchical analysis was applied to assess temporal subdivision for each locality: (1) within samples, (2) samples within transects and (3) between transects from different times of collection. The confidence intervals (CI) of the F_{ST} of the different hierachical levels were compared to verify if there were any overlapping among them, which could indicate no differentiation among F_{ST} values. Components of variance in hierarchical analyses were calculated as presented in Weir (1996).

To check for a relationship between the rock-shore distance and genetic differentiation, geographic distances and pairwise F_{ST} values within transects were obtained. F_{ST} values were transformed into $F_{ST}/(1- F_{ST})$ and plotted against geographic distances in one dimension (Rousset, 1997). The Mantel test was used to examine whether a correlation existed between the pairwise values and the corresponding pairwise geographical distances. The genetic differentiation and geographic distance matrices were permutated 10,000 times in TFPGA program (Miller, 1997). Pairwise values were also obtained for each locus and the same procedure was applied.

To determine whether there was any pattern of differentiation among localities, samples from each transect were pooled and pairwise F_{ST} and Nei's genetic distance (1978) were calculated between transects. Both matrices were used separately for non-metric multidimensional scaling (NMDS). The goodness-of-fit between the original matrix and the distances plotted on the graph was evaluated by the stress measure, for which values close to zero indicate a good fit of the points within the data set (Manly, 1994).

RESULTS

Habitat variation

Principal component analysis produced readily interpretable descriptive axes (Table 2). Scores on multivariate habitat axes were available for 58 samples (there were no data for mangrove, J_20 and J_24 samples). The first axis accounted for about 45% of the variation in the original variables and gave high scores for the presence of *Bostrychia montagnei*, *B. darwinianus*, *C. bisinuatus*, *C. subrugosa* and *E. lineolata*, species found mainly in areas highly exposed to the sea. All of the variables apparently had the same effect on this axis, which represented variation in the distance along the transect. The second axis, which

represented 12% of the original variation, distinguishes sites with *L. angulifera*, *M. coffeus* and *Bostrychia radicans* (species currently found near mangroves) from sites where *E. lineolata* specimens were found. The scores of the first two axes for all sites are plotted in Figure 2, which shows the distribution of the major variation in the habitats. There was no difference among samples from different transects and collection times, although samples from the same point on each transect had a grouped distribution. In Figure 2, the symbols refer to pooled samples collected on 0 and 4 sites (group 1), 8 and 16 sites (group 2), 32 and 64 sites (group 3) and 128 and 232 (group 4). Each group contains sites from all locations and collects.

To evaluate the variation among these groups, a Kruskal Wallis test was done using the values of their respective scores from the first two axes of the PCA. The non-parametric tests were significant for each axis, $H_{axis\ 1}$= 36.8 ($p<0.0001$), and $H_{axis\ 2}$= 20.1 ($p<0.0002$). Dunn's test was used to determine which groups were significantly different. In the first axis, groups 1 and 3, 1 and 4, 2 and 3, and 2 and 4 were significantly different ($p<0.05$); whereas axis 2 showed significant variation ($p<0.05$) among the group 1 and all the others. Corresponding sites of the transects of different localities and periods were environmentally analogous or similar in species composition, but different from adjacent sites. This finding indicated that there was horizontal structuring in the species composition along rocky shores.

Within samples variability

The average variability was high (average values for all samples and loci: $P_{95\%\ criterion}$ = 69%; H_e = 0.260; H_o = 0.172; Table 3). The F_{IS} for all samples was high and significant (over all loci: F_{IS} = 0.365, CI 95% = 0.284, 0.466). There was significant

deviation from Hardy-Weinberg equilibrium in most of the samples, always towards heterozygote deficiency. The Monte Carlo method showed that 93 out of 727 tests (~13%) were significant. Loci and their F_{IS} values are shown in Table 4.

Forty-five out of 63 samples had heterogeneous F_{IS} values across loci. Only nine out of 18 samples had significant excess of homozygotes and not significant deviation of homogeneity (Table 3). Homogeneity test for each locus across all of the 63 samples showed that most loci were heterogeneous, except for Lap, Me-1 and Gpi (F_{IS} = 0.23, 0.12 and 0.25; χ^2 = 41.4, 64.3 and 33.1; d.f. = 62, 57 and 60, respectively). In the analysis of each locus across samples within each transect, 46 out of 102 tests yielded no heterogeneous F_{IS} values (Table 4).

Of 726 Ewens-Watterson tests, 131 presented significant deviation from neutrality expectation ($p<0.00001$). All of these loci had F values greater than expected (all p-values >0.975). The association between p-values for each locus within transects showed that 79% of the combined values are above 0.975, supporting the tendency to high values of p. There was no pattern in the distribution of the deviation within loci (χ^2= 5.6, $p>0.05$) and within samples (χ^2= 3.2, $p>0.1$) among the three collections. Significant results represented an excess of homozygosity and the presence of a single, high frequency allele, together with very few alleles of intermediate frequencies and many rare alleles.

One out of 3,398 disequilibrium tests showed a significant deviation from random distribution of genotypes in the samples. The loci associations that showed disequilibrium were Ak-2 and Pep-4 in sample $D_1$128. When the samples were pooled within each transect, the number of significant associations rose to 12 out of 835 involving almost all loci.

Analysis of genetic differentiation

Plots of the most common allele frequencies at each locus showed considerable variation among samples within a transect and along time, but no distinct microgeographic trend (some of them are represented in Figure 3). Most loci had heterogeneous allelic frequencies along all transects (except for I_2, where only four loci had heterogeneous allelic frequencies): 14 loci showed significant heterogeneous allelic frequencies in D_3; 13 loci in J_1 and D_1; 12 loci in I_1; 11 loci in D_2 and J_3; 9 loci in I_3; and 8 loci in J_2.

The four-level hierarchical analysis showed no significant genetic structuring between localities, as well as between transects (Table 5). The interpopulational variability among samples was significant (F_{ST} =0.025) and the 95% CI values did not overlap with the other hierachical levels. Ninety-three percent of total variation can be ascribed to within samples variation (F_{IS} = 0.365). The next important contribution to total variability comes from within transect level of subdivision (5.6%), suggesting that there is a considerable more genetic variation between samples of a transect than between transects or localities (table 5).

There was very low (F_{ST} =0.005), but significant structuring when transects were grouped by time of collection. The values of F_{ST} obtained for transects and samples in temporal analysis were similar to those found in the previous analysis and, again, the 95% CI around F_{ST} value calculated within samples did not overlap with the interval from the remaining hierarchical levels. Again, most of variation is explained by the variation within samples (F_{IS} = 0.365), followed by variation explained by samples within transects (Table 5).

The three-level hierarchical analysis yielded the same results within localities, with no differentiation among the three collections for each locality but with significant structuring within each transect (except for I_2). The percentage of variation accounted for samples within transects is higher than beween transects in all localities (Table 5). The second and third collections at Juquehy and Dura had higher structuring values than samples from the first collection. Eight out of nine transects showed moderate but significant structuring, supporting great differentiation among samples located few meters apart. The comparison among the confidence intervals showed no difference in the level of differentiation between Juquehy transects and the Dura and Itamambuca estuaries (Table 5).

We removed the mangrove samples from Dura transects to assess the effect of the latter on structuring. The results were very similar to those with this sample: D_1, $\theta = 0.010$ (95%CI= 0.001, 0.018); D_2, $\theta = 0.051$ (95%CI= 0.010, 0.080); D_3, $\theta = 0.048$ (95%CI= 0.016, 0.109).

The Mantel test showed that only transect D_1 (without the Dm sample) had a highly significant relationship between distance and genetic differentiation (r=0.730, p=0.005; Figure 4). When the Mantel test was applied to each locus, just a few of them showed an isolation-by-distance pattern. In the first collection, Pep-1 had a significant relationship between distance and genetic differentiation (r= 0.869, p=0.001; F_{ST}=0.022) in transect D_1, whereas Lap and Me-1 in D_1 and J_1, respectively, had a marginally significant value (r=0.31 and 0.71, respectively, p< 0.07; F_{ST}=0.009 and 0.041). In the second collection, Pgm-2 showed a strong, significant relationship between distance and genetic differentiation in the J_2 transect (r=0.830, p=0.006; F_{ST}=0.050). In D_2, Ak-2 had a

significant relationship (r= 0.498, p=0.003; F_{ST}=0.034), and Pep-1, Pep-2 and Pgm-1 were marginally significant (r= 0.707, 0.682 and 0.332 respectively, p< 0.09; F_{ST}=0.320, 0.220 and 0.095). In the third collection, Pep-1 in J_3 was significant (r=0.817, p=0.007; F_{ST}=0.081) and Pgm-1 was marginally significant (r= 0.380, p=0.07; F_{ST}= 0.053); in the D_3 and I_3 transects, Pep-3 and Me-2 showed marginally significant values (respectively, r=0.351 and 0.811, p<0.09; F_{ST}=0.045 and 0.047). The F_{ST} values for each locus in each transect are given in Table 6.

In the multi-dimensional analysis, a two-dimension solution for both estimates was preferred because of the low values of stress (0.07 and 0.044 for F_{ST} and Nei's distance, respectively). For the F_{ST} data, Figure 5A shows that dimension 1 reflected differences among the collections time, especially between the second sampling (May 2002) and the other two done in November 2001 and October 2002. Dimension 2 divided the second collection into two clusters (Dura and Itamambuca separated from Juquehy). The results obtained using Nei's genetic distances were very different: dimension 1 clearly grouped each sampling site in one cluster, regardless of the time of collection (Figure 5.B), and dimension 2 separated I_1 from I_2 and I_3.

DISCUSSION

Microgeographic subdivision

Spatial and temporal effects

Even where habitats are patchy, planktonic dispersal connects populations and reduces the potential for genetic divergence (Palumbi, 1994). Disjunct populations may be more likely in marine species with direct development, because the habitat gaps are less expected to be crossed and because the rare events of colonization across a large gap have

the potential for founding a locally recruiting population (Johannesson, 1988). Studies of disjunct populations in a range of species are therefore of special interest in the search for conditions that favor genetic divergence in benthic marine organisms. Nevertheless, even species with high dispersal abilities are subjected to several factors that may limit gene flow, creating opportunities for genetic differentiation. These include abiotic factors, such as temperature, salinity gradient and wave action, isolation by distance and behavioral limits to dispersal and selection (Palumbi, 1994).

The genetic structuring in planktonic species depends not only on their dispersal ability but also on habitat characteristics and complexity. In populations of *Littoraria cingulata* with continuous distribution along Australian coast, Johnson & Black (1998) found evidence of genetic subdivision in a few kilometers, even with no apparent barrier to gene flow. Indeed, some habitats may promote local subdivision over several hundred meters. *Austrocochlea constricta*, a short-term lecitotrophic gastropod, showed higher levels of differentiation in Houtman Abrolhos than in other locations, probably because of some strong barrier to gene flow; this phenomenon resulted in endogenous recruitment in some parts of this archipelago (Parsons, 1996). Higher structuring in estuarine conditions compared to coastal populations is also common (Ayvazian *et al.*, 1994). One of our hypotheses was that estuarine habitats promoted greater genetic differentiation than coastal habitats. However, at least for the *L. flava* samples here, estuarine conditions did not affect the level of structuring, since the Juquehy, Itamambuca and Dura samples had a similar degree of structuring. Thus, there was no clear effect of the estuary, whether increasing the structuring levels or changing the allelic frequencies.

The F_{ST} value (0.054) for 4,000 km of Brazilian coastline and rare alleles shared by very distant samples (> 1,000 km apart), suggested a high gene flow among samples

(Andrade *et al.*, 2003). In this study, our main concern was to assess the subdivision of apparently continuous *L. flava* populations in horizontal transects. Despite its planktotrophic larval stage, *L. flava* showed genetic structuring on a scale from 4 m to few hundred meters. As shown in Table 5, some F_{ST} values were low, but significant (e.g., samples in transects D_1 and J_3). Considering the spatial scale, this pattern is remarkable, particularly since adults are vagile.

Both spatial F_{ST} hierarchical analyses showed higher and more significant structuring within transects than among other hierarchical levels (sampling times, localities and transects). As with the low structuring seen in some transects, the estimated degree of subdivision between localities and transects were also lower and not significant. Only samples along transect I_2 showed no significant genetic structure, perhaps because of the small number of samples (few individuals were found). There was no overlap among the confidence intervals for the F_{ST} value of all samples, transects and localities. Similarly, samples within transects showed differentiation, with the F_{ST} higher than the third and fourth -level of hierarchical analyses (Table 5). In *L. flava*, the microgeographic scale seems to be more important than the large scale concerning the distribution of genetic variability. Additional evidence of local spatial subdivision was supplied by the linkage disequilibrium test. The proportion of associated loci increased when samples within transect were pooled, suggesting that each sample behaved as a subpopulation with distinct combinations of allelic frequencies.

In littorinids, patterns and factors influencing distribution and movement on substrate are widely studied (e.g., Chapman, 1994; Saier, 2000; Pardo & Johnson, 2004). Isozonal movements, with no observed consistent directional movement, seem to describe the behavior frequently shown by littorinids (e.g., Gochfeld & Minton, 2001; Pardo &

Johnson, 2004). Several biotic and abiotic factors seem to determine the amount of movement, including topographic complexity, age, gender and reproductive phase (e.g. Saier, 2000; Pardo & Johnson 2004). Three models have been proposed to explain non-random spatial patterns of dispersion of adult marine intertidal organisms at the local scale: settlement of larvae, post-settlement mortality and behavior of adults (Underwood & Denley, 1984). Processes might vary at different spatial scales, with behavior likely to be most important for juveniles and adult snails at the smallest scale (Chapman, 1994). Moutinho & Alves-Costa (2000) showed that smaller individuals of *L. flava* are commonly found in aggregations to avoid water loss, while the largest ones are found isolated. Further studies about movement patterns in adults of *Littoraria flava* might help to elucidate if there is an association among dispersal at this level and the observed genetic structuring.

In natural populations, temporal and spatial effects are difficult to discriminate, since temporal variations may enhance the effects of spatial variations in the distribution of genetic variability (Bryant, 1976; De Wolf, Backeljau & Verhagen, 1998). The second question addressed in this study was the effect of time on the *L. flava* samples. The time of collection had a slightly more intense effect than large-scale spatial differences: when all localities were grouped, there was a low, but significant, differentiation (Table 5). Any temporal effect, such as caused by heterogeneous genotypic frequencies at different spawning times, might have been blurred by the mixing of cohorts within the sampled sites.

Mapping of genetic differentiation

In the graphical non-metric multidimensional analysis, pairwise F_{ST} and genetic distance (Nei, 1978) produced different results (Figure 5). The plot resulting from pairwise F_{ST} analysis was as expected, with more temporal than spatial differentiation among transects. By the other hand, the graphic representation of Nei's distances showed a clear

differentiation between Itamambuca and Dura/Juquehy transects, even considering that the genetic differentiation among the transects was very low (average Nei's D=0.005). This pattern can be explained by a slight differentiation in the allelic frequencies among the locations. These estimates showed different tendencies, which should be considered when choosing the analysis parameters.

Environmental data

Taking into account that species diversity may reflect the environmental heterogeneity on rocky-shores, we concluded that there was more environmental heterogeneity within transects (especially between the most distant points, Figure 2) than among localities, a few hundred kilometers apart. Species assemblages appear to be repeated at the corresponding sites in transects of different beaches. Thus, the genetic structuring observed within transects might be related to the heterogeneous species composition along the rocky shore. Further refined studies are necessary to investigate whether the local-scale genetic structure reflects the local species heterogeneity and at which scale this association could be found. Such a relationship would confirm that environmental heterogeneity has an important influence on the distribution of genetic variability.

Distribution of variability within transects

Isolation-by-distance

When the distance of individual migration is much smaller than the distribution range of a species, random local differentiation in gene frequencies is expected, as shown by the isolation-by-distance model (Wright, 1943). The multiloci Mantel test in transect D_1 was coherent with this model, and could partially explain the distribution of variability in

this transect. Mantel test showed a positive relationship between spatial distance and genetic differentiation for some loci in different collections and localities. Most of the loci that had significant (or marginally significant) correlation had a moderate to high F_{ST} at the local-scale and many also showed heterogeneous allelic frequencies within the transects. Several loci showed spatial variation in allelic frequencies along the transect, with no detectable cline or pattern (Figure 3). A temporal effect was also seen in the allelic frequencies. Notably, some loci showed moderate to high values of differentiation along transects, but these values varied among time of collections (Table 6). These results showed that there is no simple or unique process that could be explain the distribution of variability in *L. flava* samples. Thus, a singular and common demographic event might not produce such variation in allelic frequencies in this scale.

Demographic events and diversifying selection

The distribution and amount of variability within *L. flava* samples changes over time and space. At local scale, the deviation from Hardy-Weinberg equilibrium were similar to that found at macrogeographic scale (Andrade *et al.*, 2005). Most of the samples (about 76%) had heterogeneous values of the fixation index F_{IS}, thus the deviation could hardly be explained by inbreeding. While natural selection operates differently in each locus, inbreeding affects all genes simultaneously in a similar degree, as does sampling variation and migration (Lewontin & Krakauer, 1973; Gaffney *et al.*, 1990). Heterogeneous values of the F_{IS} index could also be strongly influenced by small population sizes. This, associated to outcrossing rates smaller than a unity, might result in variable $F_{IS}>0$ values in different loci within a sample (Coelho & Vencovsky, 2003). Thus, if our samples are in fact representing small and not totally panmitic populations, it is possible that the observed

heterogeneity within samples is an outcome of the finite conditions of natural *L. flava* populations.

The homogeneity test for each locus showed that Lap, Me-2, Gpi and Pgm-2 had meaningful and non heterogeneous F_{IS} within most transects. These results agree with those reported by Andrade *et al.* (2005), especially for locus Pgm-2, with a homogenous and high deviation from Hardy-Weinberg equilibrium among samples on a larger scale (4,000 km). When all of the 63 samples were analyzed together, only three loci (Lap, Me-1 and Gpi) showed homogeneity. The interesting point is that samples of each transect appear to have a particular dynamic for genotypic frequencies, that differed among localities and with time. This would imply that care should be taken when extrapolating observed patterns to different localities.

The Ewens-Watterson neutrality test indicated a number of rare alleles greater than would be expected according to the neutral theory. Nearly neutral theory (Ohta, 1992) could explain this, based on purifying selection against slightly deleterious mutations, resulting in an excess of rare alleles by reducing the frequencies of alleles that would otherwise occur at intermediate frequencies. However, it is difficult to distinguish this explanation from neutral alleles in populations that are not at a stationary distribution for purely historical reasons. Recurrent colonization could maintain rare alleles in very large populations, when followed by a subsequent and short bottleneck (Zbawicka, Wenne & Skibinski, 2003).

Differences in the genetic composition of recruits, or differential local selection through benthic existence, could explain the results of the neutrality test and also lead to populational subdivision. Some authors cited selective mortality during larval stage and in early settlement as the major determinants of allele frequencies in mollusks (Johnson &

Black, 1984; Zouros & Foltz, 1984; Fairbrother & Beaumont, 1993). Johnson & Black (1984) observed that temporal and spatial variations in genotypes at recruitment could also be responsible for microgeographic variation observed in limpets. Populational genetics studies of larvae soon after settlement showed high levels of temporal variation in allele frequencies, what may produce the spatial variation seen in adult populations of several marine taxa (Johnson & Black, 1984; Watts *et al.*, 1990; David *et al.*, 1997; Pedersen, Hunt & Scheibling, 2000). On the other hand, Gilg & Hilbish (2000) found no temporal genetic variation among newly settled mussel cohorts, but noticed a sharp change in allele frequencies within a few weeks after settlement, suggesting selection. Spatio-temporal variation in the genetic composition of larvae, settlement and post-settlement selection are, therefore, vital to the metapopulation dynamics of marine systems (Connell, 1985; Eckman, 1996). Asynchronous spawning events and variance in reproductive success (Li & Hedgecock, 1998) could produce aggregates of differing genetic composition that would show as much genetic heterogeneity within a single patch as between different patches. Also, population subdivision can affect the variance in reproductive success (Barton & Whitlock, 1997). High variance in reproductive success results in a low effective population size, because relatively few individuals will contribute with most gametes for the next generation and there is a higher chance that a random pair of alleles will be identical by descent. In contrast, repeated extinction/recolonization events are assumed to be the main processes involved in producing similar amounts of spatial and temporal genetic differentiation in some species, as seen in the hydrothermal vent populations of *Alvinella pompejana* (Jollivet *et al.*, 1998).

Occasional extinction and recolonization can enhance the rate of differentiation between demes (Wright, 1977). Ibrahim *et al.* (1996) showed that discretely subdivided

populations could occupy a continuous habitat as a result of the stochasticity of dispersal and colony establishment when there are high dispersal rates. The important point is not the possible significance of the mode of dispersal or its absolute distance, but that dispersal leads to colonization events, i.e. to the establishment of several discrete groups. Thus, asynchronous spawning associated to recurrent colonizations in *L. flava* can explain the local differentiation without a recognizable pattern. These processes could counteract the effects of genetic drift by maintaining high genetic diversity within the populations.

The mechanism by which this differentiation and the deficiency of heterozygotes are maintained on such a small scale is unclear. The chaotic and heterogeneous patterns of allelic frequencies, as well as the variable F_{ST} values in loci in space and over time are also notable and intriguing. Since similar levels of geographical variation are expected for neutral polymorphisms under drift and migration, our findings suggest that certain loci may be subject to some form of natural selection. Soft and diversifying selection could maintain the microgeographical populational structure and heterozygote deficiency in subpopulations, as well as the genetic variability in the species as a whole (Levene, 1953; Santos, 1994). Also, the model of long term selection coefficient seems plausible (Ohta, 1992). In this model, selective coefficients are stable for a certain period of time or within a certain local area, but may vary in another period or area through a change of environmental conditions, which include both the internal genetic background and external ecological factors. Analyses of microgeographical population structure have suggested that spatial heterogeneity may be one of the main factors involved in diversifying selection (Johannesson & Mikhailova, 2004). Our results also suggest that we might have identified what could be a *L. flava* populational unit and that the apparently continuous distribution along the rocky shores consists of several sub-populations. These sub-populations might be

subject to a balance among recurrent colonization and selection acting on different loci in different times and localities.

REFERENCES

Alfenas AC. 1991. *Eletroforese de Isozimas e Proteínas Afins.* Editora Universidade Federal de Viçosa, MG.

Andrade SCS, Magalhães CA, Solferini VN. 2003. Patterns of genetic variability in Brazilian littorinids (Mollusca): a macrogeographic approach. *Journal of Zoological Systematics and Evolutionary Research* **41:** 249-255.

Andrade SCS, Medeiros HF, Solferini VN. 2005. Homogeneity test of Hardy-Weinberg deviations in Brazilian littorinids: evidence for selection? *Journal of Molluscan Studies* **71**: 167-174.

Ayvazian SG, Johnson MS, McGlashan DJ. 1994. High levels of genetic subdivision of marine and estuarine populations of the estuarine catfish *Cnidoglanis macrocephalus* (Plotosidae) in southwestern Australia. *Marine Biology* **118**: 25-31.

Barton NH, Whitlock MC. 1997. The evolution of metapopulations. In Hanski IA, Gilpin ME, eds. *Metapopulation Biology. Ecology, Genetics, and Evolution.* Academic Press, London, UK.

Belkhir K. 2001. GENETIX, logiciel sous WindowsTM pour la génétique des populations Laboratoire Génome et Populations, CNRS UPR 9060, Université de Montpellier II, Montpellier (France).

Bryant EH. 1976. A comment on the role of environmental variation in maintaining polymorphisms in natural populations. *Evolution* **30**: 188-190.

Chapman MG. 1994. Small- and broad-scale patterns of distribution of the upper shore littorinids *Nodilittorina pyramidalis* in New South Wales. *Australian Journal of Ecology* **19**: 83-95.

Clayton J, Tetriak D. 1972. Amine-citrate buffers for pH control in starch gel electrophoresis. *Journal of Fisheries Research Board of Canada* **29**: 1169-1172.

Coelho ASG, Vencovsky R. 2003. Intrapopulation fixation index dynamics in finite populations with variable outcrossing rates. *Scientia Agricola* **60**: 305-313.

Connell JH. 1985. The consequences of variation in initial settlement vs. post-settlement mortality in rocky intertidal communities. *Journal of Experimental Marine Biology and Ecology* **93**:11–45.

Crow JF, Kimura M. 1970. *An Introduction to Population Genetics Theory*. Harper & Row, New York.

David P, Perdieu MA, Pernot AF, Jarne P. 1997. Fine-grained spatial and temporal genetic structure in the marine bivalve *Spisula ovalis*. *Evolution* **51**: 1318–1322

De Wolf H, Backeljau T, Verhagen R. 1998. Spatio-temporal genetic structure and gene flow between two distinct shell morphs of the planktonic periwinkle *Littorina striata* (Mollusca: Prosobranchia). *Marine Ecology Progress Series* **163**: 155-163.

De Wolf H, Verhagen R, Backeljau T. 2000. Large scale population structure and gene flow in the planktonic developing periwinkle, *Littorina striata*, in Macronesia (Mollusca: Gastropoda). *Journal of Experimental Marine Biology and Ecology* **246**: 69–83.

Eckman J. 1996. Closing the larval loop: linking larval ecology to the population dynamics of marine benthic invertebrates. *Journal of Experimental Marine Biology and Ecology* **200**: 207–237.

Ewens W. 1972. The sampling theory of selectively neutral alleles. *Theoretical Population Biology* **3**: 87-112.

Fairbrother JE, Beaumont AR. 1993. Heterozygote deficiencies in a cohort of newly settled *Mytilus edulis* spat. *Journal of Marine Biology Association of United Kingdom* **73**: 647-653.

Gaffney PM, Scott TM, Koehn RK, Diehl WJ. 1990. Interrelationships of heterozygosity, growth rate and heterozygote deficiency in the coot clam, *Mulinia lateralis*. *Genetics* **124**: 687-699.

Gilg MR, Hilbish TJ. 2000. The relationship between allele frequency and tidal height in a mussel hybrid zone: a test of the differential settlement hypothesis. *Marine Biology* **137**: 371-378.

Gochfeld DJ, Minton DT. 2001. When to move and where to go: movement behavior of the tropical littorinid *Cenchritis muricatus* (Linnaeus, 1758). *Journal of Shellfish Research* **20**: 447-452.

Guo SW, Thompson EA. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* **48**: 361-372.

Ibrahim KM, Nichols RA, Hewitt GM. 1996. Spatial patterns of genetic variation generated by different forms of dispersal during range expansion. *Heredity* **77**: 282-291.

Janson K. 1987. Allozyme and shell variation in two marine snails (*Littorina*, Prosobranchia) with different dispersal abilities. *Biological Journal of Linnean Society* **30**: 245-256.

Johannesson K. 1988. The paradox of rockall - why is a brooding gastropod (*Littorina saxatilis*) more widespread than one having a planktonic larval dispersal stage (*Littorina littorea*). *Marine Biology* **99**: 507-513.

Johannesson K, Johannesson B, Rolán-Alvarez E. 1993. Morphological differentiation and genetic cohesiveness over a microenvironmental gradient in the marine snail *Littorina saxatilis*. *Evolution* **47**: 1770–1787.

Johannesson K, Johannesson B, Lundgren U. 1995. Strong natural selection causes microscale allozyme variation in a marine snail. *Procedings of National Academy of Sciences of U.S.A.* **92**: 2602–2606.

Johannesson K, Tatarenkov, A. 1997. Allozyme variation in a snail (*Littorina saxatilis*)-deconfounding the effects of microhabitat and gene flow. *Evolution* **51**: 402-409.

Johannesson K, Lundberg J, André C, Nilsson PG. 2004. Island isolation and habitat heterogeneity correlate with DNA variation in a marine snail (*Littorina saxatilis*). *Biological Journal of the Linnean Society* **82**: 377–384.

Johannesson K, Mikhailova N. 2004. Habitat-related genetic substructuring in a marine snail (*Littorina fabalis*) involving a tight link between allozyme and a DNA locus. *Biological Journal of the Linnean Society* **81**: 301–306.

Johnson MS, Black R. 1984. Pattern beneath the chaos: the effect of recruitment on genetic patchiness in an intertidal limpet. *Evolution* **38**: 1371–1383.

Johnson MS, Black R. 1991. Genetic subdivision of the intertidal snail *Bembicium vittatum* (Gastropoda, Littorinidae) varies with habitat in the Houtman Abrolhos Islands, Western-Australia. *Heredity* **67**: 205-213

Johnson MS, Black R. 1998. Increased genetic divergence and reduced genetic variation in populations of the snail *Bembicium vittatum* in isolated tidal ponds. *Heredity* **80**: 163-172.

Johnson MS, Black R. 2000. Associations with habitat versus geographic cohesiveness: size and shape of *Bembicium vittatum* Philippi (Gastropoda: Littorinidae) in the Houtman Abrolhos Islands. *Biological Journal of the Linnean Society* **71**: 563-580.

Johnson MS, Bentley SL, Ford SS, Ladyman MT, Lambert GJ. 2001. Effects of a complex archipelago on genetic subdivision of the intertidal limpet *Siphonaria kurracheensis*. *Marine Biology* **139**: 1087-1094.

Jollivet D, Dixon LRJ, Desbruyeres D, Dixon DR. 1998. Ribosomal (rDNA) variation in a deep sea hydrothermal vent polychaete, *Alvinella pompejana*, from 13° N on the East Pacific Rise. *Journal of the Marine Biological Association of the United Kingdom* **78**: 113-130.

Lancaster A, Nelson MP, Single RM, Meyer D, Thomson G. 2003. "PyPop: a software framework for population genomics: analyzing large-scale multi-locus genotype data". In Altman RB ed. *Pacific Symposium on Biocomputing 8,* 514-525. (World Scientific, Singapore, 2003).

Levene H. 1953. Genetic equilibrium when more than one ecological niche is available. *The American Naturalist* **87**: 331-333.

Lewis PO, Zaykin D. 2001. Genetic Data Analysis: Computer program for the analysis of allelic data. Version 1.0 (d16c). Available from http://lewis.eeb.uconn.edu/lewishome/software.html.

Lewontin RC, Krakauer J. 1973. Distribution of gene frequency as a test of the theory of the selective neutrality of polymorphisms. *Genetics* **74**: 175-195.

Li G, Hedgecock D. 1998. Genetic heterogeneity, detected by PCR-SSCP, among samples of larval Pacific oysters (*Crassostrea gigas*) supports the hypothesis of large variance in

reproductive success. *Canadian Journal of Fisheries and Aquatic Sciences* **55**: 1025-1033.

Manly BFJ. 1994. *Multivariate statistical methods, a primer*. London, Chapman and Hall.

Miller MP. 1997. Tools for population genetic analyses (TFPGA) 1.3: a Windows program for the analysis of allozyme and molecular population genetic data. Available from: http://www.marksgeneticsoftware.net.

Moutinho PRS, Alves-Costa CP. 2000. Shell-size variation and aggregation behavior of *Littoraria flava* (Gastropoda: Littorinidae) on a southeastern Brazilian shore. *Veliger* **43**: 277-281.

Nei M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583-590.

Ohta T. 1992. The nearly neutral theory of molecular evolution. *Annual Review of Ecology and Systematics* **23**: 263-286.

Palumbi SR. 1994. Genetic divergence, reproductive isolation, and marine speciation. *Annual Review of Ecology and Systematics* **25**: 547–572.

Panova M, Johannesson K. 2004. Microscale variation in Aat (aspartate aminotransferase) is supported by activity differences between upper and lower shore allozymes of *Littorina saxatilis*. *Marine Biology* **144**: 1154-1167.

Pardo LM, Johnson LE. 2004. Activity and shelter use of an intertidal snail: effects of sex, reproductive condition and tidal cycle. *Journal of Experimental Marine Biology and Ecology* **301**: 175-191.

Parsons KE. 1996. The genetic effects of larval dispersal depend on spatial scale and habitat characteristics. *Marine Biology* **126**: 403-414

Pedersen EM, Hunt HL, Scheibling RE. 2000. Temporal genetic heterogeneity within a developing mussel (*Mytilus trossulus* and *M. edulis*) assemblage. *Journal of Marine Biological Association of United Kingdom* **80**: 843–854.

Raymond M, Rousset F. 1995a. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**: 248-249.

Raymond M, Rousset F. 1995b. An exact test for population differentiation. *Evolution* **49**: 1280-1283.

Reid DG. 1986. *The Littorinid Molluscs of Mangrove Forests in the Indo-Pacific Region. The Genus Littoraria*. British Museum (Natural History).

Reid DG. 1999. The genus *Littoraria* Griffith and Pidgeon, 1834 (Gastropoda: Littorinidae) in tropical eastern Pacific. *Veliger* **42**: 21-53.

Rice WR. 1989. Analyzing tables of statistical tests. *Evolution* **43**: 223-225.

Rousset F. 1997. Genetic differentiation and estimation of gene flow from *F*-statistics under isolation-by-distance. *Genetics* **145**: 1219-1228.

Saier B. 2000. Age-dependent zonation of the periwinkle *Littorina littorea* (L.) in the Wadden Sea. *Helgoland Marine Research* **54**: 224-229.

Santos M. 1994. Heterozygote deficiencies under Levene's population subdivision structure. *Evolution* **48**: 912–920.

Shaw CR, Prasad R. 1970. Starch gel electrophoresis of enzymes - a compilation of recipes. *Biochemical Genetics* **4**: 297-320.

Shepherd GJ. 1995. *FITOPAC 1. Manual de usuário*. Departamento de Botânica, UNICAMP.

Slatkin M. 1994. An exact test for neutrality based on the Ewens sampling distribution. *Genetical Research* **64**: 71-74.

Sokal RR, Rohlf FJ. 1995. *Biometry: The Principles and Practice of Statistics in Biological Research.* 3rd ed. WH Freemam Company, NY.

Sotka EE, Wares JP, Barth JA, Grosberg RK, Palumbi SR. 2004. Strong genetic clines and geographical variation in gene flow in the rocky intertidal barnacle *Balanus glandula*. *Molecular Ecology* **13**: 2143–2156.

Tatarenkov AN. 1995. Genetic heterogeneity in populations of *Littorina brevicula* (Philippi) (Mollusca: Gastropoda) in the northern part of Peter the Great Bay (Sea of Japan). *Veliger* **38**: 85–91.

Tatarenkov A, Johannesson K. 1999. Micro- and macrogeogeographic allozyme variation in *Littorina fabalis*; do sheltered and exposed forms hybridize? *Biological Journal of the Linnean Society* **67**: 199-212.

Underwood AJ, Denley EJ. 1984. Paradigms, explanations and generalisations in models for the structure of intertidal communities on rocky shores. In: Strong DR, Simberloff D, Abele LG, Thistle AB, eds. *Ecological Communities: Conceptual Issues and the Evidence*. Princeton University Press, Princeton, NJ. pp. 151–80.

Ward RD. 1990. Biochemical genetic variation in the genus *Littorina* (Prosobranchia: Mollusca). *Hydrobiologia* **193**: 53-69.

Watts RJ, Johnson MS, Black R. 1990. Effects of recruitment on genetic patchiness in the urchin *Echinometra mathaei* in western Australia. *Marine Biology* **105:**145–151.

Watterson G. 1978. The homozygosity test of neutrality. *Genetics* **88:** 405-417.

Weir BS. 1996. *Genetic Data Analysis II: Methods for Discrete Population Genetic Data.* Sinauer Assoc., Inc., Sunderland, MA, USA.

Weir BS, Cockerham CC. 1984. Estimating *F*-statistics for the analysis of population structure. *Evolution* **38:** 1358-1370.

Wright S. 1943. Isolation by distance. *Genetics* **28:** 114-138.

Wright S. 1977. *Evolution and the Genetics of Populations, vol. 3, Experimental Results and Evolutionary Deductions.* University of Chicago Press, Chicago, IL.

Zbawicka M, Wenne R, Skibinski DOF. 2003. Mitochondrial DNA variation in populations of the mussel *Mytilus trossulus* from the Southern Baltic. *Hydrobiologia* **499:** 1–12.

Zouros E, Foltz DW. 1984. Possible explanations of heterozygote deficiency in bivalve molluscs. *Malacologia* **25:** 583-591.

Table 1. Localities, collection dates and transects used in sampling *L. flava*. The mean numbers of individuals analyzed per sample are also indicated. (*) Samples located at 59 m in the transect.

Locality	Collects	Transects	Samples	Mean (s.d.)
Juquehy	1 November, 2001	J_1	J_10, J_14, J_18, J_116, J_132, J_164*, J_1128	32.8 (± 3.9)
(23°46'S/45°43'E)	2 May, 2002	J_2	J_20, J_24, J_28, J_216, J_232, J_264*, J_2128	21.8 (± 5.1)
	3 October, 2002	J_3	J_30, J_34, J_38, J_316, J_332, J_364*, J_3128	27.1 (± 5.8)
Dura	1 November, 2001	D_1	D_10, D_14, D_18, D_116, D_132, D_164, D_1128, D_1232, D_1m	36.3 (± 7.2)
(23°30'S/45°10'E)	2 May, 2002	D_2	D_20, D_24, D_28, D_216, D_232, D_264, D_2128, D_2232, D_2m	24.1 (± 6.6)
	3 October, 2002	D_3	D_30, D_34, D_38, D_316, D_332, D_364, D_3128, D_3232, D_3m	33.4 (± 8.6)
Itamambuca	1 November, 2001	I_1	I_10, I_14, I_18, I_116, I_132, I_164	32 (± 7.4)
(23°25'S/45°02'E)	2 May, 2002	I_2	I_28, I_216, I_232, I_264	15.5 (± 6.4)
	3 October, 2002	I_3	I_30, I_38, I_316, I_332, I_364	35.8 (± 12.6)

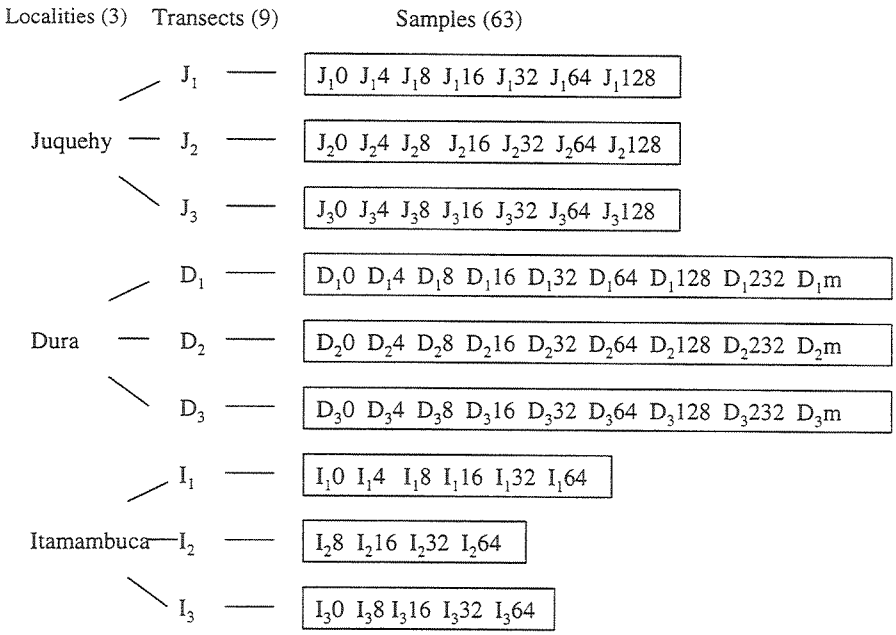

Figure 1. Graphic representation of the hierarchical classification. Abbreviations as in Table 1.

Table 2. Loadings (eigenvectors) of the habitat variables in the first five principal component axes. The portion of the original variation accounted by each axis is shown in parentheses. Loadings ≥|0.2| are in bold.

Variables	Axis 1 (44.9%)	Axis 2 (11.9%)	Axis 3 (7.6%)	Axis 4 (6.3%)	Axis 5 (6.1%)
B. radicans	0.118	**0.210**	0.064	**0.230**	**-0.407**
B. tenella	0.060	-0.040	0.112	**0.219**	0.139
B. calliptera	0.031	-0.005	-0.042	0.152	-0.043
B. montagnei	**0.221**	0.071	**0.469**	-0.189	**0.688**
P. atropurpura	0.028	-0.018	0.108	0.037	0.071
M. periclados	0.026	-0.004	-0.114	0.024	0.056
C. coelothrix	0.004	0.087	0.018	0.042	-0.049
E. compressa	0.044	0.093	-0.137	-0.039	0.149
U. lactuca	0.053	-0.022	-0.006	0.060	0.127
U. fasciata	0	0	0	0	0
R. riparium	0.055	0.029	**0.225**	0.062	0.125
C. rizhophorae	0.080	0.096	**-0.226**	-0.090	0.100
B. darwinianus	**0.228**	-0.003	**-0.449**	**0.269**	**0.284**
B. solisianus	0.176	0.091	**-0.489**	**0.248**	**0.205**
C. subrugosa	**0.281**	-0.121	**0.395**	**0.447**	**-0.241**
E. lineolata	**0.527**	**-0.230**	0.048	**0.326**	0.035
L. angulifera	0.094	**0.752**	0.085	0.081	-0.045
M. coffeus	0.032	**0.530**	0.044	0.059	0.038
C. bisinuatus	**0.684**	0.004	-0.090	**-0.606**	**-0.279**
Isopod	0.002	0.012	0.002	0.044	-0.054

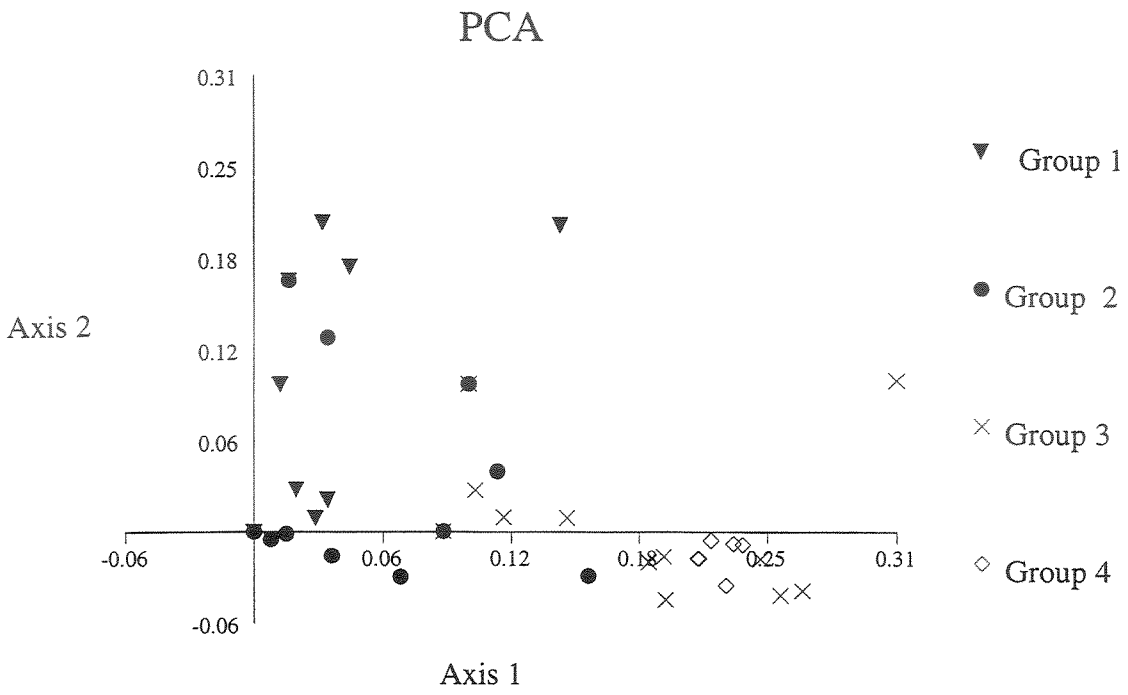

Figure 2. Principal component analysis (PCA) of the habitat variables coded by groups (pooled sites from all transects). Group 1: samples from 0 and 4 sites from all transects; Group 2: samples from 8 and 16 sites from all transects; Group 3: samples from 32 and 64 sites from all transects; Group 4: samples from 128 and 232 sites from all transects.

Table 3. The average genetic variability in samples of *L. flava*. *N*, mean sample size per locus. *P*, % polymorphic loci (95% criterion). H_e, gene diversity (unbiased estimate, Nei 1978). H_o, observed heterozygosity. F_{IS}, fixation index. χ^2 values and respective degrees of freedom (d.f.) from the homogeneity test among loci (in bold, significant values of F_{IS} and χ^2). Sample abbreviations as in Table 1. The mean values for each transect are in italics.

Sample	*N*	*P*	H_e	H_o	F_{IS}	χ^2 (d.f.)
$D_1$0	29.1	73	0.261	0.171	0.342	14.5 (11)
$D_1$4	28.4	73	0.257	0.152	0.412	45.4 (12)
$D_1$8	23.8	71	0.283	0.207	0.270	**38.0** (11)
$D_1$16	22.8	66	0.242	0.177	**0.272**	8.0 (8)
$D_1$32	26.8	73	0.290	0.203	**0.301**	**45.5** (12)
$D_1$64	13.7	66	0.266	0.197	**0.261**	7.0 (9)
$D_1$128	30.2	78	0.304	0.206	**0.324**	15.7 (11)
$D_1$232	30.9	60	0.162	0.113	**0.301**	**53.0** (11)
D_1m	29.0	71	0.264	0.190	**0.281**	**65.6** (12)
D_1	*229*	*80*	*0.260*	*0.177*	*0.317*	
$I_1$0	26.7	78	0.301	0.196	**0.352**	**28.5** (12)
$I_1$4	12.7	85	0.325	0.255	**0.231**	6.8 (11)
$I_1$8	26.0	10	0.354	0.230	**0.355**	**21.9** (12)
$I_1$16	24.9	61	0.253	0.201	**0.206**	9.4 (8)
$I_1$32	27.0	64	0.282	0.172	**0.383**	**200.3** (10)
$I_1$64	28.8	71	0.256	0.178	**0.307**	**21.5** (12)
I_1	*144*	*85*	*0.290*	*0.198*	*0.318*	
$J_1$0	24.5	85	0.271	0.170	**0.377**	**30.5** (12)
$J_1$4	28.0	71	0.273	0.152	**0.445**	**29.8** (12)
$J_1$8	27.5	64	0.200	0.128	**0.363**	**42.2** (10)
$J_1$16	28.5	78	0.264	0.149	**0.426**	**28.8** (11)
$J_1$32	24.3	76	0.233	0.140	**0.401**	**192.2** (12)
$J_1$64	24.2	71	0.256	0.146	**0.430**	**181.1** (11)
$J_1$128	23.5	85	0.271	0.169	**0.380**	**244.1** (12)
J_1	*179*	*78*	*0.260*	*0.154*	*0.406*	
$D_2$0	19.5	46	0.151	0.094	**0.381**	**84.6** (9)
$D_2$4	9.2	50	0.215	0.202	0.063	1.1 (4)
$D_2$8	14.3	66	0.203	0.164	**0.205**	**14.3** (7)
$D_2$16	14.0	50	0.202	0.177	0.125	4.9 (9)
$D_2$32	16.9	57	0.218	0.161	**0.258**	**75.9** (8)
$D_2$64	16.6	69	0.258	0.206	**0.209**	**89.1** (10)
$D_2$128	24.4	53	0.175	0.108	**0.390**	**279.6** (11)
$D_2$232	16.3	80	0.331	0.168	**0.502**	**211.5** (12)
D_2m	14.6	86	0.298	0.202	**0.323**	**207.1** (12)
D_2	*141*	*73*	*0.231*	*0.152*	*0.341*	

Sample	*N*	*P*	H_e	H_o	F_{IS}	χ^2 (d.f.)
$I_2$8	6.9	50	0.189	0.125	0.445	21.4 (3)
$I_2$16	14.4	84	0.288	0.207	0.267	12.7 (9)
$I_2$32	13.3	66	0.225	0.124	**0.467**	**86.8** (9)
$I_2$64	11.4	60	0.252	0.233	0.079	**19.84** (9)
I_2	*41.3*	*80*	*0.250*	*0.167*	*0.333*	
$J_2$0	18.0	40	0.145	0.119	**0.192**	**215.7** (9)
$J_2$4	17.2	66	0.220	0.132	**0.398**	**143.9** (9)
$J_2$8	10.2	53	0.170	0.146	0.163	4.3 (6)
$J_2$16	7.2	61	0.246	0.202	0.200	3.6 (4)
$J_2$32	12.4	76	0.265	0.159	**0.399**	**42.5** (8)
$J_2$64	14.9	64	0.243	0.155	**0.363**	**46.4** (8)
$J_2$128	11.1	64	0.248	0.141	**0.437**	**33.2** (9)
J_2	*86.9*	*66*	*0.237*	*0.145*	*0.386*	
$D_3$0	25.7	53	0.202	0.171	0.151	5.9 (8)
$D_3$4	19.0	78	0.316	0.183	**0.426**	**84.4** (11)
$D_3$8	26.2	71	0.269	0.189	**0.303**	**178.0** (10)
$D_3$16	25.0	78	0.303	0.173	**0.429**	**251.2** (12)
$D_3$32	17.0	71	0.202	0.126	**0.386**	**113.4** (12)
$D_3$64	35.2	53	0.286	0.155	**0.452**	**203.8** (11)
$D_3$128	35.0	66	0.241	0.145	**0.397**	**262.0** (12)
$D_3$232	26.9	64	0.309	0.178	**0.428**	**168.8** (10)
3D_3m	22.2	57	0.269	0.153	**0.435**	13.3 (8)
D_3	*220*	*80*	*0.281*	*0.160*	*0.431*	
$I_3$0	26.2	71	0.276	0.146	**0.475**	17.8 (10)
$I_3$8	25.9	71	0.292	0.223	**0.238**	**39.2** (11)
$I_3$16	18.5	85	0.281	0.178	**0.369**	**61.1** (11)
$I_3$32	35.2	66	0.290	0.166	**0.431**	**31.0** (12)
$I_3$64	37.0	64	0.340	0.219	**0.358**	**95.9** (12)
I_3	*135*	*73*	*0.290*	*0.178*	*0.385*	
$J_3$0	19.5	78	0.308	0.191	**0.386**	**109.5** (13)
$J_3$4	26.0	69	0.303	0.174	**0.429**	**44.8** (9)
$J_3$8	17.7	78	0.375	0.240	**0.368**	15.7 (12)
$J_3$16	22.0	85	0.311	0.210	**0.328**	**32.9** (12)
$J_3$32	14.0	57	0.256	0.156	**0.397**	**15.8** (8)
$J_3$64	21.5	71	0.217	0.145	**0.336**	**96.3** (10)
$J_3$128	26.2	85	0.319	0.194	**0.397**	**144.8** (13)
J_3	*135*	*73*	*0.290*	*0.177*	*0.388*	

Table 4. Loci and samples with deviations from Hardy-Weinberg equilibrium based on the Monte-Carlo method and sequential Bonferroni correction (p<0.002). The F_{IS} values by locus and transect are presented with their respective χ^2 values from the homogeneity test across samples (d.f., degrees of freedom). χ^2 values in bold are significant (p<0.05). Sample abbreviations as in Table 1. Tr, transect.

locus	collection 1			collection 2			collection 3		
	Tr	F_{IS} (χ^2;d.f.)	Samples	Tr	F_{IS} (χ^2;d.f.)	Samples	Tr	F_{IS} (χ^2;d.f.)	Samples
Lap	J	0.336 (5.8; 6)	J0, D4, I0	J	0.120 (3.2; 6)		J	0.234 (3.1; 6)	J128, Dm, I32, I64
	D	0.217 (3.1; 8)		D	0.102 (3.0; 8)		D	0.247 (5.2; 8)	
	I	0.283 (2.1; 5)		I	0.106 (2.3; 3)		I	0.237 (5.5; 4)	
Idh-1	J	0.620 (**103.3; 6**)	J8, J128, D8, D232, I8, I32	D	0.654 (**79.7; 6**)	D128, D232	J	0.610 (**34.7; 6**)	J16, J128, D4, D8, D232, I32,
	D	0.335 (**43.0; 8**)					D	0.739 (**287.8; 6**)	
	I	0.475 (**12.4; 5**)					I	0.550 (2.6; 4)	
Idh-2	J	0.745 (**92.3; 3**)	J4	D	0.887 (**105.9; 3**)		J	0.536 (**7.5; 3**)	
Me-1	J	0.082 (9.8; 6)		J	0.123 (3.7; 5)		J	-0.01 (1.5; 6)	I32
	D	0.222 (15.2; 8)		D	0.061 (1.7; 6)		D	0.19 (5.3; 8)	
	I	0.05 (1.4; 5)					I	0.093 (**9.6; 4**)	
Me-2	J	0.222 (2.37; 6)	Dm	J	0.281 (1.5; 4)	D128	J	0.34 (8.5; 6)	J128, D64, I64,
	D	0.149 (13.4; 8)		D	0.269 (**30.1, 7**)		D	0.281 (14.1; 8)	
	I	0.192 (2.44; 5)		I	-0.08 (0.03; 3)		I	0.370 (6.4; 4)	
Ak-1	J	0.409 (**135.7; 6**)	D32	J	0.514 (**15.2; 4**)		J	0.830 (**108.8; 5**)	J32, J128, D16, I64
	D	0.389 (**38.8; 7**)		D	0.303 (8.9; 7)		D	0.519 (**196.9; 6**)	
	I	0.231 (**12.0; 4**)					I	0.376 (**14.5; 3**)	
Ak-2	J	0.448 (**151; 6**)		J	0.485 (**57.3; 3**)	D32, I32	J	0.381 (5.36; 3)	D128
	D	0.392 (**14.6; 6**)		D	0.541 (**144.8; 6**)		D	0.530 (**89.2; 4**)	
	I	0.238 (**9.2; 4**)							
Ak-3	J	0.279 (**10.22; 4**)		J	0.194 (**43.8; 5**)	I32	J	0.100 (4.2; 4)]	
	D	0.224 (**18.6; 6**)		D	0.263 (**12.1; 6**)		D	0.124 (**150.1; 6**)	
	I	-0.040 (0.02; 3)							
Pep-1	J	0.625 (8.4; 6)	J16, J128D,4, D8, D32, I8	J	0.886 (**18.4; 3**)	J4	J	0.473 (8.4; 6)	J16, D128, D232, Dm, I0, I32
	D	0.625 (**25.2; 8**)		D	0.357 (2.1; 3)		D	0.421 (11.8; 8)	
	I	0.331 (5.4; 5)					I	0.486 (4.1; 4)	
Pep-2	D	0.498 (3.7; 3)		D	0.99 (0; 4)	D232			
Pep-3	J	0.512 (**13.7; 6**)	J16, J32, J64, J128,D8, I0, I8, I64	D	0.425 (**10.7; 3**)		J	0.620 (**13.7; 6**)	J4, J8, D8, D16, D64, D128, 232, I0, I8, I32, 164
	D	0.298 (5.4; 8)					D	0.512 (10.8; 8)	
	I	0.522 (3.4; 5)					I	0.716 (**27.6; 4**)	
Pgm-1	J	0.285 (**12.7; 4**)					J	0.331 (0.9; 3)	D4, D128, I32
	D	0.420 (**54.0; 5**)					D	0.594 (**43.6; 5**)	
	I	0.213 (**4.7; 4**)							

Table 4. Continuation.

locus		collect 1			collect 2			collect 3	
	Tr	F_{IS} (χ^2;d.f.)	Samples	Tr	F_{IS} (χ^2;d.f.)	Samples	Tr	F_{IS} (χ^2;d.f.)	Samples
Pgm-2	J	0.303 (1.2; 6)	D4, I32	J	0.316 (4.9; 6)	D64	J	0.509 (8.1; 6)	J4, J8, D64, D232, Dm, I0
	D	0.212 (7.5; 8)		D	0.302 (**19.9; 8**)		D	0.511 (13.1; 8)	
	I	0.222 (5.8; 5)					I	0.402 (4.0; 4)	
Pgm-3	J	0.676 (6.8; 3)	J0, J4				J	0.389 (**49.2; 5**)	J0, I8
	D	0.504 (**41.6; 5**)					D	0.576 (**12.8; 4**)	
							I	0.461 (**49.2; 4**)	
Gpi	J	0.306 (4.3; 6)	D0, D232, J4, J8	J	0.224 (0.8; 6)	D16, D232	J	0.283 (1.2; 6)	I32
	D	0.250 (4.5; 8)		D	0.164 (4.5; 8)		D	0.196 (3.4; 8)	
	I	0.222 (1.4; 5)					I	0.246 (4.8; 4)	

Table 5. Values of the F_{ST} for all loci. Upper table: four-level hierarchy analysis. Middle table: three-level hierarchical and analysis within transect. Lower table: variance components and percentage of variation. The number of samples analyzed is shown in parentheses. * significant genetic differentiation.

F_{ST}	Four-level hierachical analysis			
	Among and within localities			Among different sampling time
	Samples (63)	By transects (9)	By localities (3)	Collections 1,2,3 (3)
θ	0.025*	0.003	-0.001	0.005*
95% CI	0.019, 0.032	-0.000, 0.007	-0.003, 0.001	0.002, 0.009

F_{ST}	Three-level hierarchical analysis		Whithin each transect		
	Localities				
Juquehy	Transects				
	between (3)	within (21)	J_1 (7)	J_2 (7)	J_3 (7)
θ	0.006	0.028*	0.019*	0.051*	0.030*
95% CI	-0.002, 0.013	0.014, 0.042	0.007, 0.033	0.005, 0.108	0.018, 0.047
Dura	Transects				
	between (3)	within (27)	D_1 (9)	D_2 (9)	D_3 (9)
θ	0.005	0.027*	0.008*	0.048*	0.058*
95% CI	-0.001, 0.010	0.019, 0.036	0.0003, 0.016	0.027, 0.075	0.014, 0.108
Itamambuca	Transects				
	between (3)	within (15)	I_1 (6)	I_2 (4)	I_3 (5)
θ	0.001	0.015*	0.019*	0.009	0.011*
95% CI	-0.006, 0.006	0.008, 0.021	0.012, 0.026	-0.008, 0.027	0.001, 0.022

Four-level hierachical analysis	Among and within localities		Among different sampling time	
	Variance	% total variation	Variance	% total variation
Within samples	1.446	93.3	1.446	92.9
Within transects	0.086	5.6	0.086	5.6
Between transects	0.020	1.3	0.000	0.0
Between localities	-0.004	-0.2	0.023	1.5

Three-level hierachical analysis		Variance	% total variation
Juquehy	Within samples	1.466	92.9
	Within transects	0.088	5.6
	Between transects	0.023	1.5
Dura	Within samples	1.385	92.6
	Within transects	0.090	6.0
	Between transects	0.021	1.4
Itamambuca	Within samples	1.469	95.7
	Within transects	0.058	3.9
	Between transects	0.006	0.4

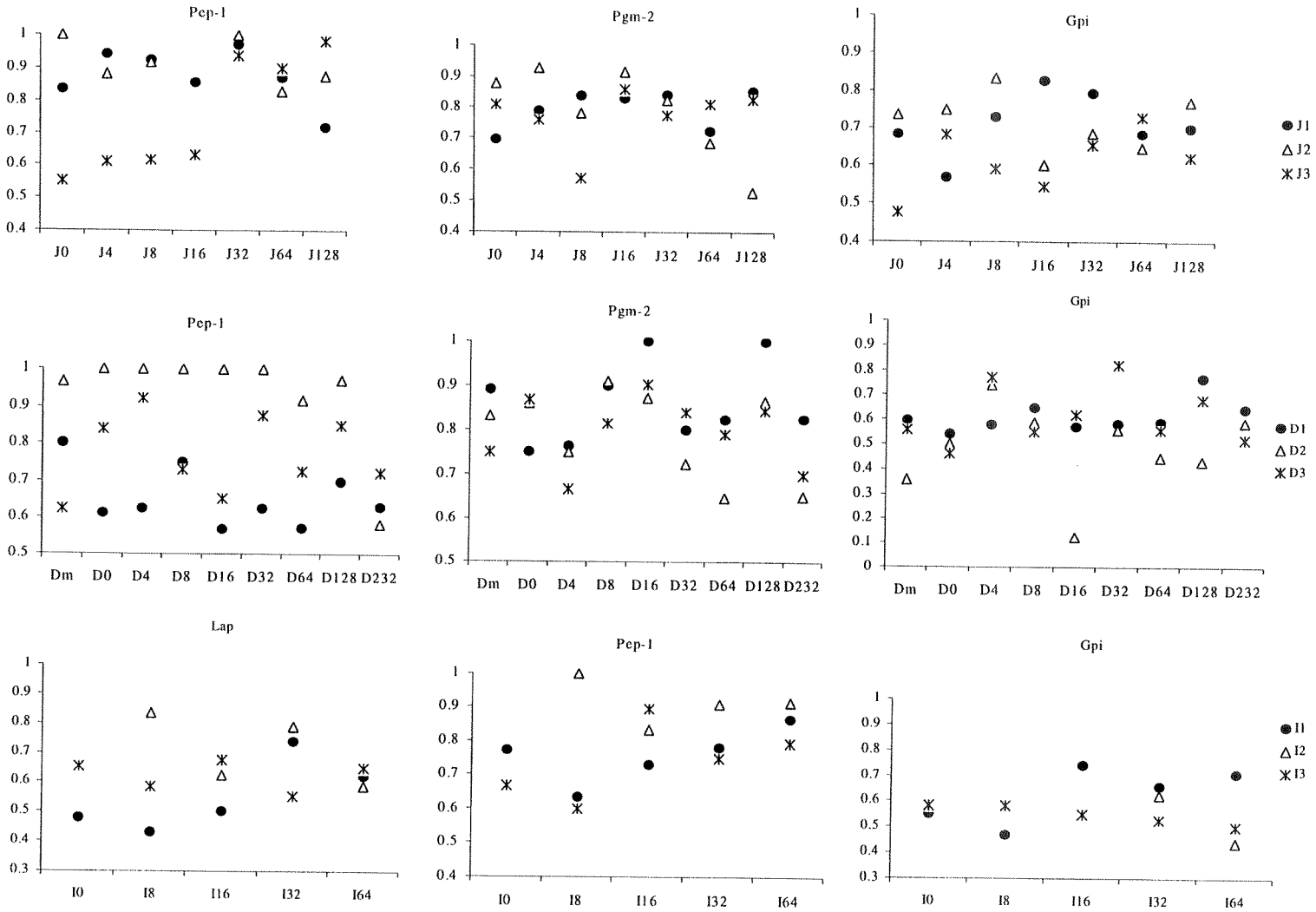

Figure 3. Allelic frequencies of the most common allele from Lap, Pep-1, Pgm-1, Pgm-2 and Gpi in all transects. Top: all collects frequencies from Juquehy. Middle: all collects frequencies from Praia Dura. Bottom: all collects frequencies from Itamabuca. Most common allele from Gpi in site J16 has the same frequency on collects I_1 e I_2. No information data for: Pep-1 in $J_2$16; Lap, Pep-1 and Gpi in $I_2$0; and Gpi in $J_2$8.

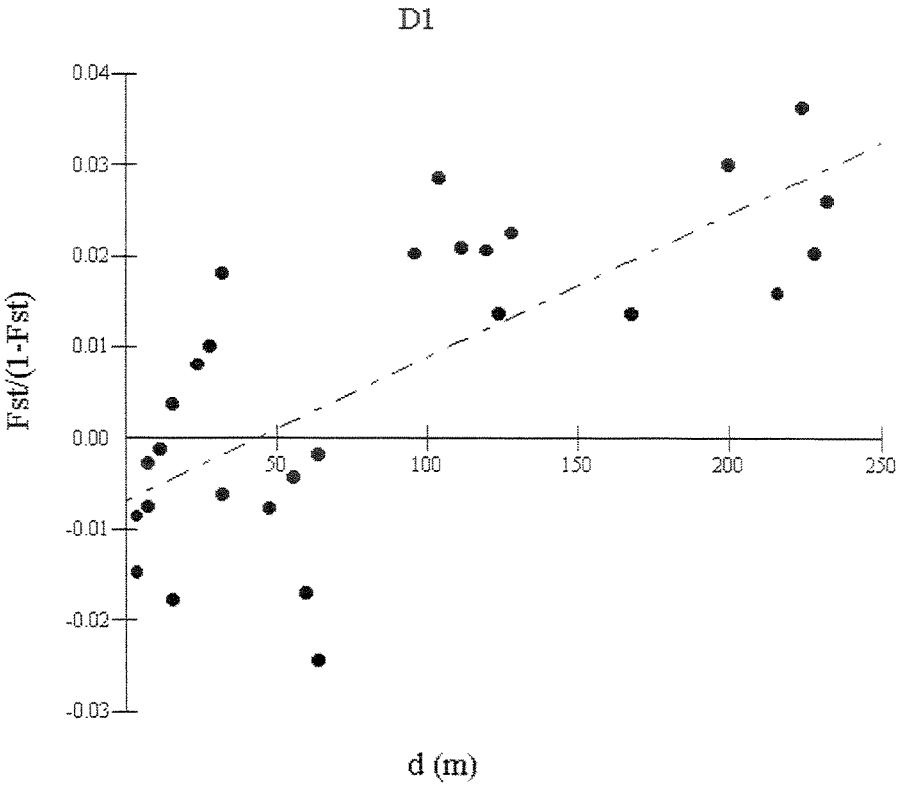

Figure 4. Plot of the Mantel test between genetic differentiation [F_{ST}/ (1-F_{ST})] and distance (in m) for transect D_1 (r=0.730, p=0.005).

Table 6. F_{ST} by loci from all samples and for all transect. Significant values are shown in bold. ns – non-significant.

	Total	J_1	D_1	I_1	J_2	D_2	I_2	J_3	D_3	I_3
Lap	**0.014**	0.004 ^{ns}	**0.009**	**0.017**	**0.004**	**0.028**	0.030 ^{ns}	**0.020**	**0.012**	0.001 ^{ns}
Idh-1	0.002 ^{ns}	-0.008 ^{ns}	-0.001 ^{ns}	**0.020**	-0.035 ^{ns}	-0.004 ^{ns}	0.033 ^{ns}	0.007 ^{ns}	0.009 ^{ns}	-0.008 ^{ns}
Idh-2	**0.015**	-0.001 ^{ns}	**0.071**	**0.020**	-0.005 ^{ns}	0.018 ^{ns}	**0.125**	-0.004 ^{ns}	0.010 ^{ns}	0.023 ^{ns}
Me-1	**0.029**	**0.041**	0.001 ^{ns}	0.001 ^{ns}	**0.060**	-0.001 ^{ns}	0.008 ^{ns}	**0.025**	**0.014**	0.006 ^{ns}
Me-2	**0.032**	**0.051**	**0.024**	-0.007 ^{ns}	0.000 ^{ns}	**0.099**	-0.001 ^{ns}	**0.052**	**0.019**	**0.047**
Ak-1	**0.022**	-0.009 ^{ns}	**0.044**	**0.027**	**0.060**	-0.003 ^{ns}	-0.027 ^{ns}	**0.038**	**0.054**	0.009 ^{ns}
Ak-2	**0.021**	0.012 ^{ns}	**0.037**	**0.033**	0.014 ^{ns}	**0.034**	0.040 ^{ns}	0.008 ^{ns}	0.003 ^{ns}	**0.031**
Ak-3	**0.028**	**0.025**	**0.008**	**0.020**	**0.018**	0.000 ^{ns}	0.061 ^{ns}	-0.003 ^{ns}	0.006 ^{ns}	-0.005 ^{ns}
Pep-1	**0.034**	0.015 ^{ns}	**0.022**	0.016 ^{ns}	-0.031 ^{ns}	**0.320**	-0.060 ^{ns}	**0.081**	0.011 ^{ns}	0.021 ^{ns}
Pep-2	**0.085**	-	-0.064 ^{ns}	-	-0.026 ^{ns}	**0.220**	0.034 ^{ns}	-	-	-
Pep-3	**0.026**	0.012 ^{ns}	0.000 ^{ns}	**0.017**	**0.167**	**0.100**	-0.028 ^{ns}	**0.019**	**0.045**	**0.024**
Pgm-1	**0.035**	0.014 ^{ns}	0.007 ^{ns}	**0.048**	0.054 ^{ns}	**0.095**	0.000 ^{ns}	**0.053**	**0.036**	**0.056**
Pgm-2	**0.011**	0.001 ^{ns}	**0.017**	**0.016**	**0.050**	**0.027**	-0.014 ^{ns}	0.013 ^{ns}	0.004 ^{ns}	-0.001 ^{ns}
Pgm-3	0.018 ^{ns}	0.057 ^{ns}	0.003 ^{ns}	0.026 ^{ns}	0.058 ^{ns}	-0.015 ^{ns}	-	0.022 ^{ns}	0.012 ^{ns}	-0.016 ^{ns}
Gpi	**0.024**	0.011 ^{ns}	0.001 ^{ns}	**0.026**	-0.006 ^{ns}	**0.032**	0.025 ^{ns}	0.010 ^{ns}	**0.019**	0.000 ^{ns}

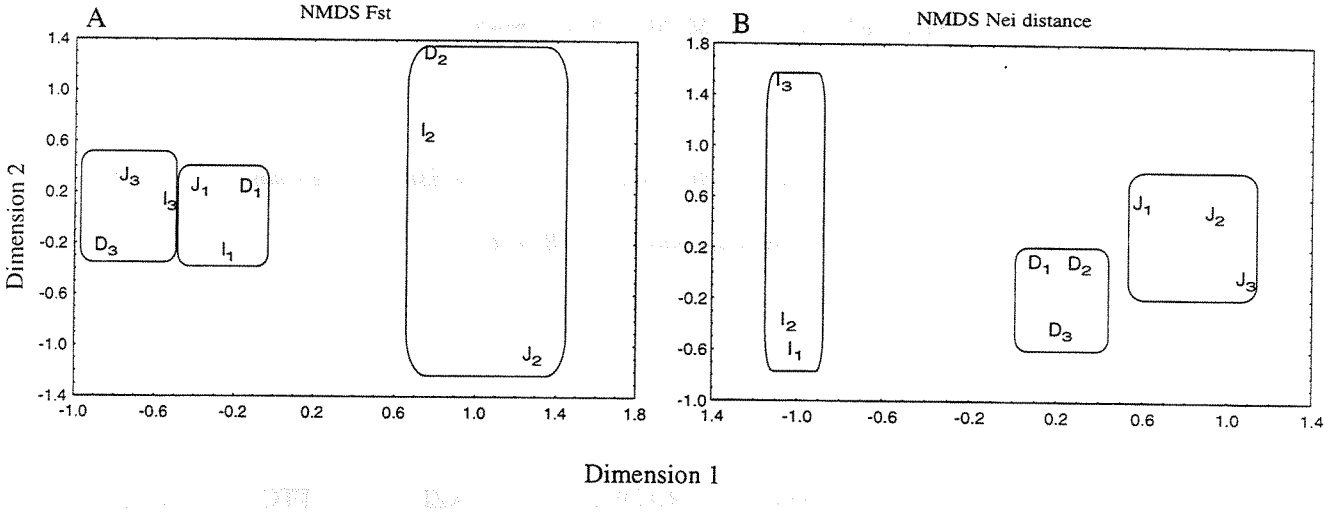

Figure 5. Graphical representation of non-metrical dimensional scaling (NMDS) on (A) the pairwise F_{ST} matrix (stress = 0.070) and (B) Nei's (1978) genetic distances (stress = 0.044) for matrix-based transects (samples grouped within each transect).

Capítulo 3

Diverse environmental effects on the radulae of *Littoraria flava* and *Littoraria angulifera* (Gastropoda: Littorinidae)

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Running Head: ENVIRONMENTAL EFFECT ON RADULA OF BRAZILIAN LITTORINIDS

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ABSTRACT

Phenotypic variation in radulae has been studied in several littorinid species because of this organ's intrinsic relationship with diet and, consequently, with the environment. In this work, we compared the radulae of two Brazilian species of *Littoraria* (*L. flava* and *L. angulifera*) found in mangroves and on rocky shores. Individuals of *L. angulifera* from mangroves showed less variation in radula shape than those from rocky shores, suggesting a differential environment effect. In addition, the radulae of *L. flava* showed more variation in shape than those of *L. angulifera*. In a natural transfer experiment with *L. flava*, the shape of the radula changed within 40 days. This alteration could be attributed to ecophenotypic plasticity. Analysis of variance showed that the length of the radula in *L. flava* was strongly influenced by the substrate ($F_{6,22}=17.13$, $p<0.001$).

INTRODUCTION

Phenotypic variation in a heterogeneous environment, whether caused by genetic variability, phenotypic plasticity, or the interaction of both of these factors, is an ongoing challenge in evolutionary ecology. In spatially or temporally fluctuating environments, many species show morphological variation under different conditions (Marchinko, 2003). Phenotypic variation has been reported for many characters of littorinids, including shell form and color, foot size and radular shape and length (Rolán-Alvarez, Rolán & Johannesson, 1996; Trussell, 1997; Padilla, 1998; Trussell, 2000; Ito, Ilano & Nakao, 2002). The radula is a suitable structure for investigating the effects of environmental heterogeneity on natural populations because of its close relationship with the feeding substrate, and also because of its susceptibility to change in response to environmental oscillations. Some species can completely replace their radula ribbon in 2-6 weeks as part of a rapid response to environmental conditions (Padilla *et al.*, 1996; Padilla, 1998). In addition, differences in the morphology of this structure have been related to sexual dimorphism (Fujioka, 1985, cited in Reid & Mak, 1999), diet (Padilla, 1998), and temporal and spatial variations in the substrate (Reid & Mak, 1999; Ito *et al.*, 2002).

The radula of littorinids is of the taenioglossate type that is characterized by a seven teeth per row in which the central rachidian is flanked on either side by lateral, inner marginal and outer marginal teeth. Although the radulae of closely related littorinid species are insufficiently differentiated to be useful for systematic studies (Reid, 1996; Reid & Mak, 1999), this organ has been used to classify littorinids into groups of species and higher taxonomic levels (Bandel, 1974; Bandel & Kadolsky, 1982; Reid, 1986). The functional significance of the form of radular teeth is still unclear, but is probably closely

associated with feeding habits (Reid, 1996). However, as pointed out by Reid & Mak (1999), few studies have addressed this issue, and most of them have inferred the function of this organ based on interspecific comparisons and correlations with diet and substrate or even with morphology (Padilla, 2004).

Littoraria (Griffith & Pidgeon, 1834) is a genus with 39 known species that occurs in mangroves, salt marshes and on rocky shores close to freshwater sources (Reid 1986, 1999, 2001). Only two species are found along the Brazilian coast: *L. flava* (King & Broderip, 1832), restricted to rocky shores near freshwater, but also found in mangroves (Reid & Mak, 1999), and *L. angulifera* (Lamarck, 1822), which occurs in mangroves and, occasionally, on nearby rocky shores (Gallagher & Reid, 1974). Kohlmeyer & Bebout (1986) suggested that the diet of *L. angulifera* was based on fungus-infested plants and fungi. In contrast, the diet of *L. flava* has not been investigated, but presumably includes epilithic and neolithic micro-algae, fungi and lichens, as in other littorinids of the littoral fringe (Reid, 1996; Christensen, 1998).

Reid & Mak (1999) reported differences in the radular form of these two species collected on rocky shores and on mangroves. In this work, we conducted an experiment in order to test if there is similar variation in Brazilian samples. The study consisted of two parts. In the first part, both species were sampled from mangrove and rocky shores and the shape of the radulae was compared among samples collected from different substrates and among animals of different sizes. In the second part, an experiment involving the reciprocal transfer of individuals of *L. flava* from a rocky shore and mangrove was done to assess possible changes in the shape of the radulae.

MATERIALS AND METHODS

Sampling sites

The samplings and the experiment were conducted on Dura beach (23°29'S/45°10'E), a sheltered location in the Escuro River estuary at Ubatuba, on the subtropical coast of São Paulo state, in southeastern Brazil. At this location there are both environments apart about 100 m: the mangrove along the estuary and a salt water rocky shore. *Littoraria flava* and *L. angulifera* were collected on the two environments. The mangrove of the Escuro River consists mainly of three species, the grass *Spartina* sp., and *Avicennia schaueriana* and *Laguncularia racemosa*, very common shrubs in Brazilian mangroves.

Experimental design

Part 1- radula shape

The sampling was done on February 20, 2003. Four samples of *L. flava* (at least 30 individuals/site) were collected on the rocky shore from Dura beach at 8, 16, 32 and 64 m along a transect from edge of the rocky shore towards the water. At the first site, point 8, almost all individuals were collected in the mud, beneath small rocks. One sample of 10 specimens of *L. angulifera* was collected on the rocky shore. In mangrove, both species were collected on *Spartina* blades and on the stems and aerial roots of *L. racemosa* and *A. schaueriana* (a total of 10 and 20 individuals of *L. flava* and *L. angulifera*, respectively, collected in mangrove).

Part 2 - transfer experiment

On the same day, a reciprocal transfer experiment was done with *L. flava*. Two hundred and nineteen individuals were collected from the first site on the rocky shore (8m,

and only snails found crawling on rocks were used) and were randomly allocated to one of two groups: rocky-control (RC, n= 108) and rocky-transferred (RT, n= 111). The controls were returned to the same site and the RT individuals were released in the *Spartina* field. A similar procedure was done in the mangrove, where 222 snails were collected on *Spartina* sp. and divided into two groups: mangrove-control (MC, n=108) and mangrove-transferred (MT, n=114), and subsequently released at the same original *Spartina* site and on the rocky shore. The snails were painted with enamel paint (Testors Co., Rockford, USA) using color codes. This high number of snails was marked in order to assure a good recapture rate since we were working in natural conditions and no previous information about recapture rates was available for this species. All marked snails were collected at the same sites after 40 days.

Laboratory procedure

Part 1 and 2

Radulae of 72 individuals were analysed, representing *Littoraria flava* and *L. angulifera.* In the laboratory, the snails were immersed in 7.5% MgCl solution and then fixed in 70% ethanol. The shell height (maximum dimension parallel to the axis of coiling) was measured to the nearest 0.01 mm with digital callipers. To isolate the radula, the snails were stored in 7.5% KOH at 50°C until the soft body was completely dissolved (10-20 minutes), followed by gentle cleaning with a fine paintbrush and rinsing with distilled water. Heating did not cause any change on the radulae, compared with non-heated samples. The radulae were mounted flat on aluminum stubs and held in place with dual adhesive tape. The structures were coated with gold and examined by scanning electron microscopy (SEM). Only fully formed and unworn teeth from the central third of the radula

ribbon were examined. The sample size used for SEM analysis and the range of shell height are shown in Table 1. Photographs were taken in two standard orientations: at 45° from front end and at 45° from side, to show shape of tooth cusps and relief.

The description of the radula characters was based on Reid & Mak (1999), with the major cusps referring only to the lateral and inner marginal teeth where the main changes had occurred. The counting of cusps was restricted to 4-12 rows based on photos for each individual. The number of cusps was counted on all visible teeth on both left and right sides of each radula. The characterization of the form of major cusp (in relation to the smallest cusps) on lateral and inner marginal teeth was assigned in following categories: subequal (Fig. 1A), blunt (Fig. 2A), sharper-subequal (cusps are subequal, with the major cusps slightly larger; Fig. 2E), and large-elongate (major cusps are very large and long; Fig. 2F).

For the results of the natural transfer experiment, the R statistical package (Ihaka & Gentleman, 1996; freely distributed on site: http://cran.r-project.org/) was used to estimate independence between the shape of the major cusps and treatment classes based on the Fisher exact test for small frequencies.

The length of the radular ribbon of *L. flava* was measured using a stereomicroscope in 29 individuals from seven groups that included: the four classes (RC, RT, MC and MT), the 64 m site on the rocky shore (RS), and the *Spartina* (S) and *L. racemosa*/*A. schaueriana* (Sh) vegetation types. Four individuals were examined from all samples, except for the RS sample, for which five specimens were used. To avoid a possible effect of size on radular length, we used only snails with a shell height between 10.5 and 14.2 mm. The differences among samples were compared using one-way analysis of variance (ANOVA) followed by a *post hoc* Tukey's multiple comparison test for unequal sample sizes.

RESULTS

Part 1: Variation within and among sites

Littoraria angulifera

This species showed little differentiation in the shape of the major cusps between environments (Fig. 1). The radula of this species had a large hood in both environments and similar number of cusps in the outer marginal teeth in mangrove and rocky shore (Table 1 and Fig. 1). There was no individual variation in the number of cusps. Among specimens from the rocky shore, individuals with different shell heights showed variation in the cusps shape on the lateral teeth (Figs. 1A and B). Of the six individuals from the rocky shore analyzed by SEM, four (with a shell height <10 mm) had blunt cusps, larger than found on specimens with larger shell height (Table 1 and Fig. 1B). There were no differences in the shape of the radulae among snails collected on shrubs and *Spartina* (Figs. 1C and D).

Littoraria flava

The specimens of *L. flava* from the two environments varied markedly in size (Table 1), but there is no apparent difference on the shape between periwinkles with different sizes (Figs. 2A and C). Also, there were no differences in radula shape among mangrove snails collected from *Spartina* and on the stems and aerial roots from the shrubs.

Snails from the two environments differed in the shape of their radulae, with individuals from the rocky shore having large, blunt cusps while those from mangroves had narrower cusps with a subequal size and shape (Fig. 2B). There was no variation within each column of teeth, and there was only slight variation between columns. In all of the periwinkles examined, the hood of the rachidian tooth was always large (Fig. 2).

Part 2: Reciprocal transfer experiment

The average recapture rate was 40%. All the snails collected (n=43 RC, 40 RT, 40 MC and 29 MT) were taken to the laboratory, where the radulae of snails with different shell heights from each class were analyzed by SEM. From these 152 snails, about 40 were analysed by SEM, but only 33 provided good images.

The teeth of snails from the control groups showed the same pattern as that of specimens examined in the first part of this study: RC snails had blunt major cusps while MC snails had subequal cusps (Figs. 2D and G, respectively). All snails from the MT class (Fig. 2H) had blunt cusps similar those of the RC class, and the RT class had sharper and narrower cusps than RC snails (Table 1). The response of RT specimens varied, with half showing larger and elongated cusps than those seen in RC snails (Fig. 2F). The other half showed sharper-subequal major cusps (Fig. 2E and Table 1). The correlation between these patterns (large-elongate or sharper-subequal) and shell height in the RT group was not significant (r_s=-0.522, P>0.1), an evidence that the variation is not related with size. The Fisher exact test showed that the forms of cusps (blunt/large-elongate and sharper-subequal/subequal) and the four treatment classes were not independent ($p<0.001$), hence confirming the relationship between cusps shape and environment.

There was no correlation between shell height and radular ribbon length when all of the snails were grouped together (r_s=-0.063, df =27, P>0.1), indicating that the length of the radula was not determined by the individual's size. An identical analysis for each class also revealed no significant correlation. Although snail size had no influence on ribbon length, we calculated the relative radular length (length/shell height) to facilitate the comparison of our findings with those of other studies (Table 2).

There was a significant influence of sample collection site on relative radular length (ANOVA, $F_{6,22}=17.13$, $MS_{effect}=0.68$, $MS_{error}=0.04$; $P<0.001$). The combined analysis among pairwise samples is presented on Table 2. The results reflected mainly differences between mangrove and the rocky shore.

Discussion

Littoraria angulifera

There was no difference in the shape of the radula between snails from the two environments, in contrast to the observations of Reid & Mak (1999). The samples used in their study were collected in different places (Belize and Florida, D.G. Reid, personal communication), what could explain the observed variation. Our samples varied less in shape than those analyzed by these authors. The only difference between the mangrove and rocky shore samples was in the number of lateral and marginal cusps (Table 1).

On the rocky shore, the cusps of the lateral teeth varied in shape according to the size of the snails, with the smallest individuals having blunt cusps. This situation could represent a normal ontogenetic process, as observed in *Conus* (Nybakken, 1990), but we do not know if the small periwinkles sampled in mangrove may have been young adults, not juveniles. Ontogenetic variation probably only occurred on the rocky shore since in mangroves the major cusps all had the same shape, regardless of the size of the snails. Explanations for this phenomenon include the possibilities 1) that specimens of different sizes from rocky shores may have distinct diets and may partition resources, 2) this pattern results from ontogenetic effect, which is dependent from environmental characteristics, 3) that distinct factors in each environment may have exerted a different selective effect on

radula shape in *L. angulifera*, and 4) that an interaction among these factors could have contributed to the divergent effect seen in these two environments.

Merkt & Ellison (1998) have already noticed that several morphological traits of *L. angulifera* populations also varied in response to different environmental conditions. These authors observed that morphological variation in some traits as shell shape, sculptural and genital morphology could be mainly ascribed to habitat characteristics and environmental heterogeneity.

Littoraria flava

Reid (1996) and Rolán-Alvarez *et al.* (1996) reported that juveniles and smaller individuals had a greater number of sharper and longer cusps that could represent an allometric relationship since juvenile-like radulae also occur in small adults. The shape of the radula in relation to shell height was examined for both environments. There was no such differentiation related to the form or number of the cusps when comparing the smallest periwinkles with larger ones. Also, in mangrove, there was no difference in the relative radular length of snails collected on *Spartina* sp. and on shrubs (Table 2).

Sharper teeth have less contact with surface and are more effective at piercing and tearing fleshy algae as blunt teeth seem to be more effective for rasping and removing loose material from surfaces or broad excavations of brittle materials, such as calcified algae (Padilla, 2004). The major cusps of *Littoraria flava* show a pattern in which snails from rocky shores have radulae with large, blunt cusps whereas snails from mangroves have sharper cusps with a subequal form, as already observed by Reid & Mak (1999) in *Littoraria* species. Rolán-Alvarez et al. (1996) reported variation in the number of cusps in *Littorina saxatilis*; Reid & Mak (1999), in the number of cusps and in the hood (a frontal

plate anterior to the cusps of the rachidian teeth) among snails from different substrates. In our samples, although there were differences in shape, the number of cusps varies slightly between environments and individual variation was very uncommon (Table 1). There was no variation in the form of the hood, which was always present and large. Radulae with a hood may be adapted for feeding upon the algae of mangroves, driftwood or salt marshes (Rosewater, 1980), although species with hooded radulae also occur no rocky shores (Reid, 1986).

Our samples showed less variation among environments than those analyzed by Reid & Mak (1999), especially in the number of cusps. These divergent findings may reflect interpopulational genetic variation, assuming that radular variation is a heritable character. In addition, samples from Reid & Mak study are from different sources (D.G. Reid, pers. communication), rocky shore sample was from Cuba and mangrove's was from Guyana, what could explain the variation observed on their samples.

Reid & Mak (1999) suggested several hypotheses to explain the morphological variation seen in snails from different environments. In our experiments, snails transferred from the rocky shore to mangroves had sharper cusps than the control snails, whereas snails transferred from mangroves to the rocky shore developed blunt cusps similar those of snails from rocky shores (Table 1). The shape of the radulae in rocky shore-control and mangrove-control snails were the same as mollusks from the two environments examined in the first part of this study. These may provide an evidence for the ecophenotypic plasticity hypothesis proposed by Reid & Mak (1999) to explain variation observed in radula from *Littoraria* species.

The radular morphology of rocky shore-transferred snails (RT) varied independently of the shell height, which could be an indication of intrapopulational variation in the

responses to environmental conditions. Another hypothesis to explain the variation in radular form is that the individuals with large-elongate cusps were still in the process of change by the time of recapture. If this change is gradual, it is likely that some snails reacted at different rates and could adapt faster to changes on environmental conditions. This was not seen in MT snails, which always had large and very elongated cusps. The three types of cusps (blunt in the RC and MT groups, large-elongate in half of the RT snails and sharper-subequal in MC and RT snails) were influenced by the environment. Even with the differences in the response to transference from one site to another, there was still a strong association between tooth shape and the environment in which the individuals were recaptured.

The rate of radular replacement in littorinids is still poorly known. In the Littorinidae as a whole, the longest radulae occur in species that graze on hard substrates, which suggests a correlation with the rate of tooth wear (Reid, 1986, 1989). Scraping wears down the teeth at a rate dependent on the hardness of the substratum so that radular length is affected by substrate hardness (Reid & Mak, 1999). The median relative radular length of mangrove samples of *L. flava* was smaller than for rocky shore samples, as expected. In RT snails, the length was the same and there was no difference in the radular length of RS, RC and RT snails (Table 2). However, snails from the 64 m site (RS) and the RC group differed significantly in radular length what may be explained by different resources along the shore, resulting in morphological variation (Padilla, 1998). If so, further studies on variation of food availability along rocky shores could be interesting to test this hypothesis.

Although MT snails had a shorter relative radular length, they were not significantly different from RC snails, as expected, but were different from RT snails. The latter snails

also showed marked variation in their relative radular length (Table 2), as well as observed in their degree of response in cusp's shape.

Other factors, in addition to substrate hardness, may have an important influence on the rate of radular replacement in mollusks. In the opisthobranch *Placida dendritica*, the species of algae eaten could determine the size and shape of the radulae (Bleakney, 1990). Isarankura & Runham (1968) reported that temperature, age and size also influence the number of tooth rows. Based on experiments with prosobranchs and pulmonates, these authors suggested that replacement rates were accelerated in newly-hatched snails, but decreased with age and size. In addition, active herbivores, such as *Littorina* species, had the fastest replacement rates.

It remains unclear if radular length of transferred snails (RT and MT) would change in a longer experiment. In addition, it is not known whether Brazilian species of *Littoraria* show seasonal variation in radular length such as that detected by Ito et al. (2002). These authors found that in *Nodilittorina radiata* the radula became shorter during intensive use while searching for food. This observation suggested that there was no direct relationship between tooth morphology and replacement rates in *L. flava*, contrary to the proposal by Reid & Mak (1999). In species of *Lacuna*, a few weeks are required for total replacement of the radula in artificial conditions (Padilla *et al.*, 1996; Padilla, 1998). If the duration of our experiments had been sufficient for *L. flava* to completely replace the radula, it should also have been enough to produce shorter radulae in mangrove specimens. Further experiments are necessary to determine whether radular length has the same plasticity as tooth shape or if the speed of response is slower.

Curiously, the relationships seen in *L. flava* individuals of different shell heights were different of those seen in *L. angulifera*, in which smaller individuals had blunter major

cusps in rocky shore. This finding indicated that the two environments exerted different effects on the radulae of this species. The greater heterogeneity of the rocky shore, with the availability of different substrates, compared with mangroves, could have resulted in a greater morphological diversity. More comprehensive experiments isolating ontogeny and environmental effects on littorinids are welcome to help solve this question. Additional experimental work will be important for understanding the processes leading to the definition of the morphological traits in littorinids.

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REFERENCES

BANDEL, K. 1974. Studies on Littorinidae from the Atlantic. *The Veliger*, **17**: 92-114.

BANDEL, K. & KADOLSKY, D. 1982. Western Atlantic species of *Nodilittorina* (Gastropoda: Prosobranchia): comparative morphology and its functional, ecological, phylogenetic and taxonomic implications. *The Veliger*, **25**: 1-42.

BLEAKNEY, J.S. 1990. Indirect evidence of a morphological response in the radula of *Placida dendritica* (Alder & Hancock, 1843) (Opistobranchia: Ascoglossa/Sacoglossa) to different algal prey. *The Veliger*, **33**: 111-115.

CHRISTENSEN, J.T. 1998. Diet in *Littoraria*. *Hydrobiologia*, **378**: 235-236.

FUJIOKA, Y. 1985. Systematic evaluation of radular characters in Thaidinae (Gastropoda: Muricidae). *Journal of Science of Hiroshima University, Serie B, Divison 1 (Zoology)*, **31**: 235-287.

GALLAGHER, S.B. & REID, G.K. 1974. Reproductive behavior and early development in *Littorina scabra angulifera* and *Littorina irrorata* (Gastropoda: Prosobranchia) in the Tampa Bay region of Florida. *Malacological Review*, **7**: 105-125.

IHAKA, R. & GENTLEMAN, R.1996. R: a language for data analysis and graphics. *Journal of Computational and Graphical Statistics*, **5**: 299-314.

ISARANKURA, K. & RUNHAM, N.W. 1968. Studies on the replacement of the gastropod radula. *Malacologia*, **7**: 71-91.

ITO, A., ILANO A.S. & NAKAO, S. 2002. Seasonal and tidal height variations in body weight and radular length in *Nodilittorina radiata* (Eydoux and Souleyet, 1852). *Journal of Molluscan Studies*, **68**: 197-203.

KOHLMEYER, J. & BEBOUT, B. 1986. On the occurrence of marine fungi in diet of *Littoraria angulifera* and observations on the behavior of the periwinkle. *Marine Ecology-Pubblicazioni Della Stazione Zoologica Di Napoli I*, **7**: 333-343.

MARCHINKO, K.B. 2003. Dramatic phenotypic plasticity in barnacle legs (*Balanus glandula* Darwin): magnitude, age dependence, and speed of response. *Evolution*, **57**: 1281-1290.

MERKT, R.E. & ELLISON, A.M. 1998. Geographic and habit-specific morphological variation of *Littoraria (Littorinopsis) angulifera* (Lamarck, 1822). *Malacologia*, **40**: 279-295.

NYBAKKEN, J. 1990. Ontogenetic change in the *Conus* radula, its form distribution among the radular types, and significance in systematics and ecology. *Malacologia*, **32**: 35-54.

PADILLA, D.K. 1998. Inducible phenotypic plasticity of the radula in *Lacuna* (Gastropoda: Littorinidae). *The Veliger*, **41**: 201-204.

PADILLA, D.K. 2004. Form and function of radular teeth of herbivorous molluscs: focus on the future. *American Malacological Bulletin*, **18**: 163-168.

PADILLA, D.K., DITTMAN, D.E., FRANZ, J. & SLADEK, R.1996. Radular production rates in two species of *Lacuna* Turton (Gastropoda: Littorinidae). *Journal of Molluscan Studies*, **62**: 275-280.

REID, D.G. 1986. *The Littorinid Molluscs of Mangrove Forests in the Indo-Pacific Region. The Genus* Littoraria. British Museum (Natural History).

REID, D.G. 1989. The comparative morphology, phylogeny and evolution of the gastropod family Littorinidae. *Philosophical Transactions of the Royal Society of London*, **B324**: 1-110.

REID, D.G. 1996. *Systematics and Evolution of* Littorina. Ray Society, London.

REID, D.G. 1999. The genus *Littoraria* Griffith & Pidgeon, 1834 (Gastropoda: Littorinidae) in tropical eastern Pacific. *The Veliger*, **42**: 21-53.

REID, D.G. 2001. New data on the taxonomy and distribution of the genus *Littoraria* Griffith and Pidgeon, 1834 (Gastropoda: Littorinidae) in Indo-West Pacific mangrove forests. *The Nautilus*, **115**: 115-139.

REID, D.G. & MAK, Y.M. 1999. Indirect evidence for ecophenotypic plasticity in radular dentition of *Littoraria* species (Gastropoda: Littorinidae). *Journal of Molluscan Studies*, **65**: 355-370.

ROLÁN-ALVAREZ, E., ROLÁN, E. & JOHANNESSON, K.1996. Differentiation in radular and embryonic characters, and further comments on gene flow, between two sympatric morphs of *Littorina saxatilis* (Olivi). *Ophelia*, **45**: 1-15.

ROSEWATER, J. 1980. A close look at *Littorina* radulae. *Bulletin of the American Malacological Union*, **1979**: 5-8.

TRUSSELL, G.C. 1997. Phenotypic plasticity in the foot size of an intertidal snail. *Ecology*, **78**: 1033-1048.

TRUSSELL, G.C. 2000. Phenotypic clines, plasticity, and morphological trade-offs in an intertidal snail. *Evolution*, **54**: 151-166.

Table 1. Specimens of *L. angulifera* and *L. flava* analyzed by SEM. The form of the major cusps and the number of cusps are shown according to the sampling site (see text and Figures 1 and 2 for explanations). The sample size is shown in parentheses. SH = range of shell height (mm). As "shrubs" we indicated the samples collected on *L. racemosa* and *A. schaueriana*.

Species and respective samples	SH	Major cusps	Lateral cusps	Inner marginal cusps	Outer marginal cusps
L. angulifera					
Rocky shore, stone (4)	6.7 - 9.4	blunt	5	4	4,5
Rocky shore, stone (2)	14.6 - 18.0	subequal	5	4	5,6
Mangrove, *Spartina* (7)	6.7 - 12.6	subequal	5,6	4	6
Mangrove, shrubs (4)	11.1 - 17.1	subequal	5,6	4	6
L. flava					
Samples from 1st part[1]					
Rocky shore, mud, 8 m (3)	4.1 - 7.9	blunt	5	4	4
Rocky shore, mud, 16 m (2)	6.1 - 7.7	blunt	5	4	4
Rocky shore, stone, 32 m (2)	16.3 - 18.3	blunt	5	4	4
Rocky shore, stone 64 m (2)	8.9 - 14.7	blunt	5	4	4,5
Mangrove, *Spartina* (4)	4.3 - 13.4	subequal	5	4	5
Mangrove, shrubs (2)	14.1 - 14.6	subequal	5	4	5
Reciprocal transfer experiment					
Rocky shore, control (8)	9.8-17.1	blunt	4,5	4	4,5*
Rocky shore, treatment (10)	9.9-15.5	large-elongate(5) subequal-sharper (5)	5	4	4,5
Mangrove, control (7)	9.7-14.5	subequal	5	4	4,5*
Mangrove, treatment (8)	8.4-15.2	blunt	5	4	4,5

[1]substrate, sampling site. *in these samples, different numbers of cusps were found in some individuals.

Table 2. Results of the *post hoc* Tukey test for unequal sample sizes. Four individuals were analysed for each sample, except for RS, for which five individuals were examined. Relative radular length (length/shell height) for samples of *L. flava* is also shown (s.d. = standard deviation). As "shrubs" (Sh) we indicated the samples collected on *L. racemosa* and *A. schaueriana*.* P<0.05, **P<0.01, *** P<0.001, and *ns*, not significant.

Sample site or class	RS	S	Sh	MC	RC	MT	Relative radular length (mean±s.d.)
Rocky shore 64 m (RS)							1.913±0.217
Spartina (S)	***						1.178±0.208
shrubs (Sh)	**	ns					1.325±0.168
MC	***	ns	*				0.767±0.145
RC	*	ns	ns	**			1.382±0.053
MT	***	ns	ns	ns	ns		0.956±0.117
RT	ns	*	ns	***	ns	***	1.683±0.348

MC, mangrove-control; MT, mangrove-transferred; RC, rocky-control; RT, rocky-transferred snails.

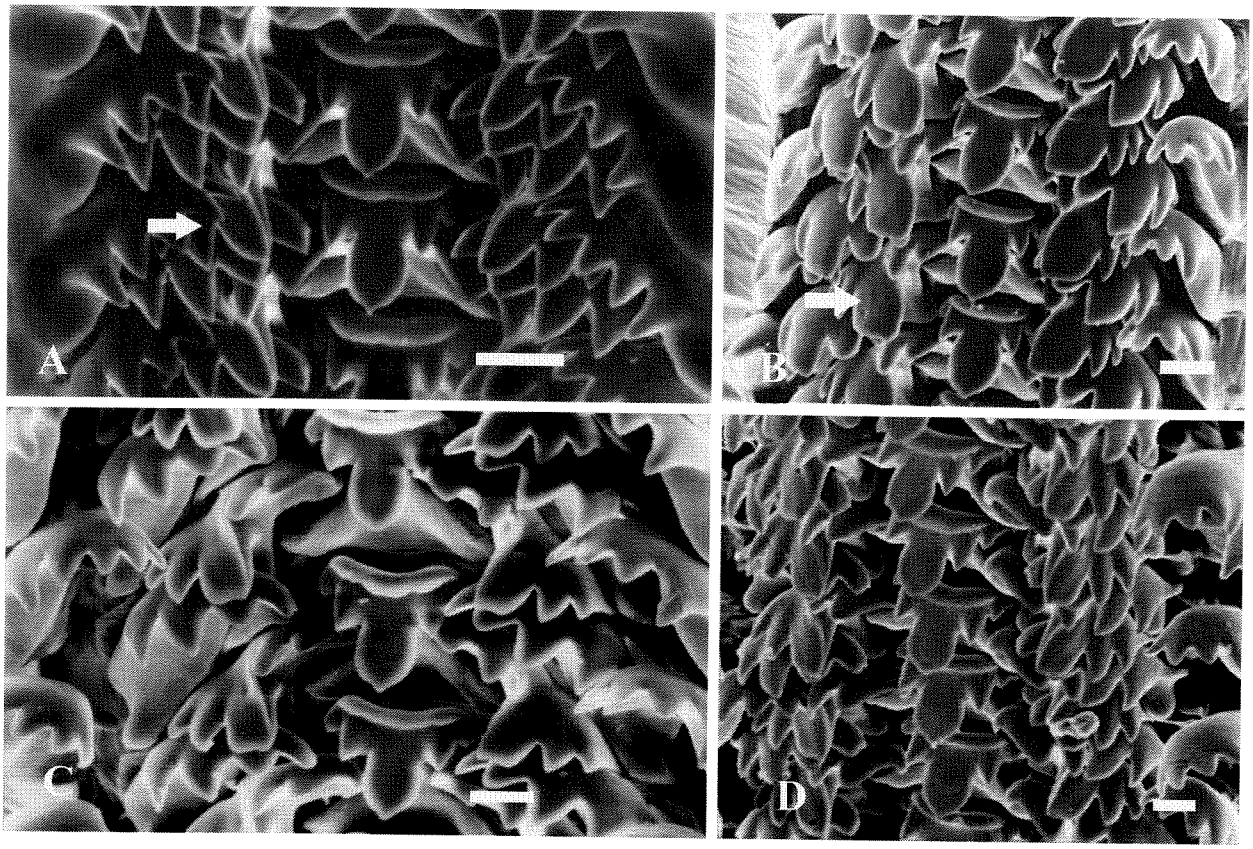

Figure 1. Radulae of *Littoraria angulifera*. A, Rocky shore (shell height=14.6 mm), lateral teeth subequal (arrowed), at 45° from front; B, Rocky shore (shell height=6.7 mm), larger lateral tooth (arrowed), at 45° from side; C, Mangrove, on *Spartina* (shell height=10.9 mm), at 45° from front; D, Mangrove, on *Laguncularia racemosa* (shell height=17.0 mm), at 45° from side. Scale bars = 10 μm.

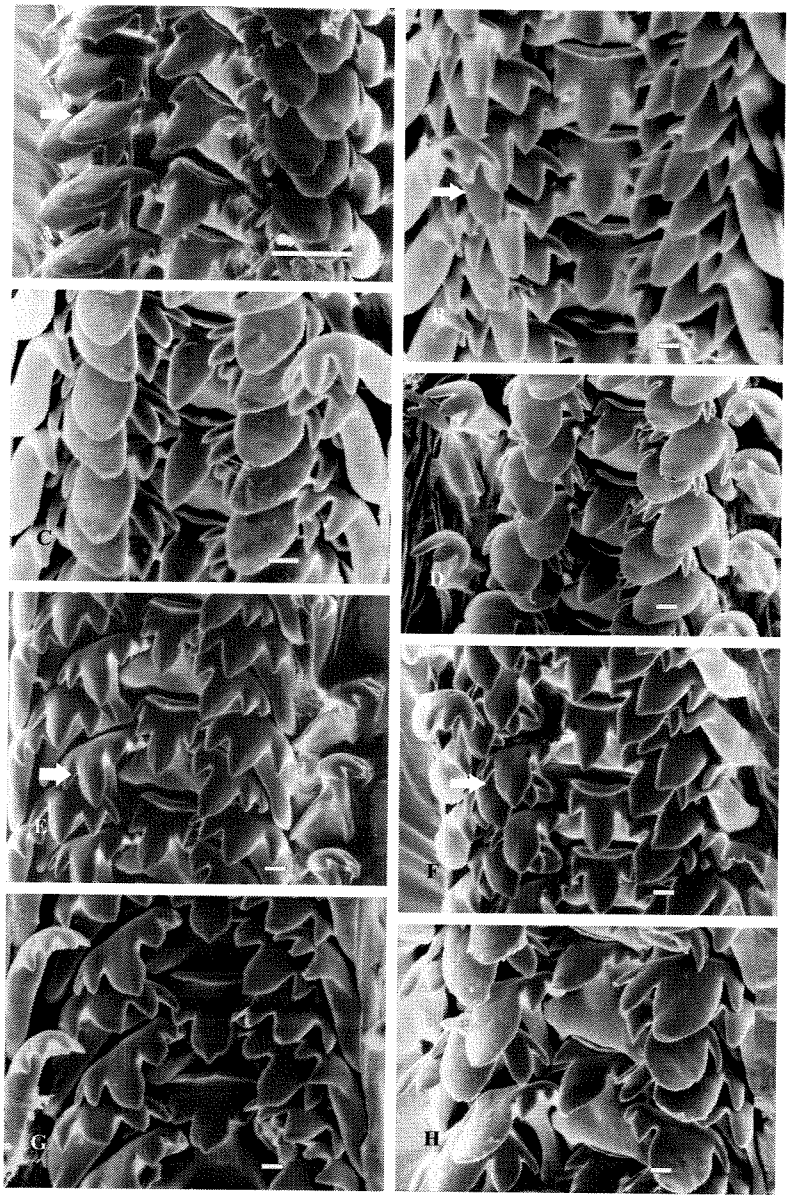

Figure 2. Radulae of *Littoraria flava.* A, Rocky shore on point 64 m (shell height=18.3 mm), blunt cusps (arrowed), at 45° from side; B, Mangrove, on *Laguncularia racemosa* (shell height=14.6 mm), subequal cusps (arrowed) and at 45° from front; C, Rocky shore on point 8 m (shell height=5.5 mm), at 45° from side; D, Rocky shore – control (shell height=14.5 mm), at 45° from side; E, Rocky shore – transferred, major cusps subequal, arrowed (shell height=13.5 mm), at 45° from side; F, Rocky shore – transferred, major cusps large-elongate, arrowed (shell height=12.7 mm), at 45° from front; G, Mangrove – control (shell height=14.3 mm), at 45° from front; H, Mangrove – transferred (shell height=10.9 mm), at 45° from side. Scale bars = 100 μm for A and 10 μm for B, C, D, E, F, G, and H.

CONSIDERAÇÕES FINAIS

- Foram encontrados desvios das proporções esperadas pelo equilíbrio de Hardy-Weinberg em todas amostras nas três espécies de litorinídeos, sempre no sentido de deficiência de heterozigotos. A fim de tentar encontrar um possível padrão nesses desvios foi realizado um teste de homogeneidade tratando os valores de F_{IS} obtidos por loco e população como coeficientes de correlação. Dentro de cada população, os resultados mostraram que a maioria dos locos apresenta heterogeneidade de valores de F_{IS} dentro de cada amostra, indicando que os desvios são característicos ou particulares de cada loco. Esse resultado exclui a possibilidade de endogamia nas populações estudadas, já que esse processo levaria a desvios uniformes em todos os locos.

- Em cada espécie, a maioria dos locos apresenta valores heterogêneos de F_{IS} entre as populações. Isso é esperado, dado que vários fatores devem estar atuando sobre as freqüências genotípicas dentro de cada loco, levando aos desvios observados nos diferentes locais amostrados e em diferentes épocas de coleta. No entanto, alguns locos apresentaram homogeneidade nos valores de F_{IS}. Os locos Pgi e Mdh em *E. lineolata*, por exemplo, apresentam valores homogêneos iguais ou muito próximos de zero. Outros locos, porém, apresentam valores altos e homogêneos, como os locos Aat-1 e Pla em *L. angulifera*, indicando que nos locais amostrados nenhum ou poucos indivíduos heterozigóticos foram observados.

- As três espécies apresentaram valores homogêneos de F_{IS} em todas as populações para pelo menos um loco do sistema enzimático PGM. Esse valor variou entre 0,21 (Pgm-3 em *L. flava*) a 0,46 (Pgm-2, em *L. angulifera*). Não foram encontrados estudos com resultados semelhantes. O mecanismo que explicaria esse padrão marcante nas três espécies não foi esclarecido, mas parece pouco provável que um fator estocástico possa ser o responsável pela homogeneidade nos locos da PGM na escala taxonômica, espacial e temporal avaliada.

- No capítulo dois, o desenho amostral planejado permitiu uma análise hierárquica de estruturação genética entre amostras de *L. flava* para testar a hipótese de que a diferenciação genética pode ocorrer em uma escala menor que a esperada para uma espécie com estágio larval planctotrófico. As amostras dentro de transectos (que variaram entre 60 e 300 mts, dependendo da praia) apresentaram maior estruturação genética que entre as três praias e entre as coletas em diferentes épocas do ano. As freqüências alélicas também variaram significativamente na maioria dos locos, sem padrão definido.

- Os dados da composição de espécies no costão rochoso mostraram um resultado consistente com os dados de estruturação genética. Houve uma maior diferença entre pontos extremos do mesmo transecto do que entre praias ou diferentes épocas de coleta. Se considerarmos que a composição de espécies é parte da caracterização do ambiente e, conseqüentemente, reflete a heterogeneidade ambiental, pode-se dizer que a estruturação genética entre as amostras parece condizente com esse padrão.

- De maneira geral, os estimadores de variabilidade encontrados nas amostras de *L. flava* foram semelhantes ao observado em estudo anterior. Ao contrário do que era esperado, as amostras e locos apresentam F_{IS} altos e significativos, mesmo na escala amostral utilizada. *Littoraria flava* não é o único litorinídeo da costa brasileira que apresenta deficiência de heterozigotos em pequena escala. O mesmo desenho amostral foi aplicado em *E. lineolata*, que também apresentou grandes desvios de Hardy-Weinberg no sentido de deficiência de heterozigotos; no entanto, não foi observada estruturação significativa em todos os níveis hierárquicos (dados em fase de análise).

- Foi feita também a análise de homogeneidade de F_{IS} que mostrou que cerca de metade dos locos com valores de F_{IS} maiores que 0,15 são homogêneos ao longo do transecto. Essa homogeneidade é perdida quando todas as 63 amostras foram analisadas, sugerindo que cada praia apresenta um padrão particular. Novamente, o loco Pgm-2 apresentou homogeneidade nos valores de F_{IS} na maioria dos transectos analisados (sete em oito transectos). Este resultado fortalece a hipótese de que esse sistema enzimático pode estar sob seleção ou ligado a algum gene sob o efeito da seleção natural.

- O teste de neutralidade mostrou um padrão comum a todos os locos analisados: um alelo muito freqüente e vários alelos raros. Esse padrão pode ser causado por seleção direcional sobre mutações levemente deletérias ("nearly neutral theory") assim como por eventos históricos, tais como expansões populacionais rápidas após colonizações recorrentes. Essa última explicação parece bastante provável já que era muito comum a

presença de juvenis durante as coletas em várias épocas do ano (observações pessoais), o que sugere recrutamento constante no costão rochoso.

- A formação de grupos de indivíduos com freqüências genotípicas diferentes no costão, a poucos metros de distância, parece ser evidente em *L. flava*. Várias hipóteses podem explicar como esses grupos são formados e mantidos. A não sincronização durante o período de recrutamento é um fenômeno bastante comum e documentado na literatura. Colonizações constantes, de grupos de recrutas com diferentes freqüências gênicas, seguidas de "efeito gargalo" de curta duração, poderiam também explicar os resultados do teste de neutralidade. No entanto, esse processo resultaria em estruturação e desvio de Hardy-Weinberg passageiros, que seriam atenuados em pouco tempo devido ao alto número de indivíduos e à grande capacidade reprodutiva e de dispersão.

- A variância no sucesso reprodutivo também seria uma explicação possível para a deficiência de heterozigotos em tantos locos e amostras. Porém, a alta variabilidade observada nas amostras de *L. flava* não parece compatível com o que seria previsto para populações com tamanhos efetivos pequenos e que, conseqüentemente, estariam sob o efeito de deriva genética. Um balanço do efeito dos eventos históricos (colonizações recorrentes) e de seleção com intensidade fraca e flutuante sobre vários locos e amostras pode ser uma explicação plausível para os padrões apresentados em populações de *L. flava*.

- *Littoraria angulifera* e *L. flava* apresentaram respostas diferentes quanto à forma da rádula em relação ao ambiente. *Littoraria angulifera* apresentou menor variação na

forma das cúspides entre ambientes que *L. flava*. Se há ação do ambiente sobre a forma deste caráter, seu efeito sobre as duas espécies é diferente. Esse resultado é diferente do que foi observado em trabalhos anteriores com *L. angulifera*, onde amostras coletadas no mangue diferiam das do costão. Além disso, observou-se variação na forma das cúspides em espécimes menores de *L. angulifera* apenas no costão, o que indica que a forma da rádula parece ser, pelo menos parcialmente, induzida pelo ambiente ocupado.

- As amostras de *Littoraria flava* apresentaram a variação como já descrita na literatura: indivíduos do costão apresentam cúspides mais arredondadas do que os do mangue. Ainda assim, as amostras não apresentaram tanta variação no número de cúspides e na forma do dente central, como já observado em outros locais. Os indivíduos menores, provavelmente juvenis, apresentaram cúspides com formato diferentes em relação aos espécimes maiores no mangue e no costão.

- Os resultados do experimento de transplante natural entre ambientes evidenciaram que algumas características da rádula podem ser plásticas. Todos os indivíduos coletados no mangue e analisados por microscopia eletrônica de varredura (MEV) apresentaram mudança na forma das cúspides. Já os indivíduos transplantados do costão para o mangue apresentaram um padrão mais complexo de resposta: alguns apresentaram alteração consistente com o esperado, enquanto outros apresentaram uma forma de cúspide intermediária entre a forma típica de costão (cúspides arredondadas) e a forma típica de mangue (cúspides pontudas e estreitas). As explicações possíveis são: (1) o caráter é plástico e a velocidade de resposta varia entre os indivíduos, (2) há variação

genética intrapopulacional quanto à resposta às mudanças do ambiente, ou seja, apenas alguns indivíduos na população respondem às mudanças de substrato e/ou dieta.

- Os resultados da análise morfológica da rádula mostram que há efeito ambiental sobre a morfologia da rádula. Este efeito pode variar entre indivíduos de uma mesma espécie e entre espécies que ocupam a mesma amplitude de condições de habitat.

Apêndice 1

Patterns of genetic variability in Brazilian littorinids (Mollusca): a macrogeographic approach

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Apêndice 1

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Patterns of genetic variability in Brazilian Littorinids (Mollusca): a macrogeographic approach

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Abstract

Macrogeographic studies are important for understanding gene flow patterns, and comparative data for related species with distinct bionomical traits may help to clarify the importance of such traits in natural populations. The aims of this study were to quantify the genetic variability and the populational structuring of three Brazilian littorinid species (*Nodilittorina lineolata*, *Littoraria flava* and *L. angulifera*) and to discuss the relationship between them, as well as each species' mode of development and spatial distribution. We also investigated the species diversity in the *ziczac* complex. Isozyme analyses were done on 20 samples of *N. lineolata*, nine of *L. flava* and 10 of *L. angulifera*, collected along 4000 km of the Brazilian coast. Sixteen polymorphic loci were analysed in *N. lineolata*, 15 in *L. angulifera* and 17 in *L. flava*. All species showed high genetic variability. At sites where more than one species was present, there was a correlation among the values of gene diversity.The degree of interpopulational differentiation (*N. lineolata*, $F_{ST} = 0.028$; *L. flava*, $F_{ST} = 0.054$; *L. angulifera*, $F_{ST} = 0.185$) was coherent with the mode of larval development of each species. No linkage disequilibrium was found in *N. lineolata*. These findings, together with morphological evidence, corroborated the existence of only one species of the *ziczac* complex along the Brazilian coast.

Key words: Littorinidae – gene flow – genetic differentiation – environmental heterogeneity

Introduction

The frequencies and distribution of genotypes in a natural population are ascribed to the population's size, breeding patterns, gene flow, mutation rates, natural selection and their interaction. Bionomical traits may influence one or more of these factors. Comparative studies of the variability and patterns of genetic structuring in different species may help in understanding the evolutionary mechanisms acting in natural populations. Phylogenetically related species with distinct dispersal potentials have often been studied to address the questions of variability and genetic structuring. In some species, the patterns of genetic differentiation are coherent with the species' dispersal mode: direct developing species usually have greater population structuring than species with planktotrophic larvae (Berger 1973; Janson 1987; Behrens Yamada 1989; Todd et al. 1998; Bohonak 1999). However, some authors argue against the relative importance of the type of development on gene flow by asserting that there is no strong evidence to support this association (Burton 1983; Hedgecock 1986; McQuaid 1996). Moreover, there is no evidence that a longer planktonic phase diminishes the genetic differentiation among populations (Burton 1983; Bohonak 1999). Hedgecock (1986) pointed out that although dispersal was indeed greater in planktotrophic species, gene flow could make distant populations genetically homogeneous only if the selective pressures were the same over the species distribution.

The Brazilian coast is about 8000 km long, and consists of sheltered and exposed rocky shores, sandy beaches and mangroves. Only a few species of the family Littorinidae occur along this coast and belong to one of two genera: *Nodilittorina* Martens 1897 and *Littoraria* Griffith and Pidgeon 1834 (Rios 1994).

Nodilittorina is the biggest littorinid genus, occurring in tropical and subtropical regions (Bandel and Kadolsky 1982; McQuaid 1996). *Nodilittorina vermeiji* occurs in Brazil, as do possibly three species of the *ziczac* complex (Borkowski and Borkowski 1969; Bandel 1974). This species complex is found on rocky shores from Florida (USA) to Uruguay. Recent studies have indicated that just one species of the *ziczac* complex, *N. lineolata*, occurs in Brazil and Uruguay and is restricted to these countries (Bandel and Kadolsky 1982; D.G. Reid, personal communication 1999).

The genus *Littoraria* is restricted to tropical zones, with two species in Brazil: *L. flava* (King and Broderip 1832) and *L. angulifera* (Lamarck 1822). *Littoraria angulifera* occurs in mangroves, but may sometimes be found on rocky shores; *L. flava* occupies rocky shores near to freshwater, but may be found in mangroves (Gallagher and Reid 1974; Reid 1986; personal observations).

All of these molluscs have planktotrophic larvae (Gallagher and Reid 1974; Mileikovsky 1975; Bandel and Kadolsky 1982; Reid 1999) and are oviparous, except for *L. angulifera*, which has incomplete ovoviviparity and retains the eggs until the swimming veliger stage (Bandel 1974; Gallagher and Reid 1974).

In Littorinidae, isozyme electrophoresis has provided information about intra- and inter-specific differentiation (Ward and Warwick 1980; Berger 1983; Janson 1985a; Ward 1990; Boulding et al. 1993; Zaslavskaya 1995).

In this study, the amount and distribution of genetic variability in all species of Littorinidae along the Brazilian coast, except for *N. vermeiji*, were investigated. The possible presence of other species of the *ziczac* complex were also checked, apart from *N. lineolata*.

Materials and methods

Sampling and identification

Twenty-one localities, covering about 4000 km of the Brazilian coast (Fig. 1), were sampled between May 1996 and January 2000. Twenty samples of *N. lineolata*, nine of *L. flava* and 10 of *L. angulifera* were collected; some sites were sampled more than once (Table 1). Approximately 100 individuals were usually collected per sample.

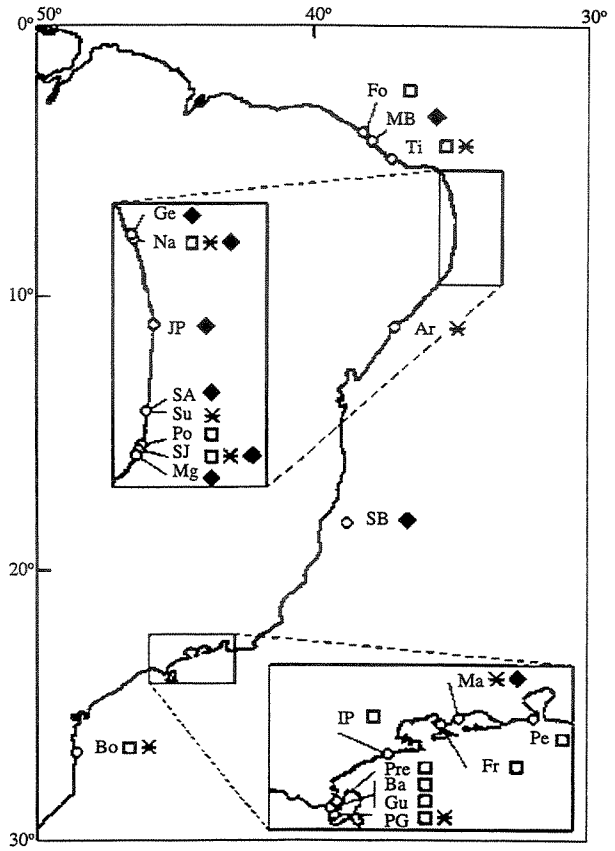

Fig. 1. Outline of Brazilian coast, showing sampling sites for *Nodlittorina lineolata* (□), *Littoraraia flava* (✱) and *L. angulifera* (◆)

although some samples were smaller because of the low abundance of *L. flava* and *L. angulifera* at some sites.

All *L. angulifera* specimens were collected in mangroves, except those from Santa Barbara Island and Natal, which came from rocky shores. *N. lineolata* was collected in the mid-intertidal and supralittoral zones. *L. flava* was found only in the latter zone, frequently close to estuaries or brackish water.

The periwinkles were transported alive to the laboratory and frozen in liquid nitrogen. Fifteen individuals from four samples of *N. lineolata* (Tibau, Guaecá, Preta and Barequeçaba, collected in 1996) were fixed in alcohol and sent to Dr David G. Reid at the British Museum of Natural History, for confirmation of species identification.

Electrophoresis

A total of 1109 individuals of *N. lineolata*, 239 of *L. flava* and 271 *of L. angulifera* were analysed. The entire soft body was squashed in extraction buffer (0.1 M Tris pH 8.0, 0.5% β-mercaptoethanol). The extracts were blotted onto Whatman no. 3 paper wicks and loaded on to 8.5% starch gels (hydrolysed potato starch, Sigma, St. Louis, MO, USA). Three buffer systems were used: (1) electrode: 0.3 M boric acid, 60 mM NaOH, pH 8.0, and gel: 10 mM Tris, pH 8.5 (Shaw and Prasad 1970, with modifications); (2) electrode: 0.04 M citric acid and N (3 aminopropyl) morpholine adjusted to pH 6.1, and gel: electrode solution diluted 1:20 (Clayton and Tetriak 1972); (3) electrode: 0.25 M Tris and 0.057 M citric acid, pH 8.0, and gel: electrode solution diluted 1:25 (Ward and Warwick 1980).

Thirty-one enzyme systems were tested, and resolution was obtained with 14 systems for *N. lineolata*, and with 12 for *L. flava* and *L. angulifera* (Table 2). Staining procedures were adapted from Shaw and Prasad (1970), Soltis and Soltis (1989) and Alfenas (1991), with modifications (the recipes can be provided on request).

Data analysis

Alleles were identified by their mobility relative to the most common allele of each genus. The BIOSYS-1 program (Swofford and Selander 1981) was used to estimate the genetic variability, namely, the percentage of polymorphic loci (P, 95% criterion), the mean number of alleles per locus (Ap), and the gene diversity (H_e) per locus and sample (unbiased estimate; Nei 1978). This program was also used to calculate distance matrices and genetic similarity (Nei 1972). A UPGMA ('unweighted pairgroup method with arithmetic means') cluster analysis was obtained based on genetic distances (Nei 1972).

The F_{ST} coefficient was calculated using the Weir and Cockerham (1984) parameter (θ). This is the most suitable for these populations, which fit the 'random populations' model (Weir 1996). Ninety-five per cent confidence intervals were determined for the total F_{ST} value by bootstrapping 10 000 times across the loci using the GDA program (Lewis and Zaykin 1999).

Geographical distances and F_{ST} were obtained for each pair of *L. angulifera* samples. The two matrices were compared by the Mantel test using the TFPGA program (Miller 1997). The GENETIX v.4.02 program (Belkhir 2001) uses random permutations on the matrix of individuals versus genotypes to find the expected distribution of F_{ST} under the null hypothesis ($F_{ST} = 0$). The probability that a value larger than or equal to the estimated occurs was calculated by $p = (n + 1)/(N + 1)$, where n is the number of pseudo-values larger than or equal to the estimate and N is the number of random permutations (Sokal and Rohlf 1995). As multiple tests enhance type 1 errors, the sequential Bonferroni procedure (Rice 1989) was used.

To check whether the studied samples consisted of more than one species of the *ziczac* complex, genotypic data were tested for linkage disequilibrium using the GENEPOP program (Raymond and Rousset 1995a,b). The default program, 100 batches of 1000 iterations per batch with 1000 dememorization steps, was used. Again, the sequential Bonferroni procedure was applied.

In some cases, more than one species was sampled at the same locality (Table 1). As the values of H_e followed a normal distribution, Pearson correlation analysis was used to compare the gene diversity (H_e) of *L. flava* with *N. lineolata* and of *L. flava* with *L. angulifera* at such sites (*N. lineolata* and *L. angulifera* overlapped at only two sites). In the case of more than one sample from the same locality, those collected in the same year or in near years were chosen. A miscellaneous method for combining probabilities, as developed by Fisher (1954, in Sokal and Rohlf 1995), was then applied. According to Sokal and Rohlf (1995), this analysis is appropriate when 'separate significance tests on different sets of data may test the same scientific (but not statistical) hypothesis'. With this analysis, correlations between *L. flava* gene diversity and the other two species, and hence, an association among these species estimates were tested. Such an association could point to a possible environmental effect on genetic variability.

Results

Nodilittorina lineolata

All individuals analysed by Dr D. G. Reid (personal communication) belonged to *N. lineolata*. The occurrence of only one species was also supported by linkage disequilibrium analysis: none of the loci combinations out of 1055 tests were significant.

Two of 18 loci scored were monomorphic (*Mdh*-1 and *Idh*-1). The percentage of polymorphic loci ranged from 11.1% (Nl Pre97) to 72.2% (Nl Pre96), with an average of 45% (Table 3). *Pgm*-1 and *Lap*-1 were the most polymorphic, with 10 alleles each (Table 2).

There was high gene diversity, the average for populations and the species being 0.155 and 0.190, respectively; the values ranged from 0.042 (Nl Pre97) to 0.244 (Nl IP).

The samples studied had a low genetic structure (F_{ST} = 0.028, 95% CI = 0.016–0.043). The genetic distances (D) ranged from 0 (Nl Ba96 versus Nl SJ and Nl Ba98 versus Nl

Table 1. Localities sampled for *Littoraria flava*, *Littoraria angulifera* and *Nodilittorina lineolata*

Species	N^1	Abbr.[2]	Locality	Collection date	Lat/long
L. flava	34	Fl Ti	Tibau	Jul/1999	4°48′S/37°15′E
	21	Fl Na	Natal	Nov/1999	5°48′S/35°10′E
	40	Fl Su	Suape	Oct/1999	8°28′S/34°57′E
	17	Fl SJ97	S. José da Coroa Grande	Jan/1997	8°54′S/35°8′E
	24	Fl SJ00		Jan/2000	
	35	Fl Ar	Aracaju	Jul/1997	10°55′S/37°2′E
	48	Fl Ma	Mangaratiba	Sept/1998	22°56′S/44°4′E
	10	Fl PG	Praia Grande (Ilhabela)	Jun/1998	23°52′S/45°25′E
	10	Fl Bo	Bombas	Oct/1998	27°8′S/48°29′E
L. angulifera	17	An MB	Morro Branco	Sept/1997	4°9′S/38°7′E
	24	An Ge	Genipabu	Feb/1998	5°41′S/35°12′E
	12	An Na	Natal	Nov/1999	5°48′S/35°10′E
	33	An JP	João Pessoa	Aug/1997	7°8′S/34°57′E
	13	An SA	Cabo de Sto. Agostinho	May/1997	8°21′S/34°57′E
	20	An SJ97	S. José da Coroa Grande	Jan/1997	8°54′S/35°8′E
	25	An SJ00		Jan/2000	
	41	An Mg	Maragoji	Nov/1999	9°1′S/35°13′E
	41	An SB	Abrolhos (Sta. Bárbara Is.)	Oct/1998	18°21′S/38°36′E
	45	An Ma	Mangaratiba	Sept/1998	22°56′S/44°4′E
N. lineolata	57	Nl Fo	Fortaleza	Jul/1998	3°43′S/38°30′E
	94	Nl Ti96	Tibau	Aug/1996	4°48′S/37°15′E
	69	Nl Ti97		Feb/1997	
	30	Nl Na	Natal	Apr/1997	5°48′S/35°10′E
	64	Nl Po	Porto (Barreiro)	Jan/1997	8°49′S/35°7′E
	61	Nl SJ	S. José da Coroa Grande	Jan/1997	8°54′S/35°8′E
	50	Nl Pe	Pepino	Jan/1998	22°56′S/43°16′E
	67	Nl Fr	Frade	Aug/1997	23°00′S/44°16′E
	51	Nl IP	Ilha dos Porcos Pequena	Feb/1999	23°23′S/44°53′E
	89	Nl Pre96	Preta	Nov/1996	23°48′S/45°23′E
	53	Nl Pre97	(S. Sebastião)	Jun/1997	
	31	Nl Pre98		Jun/1998	
	78	Nl Ba96	Barequeçaba	Nov/1996	23°50′S/45°26′E
	19	Nl Ba98	(São Sebastião)	Jun/1998	
	52	Nl Gu96	Guaecá	May/1996	23°50′S/45°27′E
	58	Nl Gu3/97	(S. Sebastião)	Mar/1997	
	45	Nl Gu6/97		Jun/1997	
	45	Nl PG97	Praia Grande	Feb/1997	23°52′S/45°25′E
	36	Nl PG98	(S. Sebastião, IlhaBela)	Jun/1998	
	60	Nl Bo	Bombas	Oct/1998	27°8′S/48°29′E

[1]Sample size used in gels.
[2]Abbreviation of localities.

Ti96) to 0.132 (Nl Gu96 versus Nl Ti97), with an average of 0.023 (Fig. 2).

Littoraria flava

The mean locus variability was high (H_e = 0.160, Table 2). The gene diversity of the samples was also high (mean H_e = 0.172), ranging from 0.104 (sample Fl Ar) to 0.235 (sample Fl Na). The mean percentage of polymorphic loci was high (48.9%), and sample Fl Na was the most polymorphic (68.4%). Excluding *Mdh*-1 and *Mdh*-2, all loci were polymorphic (95% criterion). The most polymorphic ones were *Pgm*-2 and *Gpi*, with 13 and 12 alleles, respectively.

There was low to moderate genetic differentiation (F_{ST} = 0.054, 95% CI = 0.025–0.084). The mean genetic distance (Nei 1972) was low (D = 0.022), and ranged from 0.007 (Fl Su versus Fl Ti) to 0.068 (Fl Na versus Fl PG) (Fig. 3).

Littoraria angulifera

This species had a mean frequency of polymorphic loci of 41.5% (Table 3), and the An Na sample (like that of *L. flava*) was the most polymorphic (68.4%). The *Pgm*-2 locus was the most polymorphic, with 16 alleles; the *Mdh*-1, *Mdh*-2, *Idh*-2 and *Ak*-2 loci were monomorphic (95% criterion).

The averages of H and H_e, for locus and gene diversity, were equal (0.180). As with *L. flava*, the *L. angulifera* sample collected in Natal had the greatest gene diversity (H_e = 0.274). The *Pgm*-2 and *Gpi* loci showed the greatest variability, with H = 0.726 and 0.646, respectively (Table 2).

The F_{ST} coefficient for all samples was high (F_{ST} = 0.185; 95% CI = 0.11–0.27). The correlation between geographic distance and genetic structuring was not significant (R = 0.2324, p = 0.24). The F_{ST} parameter ranged from 0.056 (An Ge versus An SB) to 0.394 (An MB versus An Ma). Geographical distances ranged from 12.8 to 2717 km. The S. José 1997 and S. José 2000 collections were probably made a few meters apart, but showed a high F_{ST} value (0.209). The F_{ST} estimates calculated for each pair of populations are shown in Table 4. All were significant, even when the Bonferroni correction was applied.

Among *L. angulifera* samples, the mean genetic distance (Nei 1972) was 0.103 and ranged from 0.024 (An Ge versus An SB) to 0.244 (An Ma versus An SJ97). The mean genetic distance among *Littoraria* species was very high (1.86) (Fig. 3).

Table 2. Enzyme systems and their respective variabilities

Enzyme system	E.C. number	Buffer	Locus	Heterozygosity[1]		
				Nodilittorina lineolata	*Littoraria flava*	*Littoraria angulifera*
Leucine aminopeptidase	3.4.11.1	(I)	*Lap*-1	0.530	0.026	–
			Lap-2	–	0.270	0.350
L-leucyl-L-glycylglycine peptidase	3.4.11	(I)	*Plgg*	0.222	0.129	0.242
L-leucyl-L-alanine peptidase	3.4.13	(I)	*Pla*	0.116	–	–
Pyrophosphatase	3.6.1.1	(I), (II)[2]	*Pp*	0.163	0.039	0.082
Aspartate aminotransaminase	2.6.1.1	(II)	*Aat*-1	–	0.030	0.113
			Aat-2	–	0.037	0.053
Lactate dehydrogenase	1.1.1.27	(II)	*Ldh*	0.058	–	–
Mannose phosphate isomerase	5.3.1.8	(II)	*Mpi*	0.023	0.039	0.150
Sorbitol dehydrogenase	1.1.1.14	(II)	*Sdh*-1	0.120	0.095	0.049
			Sdh-2	0.280	–	0.094
Malic enzyme (NADP)	1.1.1.40	(II)	*Me*	0.015	0.185	0.267
Phosphoglucose isomerase	5.3.1.9	(III)	*Gpi*	0.108	0.590	0.646
Phosphoglucomutase	2.7.5.1	(III)	*Pgm*-1	0.553	0.330	–
			Pgm-2	0.042	0.258	0.726
			Pgm-3	–	0.301	0.187
Malate dehydrogenase (NAD)	1.1.1.37	(III)	*Mdh*-1	0.007	0.003	0.008
			Mdh-2	–	0.007	0.008
Isocitric dehydrogenase	1.1.1.42	(III)	*Idh*-1	0.004	0.338	0.011
			Idh-2	–	–	0.016
Adenylate kinase	2.7.4.3	(III)	*Ak*-1	0.056	0.224	0.333
			Ak-2	0.221	0.021	0.008
			Ak-3	–	0.125	0.078
Hexokinase	2.7.1.1	(III)	*Hk*-1	0.112	–	–
			Hk-2	0.140	–	–

[1]Values obtained by calculation of the expected heterozygosity averages of all samples for locus and species (H).
[2]Buffer (I) for *Nodilittorina lineolata* and (II) for species of *Littoraria*. Locus not observed. Note that loci of *N. lineolata* do not correspond to those of other species.

Pearson correlation analysis between the H_e values of *L. flava* and *L. angulifera* was significant ($R^2 = 0.995$, p = 0.002) but that between *L. flava* and *N. lineolata* was not ($R^2 = 0.6355$, p = 0.106), although the tendency was similar to that of *L. flava* and *L. angulifera* (Fig. 4). Based on the miscellaneous method, the association tendency among values of heterozygosity was significant (p = 0.004). To test whether these results were due to similar population densities, correlation tests between sample size and gene diversity were performed. None of the tests was significant: for *L. angulifera*, $R^2 = 0.154$ (p = 0.607); for *N. lineolata*, $R^2 = 0.312$(p = 0.328); for *L.flava*,$R^2 = 0.0001$ (p = 0.985), and $R^2 = 0.025$ (p = 0.801) for sites shared with *L. angulifera* and *N. lineolata*, respectively.

Discussion

The *ziczac* complex along the Brazilian coast

An absence of diagnostic loci has frequently been reported among closely related species of Littorinidae (Ward and Warwick 1980; Mastro et al. 1982; Janson 1985a; Knight et al. 1987). Thus, the fact that no locus indicative of more than one species was detected does not necessarily mean that only a single species was dealt with. However, the absence of linkage disequilibrium in all *Nodilittorina* samples was evidence for only one species belonging to the *ziczac* complex

Table 3. Average genetic variability for the three littorinid species. *N*, mean sample size per locus. Ap, mean number of alleles per locus. P, % polymorphic loci (95% criterion). H_e, gene diversity (unbiased estimate, Nei 1978). Standard deviations are shown in parenthesis

Species	*N*	Ap	P	H_e
N. lineolata	30.0 (11.7)	2.7 (0.6)	45.0 (17.2)	0.155 (0.058)
Species level	600.7	6.2	61.1	0.190
L. flava	23.3 (11.9)	2.6 (0.8)	48.9 (11.1)	0.172 (0.039)
Species level	210.2	6.8	52.6	0.167
L. angulifera	23.7 (9.8)	2.4 (0.6)	41.5 (13.2)	0.180 (0.052)
Species level	236.8	5.9	47.4	0.226

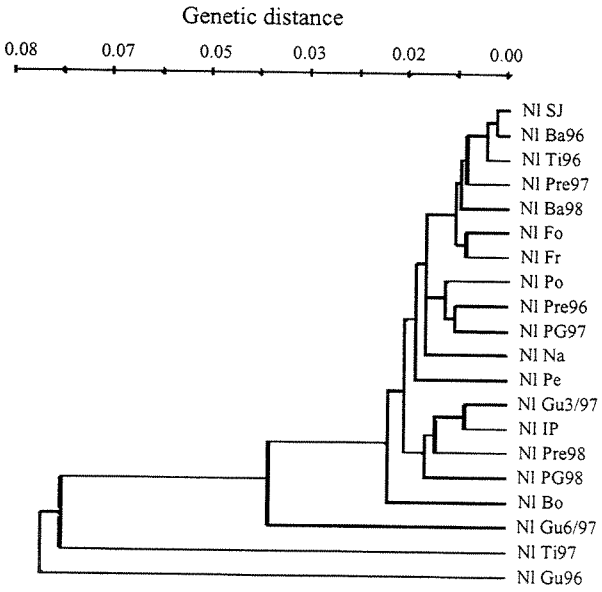

Fig. 2. UPGMA cluster analysis based on Nei's (1972) unbiased genetic distance for samples of *Nodilittorina lineolata* (cophenetic correlation = 0.957)

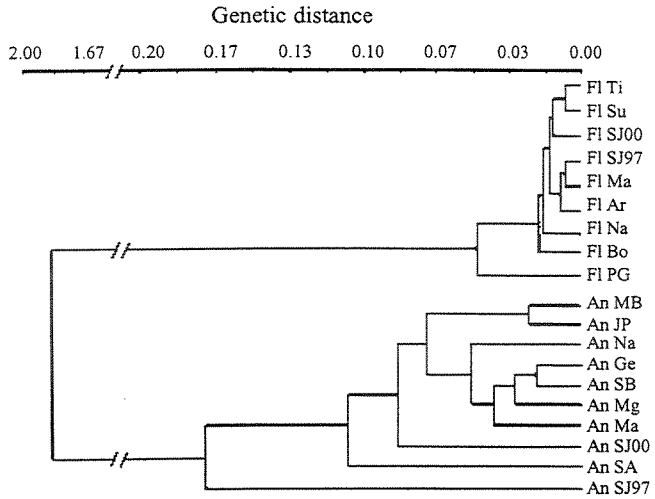

Fig. 3. UPGMA cluster analysis based on Nei's (1972) unbiased genetic distance for samples of *Littoraria flava* (identified as Fl) and *L. angulifera* (identified as An). Cophenetic correlation = 0.997

along the coast (linkage disequilibrium would be generated by preferential intraspecific crosses). This finding agrees with the morphological diagnosis by Dr Reid of only *N. lineolata* in Brazil, as proposed by Bandel and Kadolsky (1982).

Genetic distance and structuring

High dispersal rates have been related to low interpopulational differentiation in marine invertebrates. Indeed, *N. lineolata* and *L. flava* (both with planktotrophic larvae) showed low populational structuring and low mean intraspecific genetic distances (F_{ST} = 0.028 and 0.054, and D = 0.023 and 0.022, respectively). These results are very similar to those reported by Janson (1985a) for species of the *ziczac* complex. In contrast, *L. angulifera*, (incompletely ovoviviparous) had a higher mean genetic distance among its populations (D = 0.103) and a high degree of genetic structuring (F_{ST} = 0.185). Janson (1985b) found lower values of distance among Florida populations of *L. angulifera* (mean D = 0.014), but her samples were taken from a smaller geographical scale than ours.

There is no information on the duration of the planktonic stage of *N. lineolata* or *L. flava*, but it is probably long enough to promote gene flow between distant places, a conclusion reinforced by the fact that geographically distant samples shared the same rare alleles.

In addition to larval dispersal, the three species had different distributional patterns: *N. lineolata* had an almost continuous distribution, always with a great number of individuals. *L. flava* was more discontinuous because of its association with brackish environments and freshwater flows, and *L. angulifera*, which occurred in mangroves, was even more discontinuous. *Littoraria* species have smaller populations than *N. lineolata*. Thus, the different genetic structuring among these species probably represented a combination of gene flow (influenced by the continuity of each particular environment) and of their effective population sizes.

The lack of correlation between geographical distance and genetic differentiation (F_{ST}) in *L. angulifera* suggested random geographical dispersal of individuals. Hutchinson and Templeton (1999) suggested that this pattern was a consequence of strong genetic drift which overlapped the effects of gene flow, as may have occurred here.

The planktonic phase estimated for *L. angulifera* was 8–10 weeks (Gallagher and Reid 1974), which would be enough for efficient dispersal over long distances. Indeed, this species is found on both sides of the Atlantic (Reid 1986). In contrast, the discontinuity of mangroves along the coast means that *L. angulifera* larvae would be expected to remain at the same site as their parents, thus explaining the great divergence observed between sampling sites (Table 4).

Nodilittorina lineolata and *L. angulifera* samples collected from the same site on different occasions were not genetically closer than those from different sites (Figs 2 and 3). Thus, these species show spatial and temporal differentiation. Todd et al. (1998) also observed differences in the allele frequencies of *Acon*-2 locus in samples of *Adalaria proxima* taken from the same site in different years. These temporal differences may reflect the recruitment of different genotypes on different occasions. According to Borkowski (1971), species of the *ziczac* complex lay eggs for about 8 months during the year. If groups with similar genotypes lay eggs at the same time, there will be temporal differentiation among groups of recruits. This temporal pattern could be reinforced by microgeographical differences and result in differentiation among samples.

Interspecific genetic distances in *Littoraria*

Littoraria angulifera and *L. flava* belong to different subgenera (*Littorinopsis* and *Littoraria*, respectively). Nevertheless, only the *Mdh*-1 locus was diagnostic between them; *Pgm*-1 and *Lap*-1 were detected only in *L. flava*, whereas *Idh*-2 and *Sdh*-2 were detected only in *L. angulifera*. Both species shared alleles at the remaining loci.

The genetic distance between *L. angulifera* and *L. flava* was high (D = 1.86) and the genetic identity was 0.22. Likewise, Johnson and Black (1998) reported I = 0.26 between *Littorina sulculosa* and *L. cingulata*, which also belong to different

Table 4. F_{ST} estimates between pairs of *Littoraria angulifera* samples. All values were significantly different from zero (1000 pseudo-values obtained by permutations of genotypes in the total sample) with the significance level adjusted using the sequential Bonferroni procedure (Rice 1989)

	An Ge	An Na	An JP	An SA	An SJ97	An SJ00	An Mg	An SB	An Ma
An MB	0.335	0.255	0.059	0.331	0.351	0.349	0.129	0.251	0.394
An Ge		0.145	0.148	0.207	0.306	0.302	0.110	0.056	0.135
An Na			0.119	0.133	0.182	0.121	0.068	0.090	0.177
An JP				0.160	0.145	0.171	0.067	0.115	0.248
An SA					0.211	0.222	0.175	0.179	0.268
An SJ97						0.209	0.234	0.258	0.338
An SJ00							0.208	0.223	0.326
An Mg								0.061	0.166
An SB									0.085

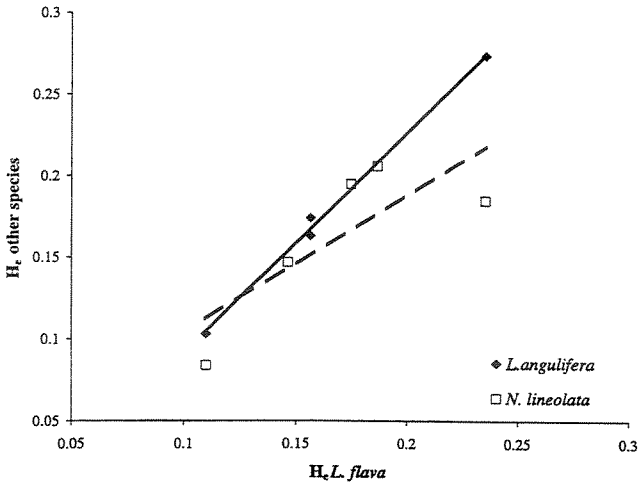

Fig. 4. Correlation among the gene diversity values for *Littoraria flava* and *Nodilittorina lineolata* (R^2 = 0.6355, p = 0.106) and *L. flava* and *L. angulifera* (R^2 = 0.995, p = 0.002)

subgenera, and Zaslavskaya (1995) found a similar distance between *Littorina kasatka* and *L. sitkana* (D = 2.087). However, Janson (1985b) calculated I = 0.178 between *L. angulifera* and *N. angustior*. The two *Littoraria* species studied here were genetically very divergent, but this may be a common pattern in Littorinidae.

Intraspecific genetic variability

The population genetics of the Littorinidae were reviewed by Ward (1990) and McQuaid (1996). The average of polymorphic loci (P) in the family is 35%, while the average gene diversity (H_e) is 0.15. Similar values of H_e and higher values of P in the samples of the three species (Table 3) were found. Janson (1985a,b) studied populations of the *ziczac* complex and of *L. angulifera* from Florida. The genetic variability (P and H_e) of *L. angulifera* populations from Brazil was very similar to that of North American populations. In contrast, *N. lineolata* had higher values of P (45%) than the Florida species of the *ziczac* complex (P = 34%).

The genetic variability in several species is correlated with environmental heterogeneity (Levene 1953; Levinton 1973; Levinton and Suchanek 1978; Nevo 1978; Mitchell-Olds 1992; for the Littorinidae, see Ward and Warwick 1980; Noy et al. 1987; McQuaid 1996). However, the patterns of genetic diversity may be explained by many factors, in addition to spatial heterogeneity. Bryant (1976) pointed out that the effects of temporal variation might improve the consequences of spatial variation in natural populations, and that it could be very difficult to separate these two components.

There was a correlation among the gene diversity (H_e) values of the different species (Fig. 4). Despite the low number of common sampling sites, the correlation was well supported. This may be indicative of the importance of local environmental conditions in the amount and distribution of gene diversity. Alternatively, the sampled sites may have similar features affecting both species in the same way. If the site is restricted, as in the case of an inlet for example, the dispersal capability could be limited. However, this would result in higher degrees of genetic structuring than that observed in *N. lineolata* and *L. flava*.

The influence of habitat conditions on isozyme variation is controversial (Hedrick 1986; Solé-Cava and Thorpe 1991; Prout and Savolainen 1996). Our data indicate that this relationship deserves detailed studies to determine which environmental features affect the distribution of genetic variability in the studied species here.

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Zusammenfassung

Muster genetischer Variabilität brasilianischer Littoriniden (Mollusca): Ein makrogeographischer Ansatz

Makrogeographische Studien sind wichtig für das Verständnis von Genflussmustern, und komparative Daten von verwandten Arten mit verschiedenen bionomischen Merkmalen können helfen, die Bedeutung solcher Merkmale in natürlichen Populationen aufzuklären. Das Ziel der vorliegenden Untersuchung war es, die genetische Variabilität und die Populationsstruktur dreier brasilianischer Littoriniden Arten (*Nodilittorina lineolata, Littoraria flava* und *Littoraria angulifera*) festzustellen, und die Beziehung zwischen diesen Abschätzungen und dem Entwicklungsmodus und der räumlichen Verteilung jeder Art zu diskutieren. Wir haben ausserdem das Vorhandensein weiterer Arten des *ziczac* Komplexes (abgesehen von *N. lineolata*) untersucht. Isoenzym-Analysen von 1619 Individuen von 20 Aufsammlungen von *N. lineolata*, neun von *L. flava* und 10 von *L. angulifera* wurden durchgeführt, basierend auf Material, gesammelt entlang 4000 km brasilanischer Küste. Alle Arten zeigten eine hohe genetische Variabilität, möglicherweise verursacht durch die Heterogenität der Habitate. An Lokalitäten, wo mehr als eine Art vertreten war, existiert eine Korrelation zwischen den Werten der genetischen Variabilität (H_e). Wir diskutieren dieses Ergebnis als einen möglichen Hinweis auf die Rolle des Lebensraums in der Verteilung genetischer Variabilität. Der Umfang der Differenzierung zwischen den Populationen (*N. lineolata*, F_{ST} = 0.028; *L. flava*, F_{ST} = 0.054; *L. angulifera*, F_{ST} = 0.185) war koherent mit dem Entwicklungsmodus. Wir fanden kein Kopplungsungleichgewicht bei *N. lineolata*; dies, zusammen mit morphologischen Daten, bestätigt die Existenz von nur einer Art des *ziczac* Komplexes entlang der brasilianischen Küste.

References

Alfenas, A. C., 1991: Eletroforese de Isozimas e Proteínas Afins. Edn. Minas Gerais: Universidade Federal de Vicosa.

Bandel, K., 1974: Studies on littorinidae from the Atlantic. Veliger **17**, 92–114.

Bandel, K.; Kadolsky, D., 1982: Western Atlantic species of *Nodilittorina* (Gastropoda: Prosobranchia): comparative morphology and its functional, ecological, phylogenetic and taxonomic implications. Veliger **25**, 1–42.

Behrens Yamada, S., 1989: Are direct developers more locally adapted than planktonic developers? Mar. Biol. **103**, 403–411.

Belkhir, K., 2001: GENETIX, logiciel sous WindowsTM pour la génétique des populations. Laboratoire Génome et Populations, CNRS UPR 9060. Montpellier (France): Université de Montpellier II.

Berger, E. M., 1973: Gene-enzyme variation in three sympatric species of Littorina. Biol. Bull. **145**, 83–90.

Berger, E. M., 1983: Chap. 13: Population genetics of marine gastropods and bivalves. In: The Mollusca, vol. 6 Ecology. W. D. Russel-Hunter (ed.) New York, NY: Academic Press, pp. 563–596.

Bohonak, A. J., 1999: Dispersal, gene flow, and population structure. Q. Rev. Biol. **74,** 21–45.

Borkowski, T. V., 1971: Reproduction and reproductive periodicities of South Floridian Littorinidae. B. Mar. Sci. **21,** 826–840.

Borkowski, T. V.; Borkowski, M. R., 1969: The Littorina ziczac species complex. Veliger **11,** 408–415.

Boulding, E. G.; Buckland-Nicks, J.; Van Alstyne, K.L., 1993: Morphological and allozyme variation in *Littorina sitkana* and related Littorina species from the northeastern Pacific. Veliger **36,** 43–68.

Bryant, E. H., 1976: A comment on the role of environmental variation in maintaining polymorphisms in natural populations. Evolution **30,** 188–190.

Burton, R. S., 1983: Protein polymorphisms and genetic differentiation of marine invertebrate populations. Mar. Biol. Lett. **4,** 193–206.

Clayton, J.; Tetriak, D., 1972: Amine-citrate buffers for pH control in starch gel electrophoresis. J. Fish. Res. Bd. Can. **26,** 1169–1172.

Fisher, R. A., 1954: Statistical Methods for Research Workers, 12th edn. Edinburgh: Oliver & Boyd.

Gallagher, S. B.; Reid, G. K., 1974: Reproductive behavior and early development in *Littorina scabra angulifera* and *Littorina irrorata* (Gastropoda: Prosobranchia) in the Tampa Bay region of Florida. Malacol. Rev. **7,** 105–125.

Hedgecock, D., 1986: Is gene flow from pelagic larval dispersal important in the adaptation and evolution of marine invertebrates? B. Mar. Sci. **39,** 550–564.

Hedrick, P. H., 1986: Genetic polymorphism in heterogeneous environments: a decade later. Annu. Rev. Ecol. Syst. **17,** 535–566.

Hutchinson, D. W.; Templeton, A. R., 1999: Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variabilidade. Evolution **53,** 1898–1914.

Janson, K., 1985a: Genetic variation in three species of Caribbean periwinkles, *Littorina angustior*, *L. lineolata* and *L. ziczac* (Gastropoda: Prosobranchia). B. Mar. Sci. **37,** 871–879.

Janson, K., 1985b: Genetic and morphologic variation within and between populations of *Littorina angulifera* from Florida. Ophelia **24,** 125–134.

Janson, K., 1987: Allozyme and shell variation in two marine snails (*Littorina*, Prosobranchia) with different dispersal abilities. Biol. J. Linn. Soc. **30,** 245–256.

Johnson, M. S.; Black, R., 1998: Effects of isolation by distance and geographical discontinuity on genetic subdivision of *Littoraria cingulata*. Mar. Biol. **132,** 295–303.

Knight, A.J.; Hughes, R.N.; Ward, R.D., 1987: A striking example of the founder effect in the mollusc *Littorina saxatilis*. Biol. J. Linn. Soc. **32,** 417–426.

Levene, H., 1953: Genetic equilibrium when more than one ecological niche is available. Am. Nat. **87,** 331–333.

Levinton, J., 1973: Genetic variation in a gradient of environmental variability: marine bivalvia (Mollusca). Science **180,** 75–76.

Levinton, J. S.; Suchanek, T. H., 1978: Geographic variation, niche breadth and genetic differentiation at different geographic scales in the mussels. *Mytilus californianus* and *M. edulis*. Mar. Biol. **49,** 363–375.

Lewis, P. O.; Zaykin, D., 1999: Genetic data analysis: computer program for the analysis of allelic data, version 1.0 (d12). Free program distributed by the authors over the internet from the GDA Home Page at http://chee.unm.edu/gda/

Mastro, E.; Chow, V.; Hedgecock, D., 1982: *Littorina scutulata* and *Littorina plena*: sibling species status of two prosobranch gastropod species confirmed by electrophoresis. Veliger **24,** 239–246.

McQuaid, C. D., 1996: Biology of the gastropod family Littorinidae. I. Evolutionary aspects. Oceanogr. Mar. Biol. **34,** 233–262.

Mileikovsky, S. A., 1975: Types of larval development in Littorinidae (Gastropoda: Prosobranchia) of the world ocean, and ecological patterns of their distribution. Mar. Biol. **30,** 129–135.

Miller, M. P., 1997: Tools for population genetic analyses (TFPGA) 1.3: a Windows program for the analysis of allozyme and molecular population genetic data. Computer software distributed by author.

Mitchell-Olds, T., 1992: Does environmental variation maintain genetic variation? A question of scale. Trends Ecol. Evol. **7,** 397–398.

Nei, M., 1972: Genetic distance between populations. Am. Nat. **106,** 283–293.

Nei, M., 1978: Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics **89,** 583–590.

Nevo, E., 1978: Genetic variation in natural populations: patterns and theory. Theor. Popul. Biol. **13,** 127–177.

Noy, R.; Lavie, B.; Nevo, E., 1987: The niche-width variation hypothesis revisited: genetic diversity in the marine gastropods *Littorina punctata* (Gmelin) and *L. neritoides* (L.). J. Exp. Mar. Biol. Ecol. **109,** 109–135.

Prout, T.; Savolainen, O., 1996: Genotype-by-environment interaction is not sufficient to maintain variation: Levene and the leafhopper. Am. Nat. **148,** 930–936.

Raymond, M.; Rousset, F., 1995a: GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. **86,** 248–249.

Raymond, M.; Rousset, F., 1995b: An exact test for population differentiation. Evolution **49,** 1280–1283.

Reid, D. G., 1986: The littorinid molluscs of mangrove forests in the Indo-Pacific region. The genus Littoraria. British Museum (Natural History), London.

Reid, D. G., 1999: The genus Littoraria Griffith & Pidgeon, 1834 (Gastropoda: Littorinidae) in tropical eastern Pacific. Veliger **42,** 21–53.

Rice, W. R., 1989: Analyzing tables of statistical tests. Evolution **43,** 223–225.

Rios, E. C., 1994: Seashells of Brazil. Edn. Rio Grande do Sul: UFRG.

Shaw, C. R.; Prasad, R., 1970: Starch gel electrophoresis of enzymes – a compilation of recipes. Biochem. Genet. **4,** 297–320.

Sokal, R. R.; Rohlf, F. J., 1995: Biometry: the principles and practice of statistics in biological research. NY: WH Freemam Company, 885 pp.

Solé-Cava, A. M.; Thorpe, J. P., 1991: High levels of genetic variation in natural populations of marine lower invertebrates. Biol. J. Linn. Soc. **44,** 65–80.

Soltis, D. E.; Soltis, P. S., 1989: Advances in Plant Sciences Series in T.R. Dudley edn, vol. 4. Isozymes in Plant Biology. Portland, Oregon: Discorides Press.

Swofford, D. L.; Selander, R. B., 1981: BIOSYS-1: a FORTRAN program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J. Hered. **72,** 281–283.

Todd, C. D.; Lambert, W. J.; Thorpe, J. P., 1998: The genetic structure of intertidal populations of two species of nudibranch molluscs with planktotrophic and pelagic lecithotrophic stages: are pelagic larvae "for" dispersal? J. Exp. Mar. Biol. Ecol. **228,** 1–28.

Ward, R. D., 1990: Biochemical genetic variation in the genus *Littorina* (Prosobranchia: Mollusca). Hydrobiologia **193,** 53–69.

Ward, R. D.; Warwick, T., 1980: Genetic differentiation in the molluscan species *Littorina rudis* and *L. arcana* (Prosobranchia: Littorinidae). Biol. J. Linn. Soc. **14,** 417–428.

Weir, B. S., 1996: Genetic data analysis II. Methods for discrete population genetic data. Sunderland, Massachusetts: Sinauer Associates, Inc. Publishers.

Weir, B. S.; Cockerham, C. C., 1984: Estimating F-statistics for the analysis of population structure. Evolution **38,** 1358–1370.

Zaslavskaya, N. I., 1995: Allozyme comparison of four littorinid species morphologically similar to *Littorina sitkana*. Hydrobiologia **309,** 123–128.

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APÊNDICE 2

Protocolos dos sistemas enzimáticos modificados para *E. lineolata*. As abreviaturas das enzimas e suas respectivas identificações estão no capítulo 1 (*). Referências citadas podem ser encontradas no capítulo 1.

LAP (modificada a partir de Shaw e Prasad, 1970)

tampão: tris maleato 0,2M pH 6,0 - 25ml

l-leucina β-naftilamida HCL - 35mg

n-dimetil formamida - 900µl

Fast-garnet GBC - 30mg

PEP leu -ala (modificada a partir de Shaw e Prasad, 1970)

tampão: tris HCL 0,1M pH8,0 - 30ml

leu-ala - 30mg

$MnCl_2$ - 6mg

veneno de cobra (*Crothalus atrox*) - 8mg

peroxidase - 8mg

dianisidina - 8mg

PEP leu-gly-gli (modificada a partir de Shaw e Prasad, 1970)

tampão: DH pH 8,4- 30ml

leu-gly-gli - 30mg

$MnCl_2$ - 6mg

veneno de cobra (*Crothalus atrox*) - 8mg

peroxidase - 8mg

dianisidina - 16mg

PP (modificada a partir de Shaw e Prasad, 1970)

solução 1: tampão tris HCL 0,1M pH 8,0 - 25ml

pirofosfato de sódio - 112mg

$MgCl_2$ - 0,5ml

Incubar o gel nesta solução durante 1h, retirar e colocar a solução 2:

solução 2: H_2O - 25ml

molibdato de amônia - 625 mg

ácido ascórbico - 1,25 g

LDH (modificada a partir de Shaw e Prasad, 1970)

tampão: DH pH 8,4 - 30ml

NaD lactato - 15ml (ajustar para pH 8,2)

NAD - 0,5ml

MTT - 0,5ml

NBT - 0,5ml

PMS - 0,6ml

MPI (modificada a partir de Alfenas *et al*,. 1991)

tampão: tris HCL 0,2M pH 8,0 - 30ml

manose 6-fosfato - 6 mg

NADP - 0,3ml

$MgCl_2$ - 0,3ml

MTT - 0,3ml

fosfoglicose isomerase - 40U (1U= 2µl)

glicose 6-fosfato desidrogenase - 10U (1U= 5µl)

PMS - 0,3ml

SDH (modificada a partir de Alfenas *et al*., 1991)

tampão: tris HCL 0,05M pH8,0 - 30ml

sorbitol - 275mg (ajustar para pH 8,0)

NAD - 0,5ml

MTT - 0,3ml

NBT - 0,3ml

PMS - 0,5ml

ME (modificada a partir de Shaw e Prasad, 1970)

tampão: DH pH 8,4- 30ml

substrato MDH - 10ml

NADP - 0,6ml

$MgCl_2$ - 0,3ml

NBT - 0,3ml

PMS - 0,6ml

HK (modificada a partir de Shaw e Prasad, 1970)

tampão: DH pH 8,4 - 30ml

glicose - 60mg

ATP - 25mg

NADP - 0,6ml

$MgCl_2$ - 0,3ml

MTT - 0,2ml

NBT - 0,15ml

glicose 6-fosfato desidrogenase - 40U (1U= 5µl)

PMS - 0,3ml

PGI (modificada a partir de Shaw e Prasad, 1970)

tampão: DH pH 8,4 - 30ml

frutose 6-fosfato - 5mg

NADP - 0,3ml

$MgCl_2$ - 0,3ml

MTT - 0,1ml

glicose 6-fosfato desidrogenase - 9U (1U= 5µl)

PMS - 0,3ml

PGM (modificada a partir de Shaw e Prasad, 1970)

tampão: tris HCL 0,05M pH8,0 - 30ml

glicose 1-fosfato - 60mg

NADP - 0,3ml

$MgCl_2$ - 0,6ml

MTT - 0,3ml

glicose 6-fosfato desidrogenase - 30U (1U= 5µl)

PMS - 0,3ml

MDH (modificada a partir de Shaw e Prasad, 1970)

tampão: DH pH 8,4 - 30ml

substrato MDH - 5ml

NAD - 0,3ml

MTT - 0,4ml

PMS - 0,5ml

AK (modificada a partir de Shaw e Prasad, 1970)

tampão: tris HCl 0,1M pH8,0 - 30ml

glicose - 30mg

ADP - 6mg

NADP - 0,3ml

$MgCl_2$ - 0,3ml

MTT - 0,25ml

hexoquinase - 20U (18U= 1mg)

glicose 6-fosfato desidrogenase - 15U (1U= 5µl)

PMS - 0,3ml

IDH (modificada a partir de Alfenas *et al.*, 1991)

tampão: DH pH 8,4 - 30ml

ácido isocítrico - 70mg

$MgCl_2$ - 0,3ml

NADP - 0,6ml

MTT - 0,3ml

PMS - 0,6ml

Protocolos dos sistemas enzimáticos modificados para *Littoraria flava* e *L. angulifera* (os sistemas cujo protocolo é o mesmo de *E. lineolata* não foram repetidos). As abreviaturas das enzimas e suas respectivas identificações estão nos capítulos 1 e 2. Referências citadas podem ser encontradas no capítulo 1.

AAT (modificada a partir de Soltis e Soltis, 1989)

tampão: C-NaH_2PO_4 0,2M pH4,3 - 4ml

D- Na_2HPO_4 0,2M pH9,2 - 21ml

misturar quantidades indicadas dos tampões C e D.

BSA - 30mg

PVP - 125mg

ácido L-aspártico - 113mg

ácido α-cetoglutárico - 18mg

piridoxal 5-fosfato - 2mg

Fast-blue BB - 60mg

PGI (modificada a partir de Shaw e Prasad, 1970)

tampão: DH pH 8,4 - 30ml

frutose 6-fosfato - 5mg

NADP - 0,3ml

$MgCl_2$ - 0,3ml

MTT - 0,1ml

glicose 6-fosfato desidrogenase - 8U (1U= 5µl)

PMS - 0,3ml

AK (modificada a partir de Shaw e Prasad, 1970)

tampão: tris HCl 0,1M pH8,0 - 30ml

glicose - 25mg

ADP - 6mg

NADP - 0,3ml

$MgCl_2$ - 0,3ml

MTT - 0,2ml

hexoquinase - 20U (18U= 1mg)

glicose 6-fosfato desidrogenase - 10U (1U= 5µl)

PMS - 0,3ml

* As concentrações das soluções utilizadas foram:

$MgCl_2$ - 100mg/ ml

NAD - 25mg/ ml

NADP - 10mg/ ml

MTT - 10mg/ ml

NBT - 20mg/ ml

PMS - 1mg/ ml