Gustavo Maruyama Mori

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Characterization of natural populations of Avicennia germinans *and* A. schaueriana *(Acanthaceae) from mangrove forests along the Brazilian coast and analysis of a hybridization zone: Phylogeography, population and community genetics*

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Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Genética e Biologia Molecular na área de Genética Vegetal e Melhoramento

Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Genetics and Molecular Biology, in the area of Plant Genetics and Genetic Breeding

Orientadora/ Supervisor: Profa. Dra. Anete Pereira de Souza
Co-orientadora/ Co-supervisor: Dra. Maria Imaculada Zucchi

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Resumo

Manguezais são comunidades lenhosas encontradas na faixa entremarés das regiões tropical e subtropical ao redor do mundo. Eles apresentam uma baixa riqueza de plantas superiores dominantes, algumas ordens de grandeza menor em comparação com, por exemplo, a Mata Atlântica e o Cerrado. No hemisfério ocidental, por exemplo, há um total de oito espécies, três das quais pertencem ao gênero *Avicennia* (Acanthaceae): *A. germinans, A.bicolor,* e *A. schaueriana*. A primeira é encontrada em praticamente toda a extensão de florestas de mangue deste hemisfério enquanto que a segunda é restrita ao litoral do Oceano Pacífico da América Central e a última se distribui do sul do Caribe até o sul do Brasil.

Diversos aspectos da Biologia destas e de outras espécies de mangue vem sendo estudados por meio de marcadores moleculares em um conjunto de questões de interesse da disciplina Ecologia Molecular. Neste contexto, nosso objetivo foi compreender a organização da diversidade genética de espécies de *Avicennia* do hemisfério ocidental, com particular atenção para aquelas encontradas no litoral brasileiro. Também procuramos entender como fatores históricos e contemporâneos influenciaram e continuam atuando na variação genética e se esta está relacionada à riqueza de espécies de mangue.

Para isso, desenvolvemos novos marcadores microssatélites específicos para *A. germinans* e *A. schaueriana*. O estudo utilizando estas ferramentas moleculares revelou a organização da diversidade genética em diferentes escalas geográficas no litoral do Brasil. Também foi possível avaliar fatores contemporâneos que moldam esta diversidade, tais como o sistema misto de cruzamento, a limitação de dispersão de pólen e de propágulo (intrínsecos a estes organismos) e o sentido e a intensidade de correntes marinhas além do regime de marés (extrínsecos a essas plantas). Além disso, encontram os evidências de hibridação entre estas espécies.

Ao analisar marcadores de genomas nuclear e cloroplastidial e sequências disponíveis em bancos de dados públicos, confirmamos a ocorrência de hibridação interespecífica entre *A. germinans* e *A. schaueriana* e encontramos novas evidências de dispersão transatlântica para a primeira. Associando informações obtidas por meio destes marcadores às geradas por meio dos microssatélites, explicamos o padrão de distribuição atual destas duas espécies no litoral atlântico da América do Sul com base em diferenças históricas entre elas em termos de dispersão por propágulos e por pólen e de expansões demográficas após a última glaciação.

Esse crescimento populacional pode ser um dos motivos pelo qual não encontrarmos congruência entre diversidade genética e riqueza de espécies de árvores de mangue no litoral do Brasil, como era esperado com base em modelos teóricos. Fica então uma incógnita no entendimento de como a informação é transmitida entre o nível genético e o de comunidades neste sistema de florestas de mangue, o que será melhor avaliado em estudos futuros incluindo outra espécie desta comunidade, *Rhizophora mangle.*

Abstract

Mangrove forests are woody plants communities found within the intertidal zone of tropical and subtropical regions worldwide. They present a distinctive feature, its low superior dominant plants richness, some orders of magnitude lower compared to other communities as the Atlantic Rainforest of the Brazilian savannah. For example, in the western hemisphere, there are only eight species of strict mangrove plants, three of them belong to the genus *Avicennia* (Acanthaceae): *A. germinans, A. bicolor* and *A. schaueriana.* The first is a widespread species found across almost the entire hemisphere while the second is restricted to the Pacific Coast of Central America and the last ranges from South Caribbean to the South of Brazil.

Several issues regarding the biology of *Avicennia* and other mangrove species have been studied using molecular markers to answer different questions within the Molecular Ecology discipline. In this context, our objective is to understand the genetic diversity structure of western hemisphere *Avicennia* species, with particular attention to those found on the Brazilian coast. We also aimed to understand the contemporary and historical factors that shape and have shaped the genetic variation and whether this diversity is related to the mangrove species richness along the littoral of Brazil.

For this purpose, we developed new specific microsatellite markers for *A. germinans* and *A. schaueriana.* The study using these molecular tools revealed a multiple-geographic-scale genetic structure in the Brazilian coast. We then evaluated the role of contemporary factors that influence this diversity such as the species mixed mating system, the limited pollen and propagule dispersal (features intrinsic to these plants) and the marine currents velocity and direction and the tide regimen (extrinsic to the organisms). Moreover, we found evidence of hybridization between these species.

Evaluating nuclear and chloroplastidial markers coupled with sequences available in public databases, we confirmed the occurrence of interspecific hybridization between *A. germinans* and *A. schaueriana* and found novel signs of transatlantic dispersal for the former. Coupling the results obtained by means of these markers with those acquired by microsatellites we explained the current pattern of distribution of these species in the Atlantic basin of South America based on the historical differences of pollen and propagule dispersal and postglacial demographic expansions.

These population expansions may be one of the reasons we were not able to observe a positive correlation between genetic diversity and mangrove species richness along the Brazilian coast. The question on how the information is transferred between the genetic and community levels in the mangrove forest system remains to be explained. We argue that this matter will be better understood when we consider the genetic data from another mangrove species, *Rhizophora mangle*.

Sumário

Dedicatória

Dedico este trabalho a meus pais, **Tadasi** e **Marina**,

Pelo exemplo de cuidado parental,

pelos valores passados,

por mostrar a importância e a beleza da vida.

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meu mais sincero “Muito obrigado! Mesmo!”.

Organização da Tese

Esta tese é fruto de um intenso e extenso trabalho de geração de dados, análise e interpretação de resultados. Para facilitar a compreensão do leitor de forma a tornar o texto mais fluido, o trabalho foi organizado em capítulos.

Inicialmente apresentamos ao leitor quais as questões que este trabalho se dispôs a responder e o sistema biológico de interesse, espécies de árvores de mangue do gênero *Avicennia.* Além disso, por meio de uma revisão bibliográfica, ilustramos como o estudo da variação genética tem sido utilizado para auxiliar a compreensão de diversos aspectos da Biologia de espécies de mangue ao redor do mundo, com especial atenção para as espécies do hemisfério ocidental.

Em seguida, de forma sucinta, são apresentados os objetivos geral e específicos, deste trabalho.

No primeiro Capítulo, apresenta-se a reprodução de um artigo científico já disponível para a comunidade no qual descrevemos como foram desenvolvidos os marcadores microssatélites utilizados para avaliar a diversidade genética de *A. germinans,* os quais se mostraram úteis também para o estudo de *A. schaueriana.* Neste Capítulo, adiantamos também algumas das questões biológicas que pretendíamos responder por meio destas ferramentas moleculares, como a ocorrência de hibridação interespecífica e o estudo do sistema reprodutivo dessas espécies de *Avicennia*.

Posteriormente, no Capítulo II, apresentamos um artigo submetido para publicação. Nele, descrevemos o primeiro conjunto de microssatélites específicos para *A. schaueriana*, o qual também se mostrou valioso para o estudo de *A. germinans.* Utilizando esses marcadores conjuntamente com os descritos no Capítulo I, descrevemos como a variação genética dessas espécies está distribuída ao longo da costa brasileira. Avaliamos também o

papel de diversos fatores como o sistema reprodutivo, a ocorrência de hibridação interespecífica, e a ação da maré e de correntes marinhas na dispersão dos propágulos na estrutura genética em distintos níveis de organização.

O Capítulo III está apresentado em formato de artigo, o qual em breve será submetido a periódico científico especializado. Nele avaliamos marcadores moleculares baseados em sequencias tanto de genoma nuclear (nDNA) quanto cloroplastidial (cpDNA), cujas taxas de mutação são mais lentas do que a dos microssatélites. A escolha destas ferramentas moleculares permitiu-nos avaliar não apenas amostras do litoral brasileiro, mas também material proveniente do litoral africano do Oceano Atlântico e litoral americano do Pacífico, bem como indivíduos provenientes do Caribe. Discutimos a ocorrência de hibridação introgressiva, e dispersão a longa distância, em seguida e propomos uma explicação à distribuição espacial de *A. germinans* e de *A. schaueriana* no litoral da América do Sul.

Posteriormente, no Capítulo IV, expomos e discutimos a investigação preliminar da relação entre a diversidade genética de *A. germinans* e de *A. schaueriana* e a riqueza de espécies verdadeiras de mangue, buscando entender como a informação genética poderia se relacionar com outro nível da hierarquia biológica.

Finalmente, na última seção, apresentamos de modo sucinto as conclusões obtidas após o desenvolvimento deste trabalho e apresentamos algumas aplicações decorrentes dos resultados e que podem ser valiosas para medidas de conservação e manejo de florestas de mangue. Além disso, como esta é uma linha de investigação que continuará em desenvolvimento, indicaremos, de maneira concisa, perspectivas para aprofundarmos a compreensão deste sistema biológico. Desta maneira acreditamos que, neste trabalho de tese, exploramos os desafios científicos envolvidos no projeto, os resultados alcançados e as perspectivas para resolver as questões referentes a esses organismos tão interessantes.

Introdução

"I have a friend who's an artist and he's sometimes taken a view which I don't agree with very well. He'll hold up a flower and say, "look how beautiful it is," and I'll agree, I think. And he says, "you see, I as an artist can see how beautiful this is, but you as a scientist, oh, take this all apart and it becomes a dull thing." And I think he's kind of nutty.

First of all, the beauty that he sees is available to other people and to me, too, I believe, although I might not be quite as refined aesthetically as he is. But I can appreciate the beauty of a flower.

At the same time, I see much more about the flower that he sees. I could imagine the cells in there, the complicated actions inside which also have a beauty. I mean, it's not just beauty at this dimension of one centimeter: there is also beauty at a smaller dimension, the inner structure... also the processes.

The fact that the colors in the flower are evolved in order to attract insects to pollinate it is interesting - it means that insects can see the color.

It adds a question - does this aesthetic sense also exist in the lower forms that are... why is it aesthetic, all kinds of interesting questions which a science knowledge only adds to the excitement and mystery and the awe of a flower.

It only adds. I don't understand how it subtracts."

Richard Feynman, 1981, em entrevista à BBC

Introdução

As florestas de mangue, comunidade de arbustos e árvores restritos aos ambientes entremarés e adjacências de regiões tropicais e subtropicais (Tomlinson 1986), ocupavam no ano 2000 uma área estimada de 137760km^2 ao redor do globo (Giri *et al.* 2011). Embora geralmente se encontrem em uma estreita faixa de elevação onde há a influência da maré na interface continente-mar, estas comunidades se distribuem em uma amplitude latitudinal bastante extensa, entre 31°22'N e 32º20'N no Japão e na Bermuda, respectivamente, e 38º49'S e 32º59'S na Nova Zelândia e África do Sul (Giri *et al.* 2011).

Apesar desta ampla distribuição latitudinal e desta vasta área de cobertura, essas florestas apresentam uma particularidade que as diferencia de outras comunidades tropicais e subtropicais: sua baixa diversidade de espécies dominantes (Tomlinson 1986; Duke *et al.* 1998). Estas plantas foram arbitrariamente categorizadas por Tomlinson (1986) em dois grupos com base em atributos fisiológicos, ecológicos e evolutivos: plantas verdadeiras ou estritas de mangue e elementos menores do manguezal. Em relação às primeiras, globalmente, há apenas 38 gêneros contendo aproximadamente 60 espécies (Duke *et al.* 1998) o que é algumas ordens de grandeza menor quando comparado com comunidades de árvores encontradas, por exemplo, na Mata Atlântica ou no Cerrado (Myers *et al.* 2000; Joppa *et al.* 2011; Zachos & Habel 2011). As poucas espécies de mangue se distribuem em duas grandes regiões biogeográficas: o hemisfério oriental (região Indo- Oeste Pacífico – IWP da sigla em inglês) e ocidental (região do Atlântico – Caribe e Leste do Pacífico – ACEP da sigla em inglês), sendo que o primeiro apresenta cinco vezes o número de espécies quando comparado com o segundo (Tomlinson 1986; Ellison *et al.* 1999; Ricklefs *et al.* 2006). Do total de espécies de árvores estritas de mangue, 48 (pertencentes a 22 gêneros) são encontradas na região IWP enquanto que na região ACEP há oito espécies de quatro gêneros (Tomlinson 1986; Duke *et al.* 1998; Ricklefs *et al.* 2006) (Figura 1). Além

disso, dentro de cada região biogeográfica, a diversidade de espécies também não é homogênea, de modo que, em geral, há um aumento no número de espécies em direção ao equador (Tomlinson 1986; Duke *et al.* 1998; Ellison *et al.* 1999; Ricklefs *et al.* 2006).

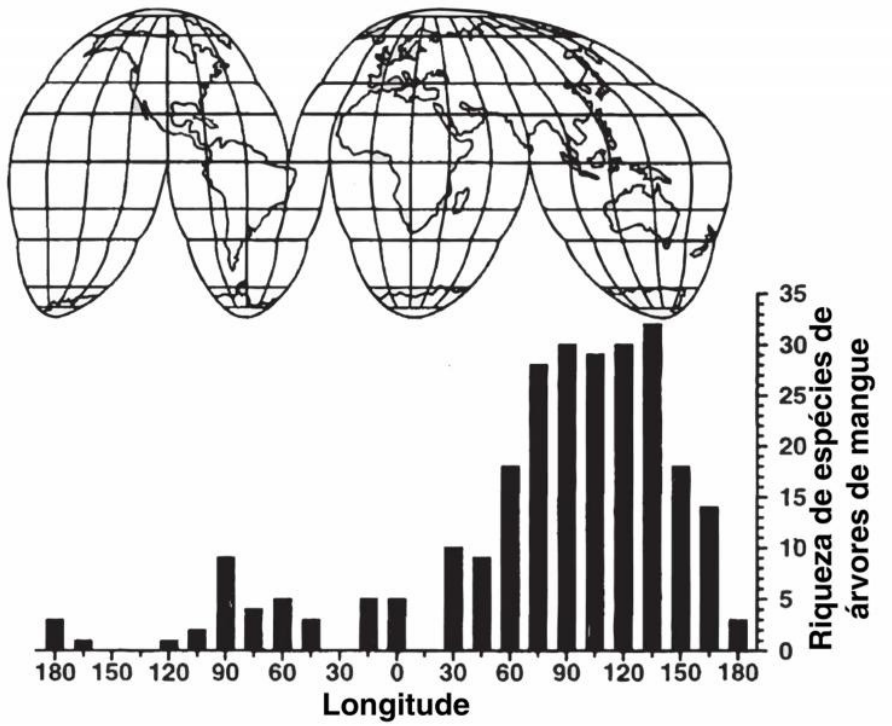

Figura 1. Riqueza de espécies de árvores de mangue em função da longitude, indicada a cada 15°. Adaptado de Ellison *et al.* (1999).

Distintas linhas de raciocínio e evidências sugerem os processos que levaram a este padrão global de distribuição de espécies (Tomlinson 1986; Saenger 1998; Duke *et al.* 1998; Ellison *et al.* 1999; Ricklefs *et al.* 2006). Por exemplo, o fato de *Rhizophora mangle* L. (Rhizophoraceae) ser a única espécie compartilhada entre as regiões ACEP e IWP (Takayama *et al.* 2013) e, associado a diversas evidências paleontológicas, sugere-se que a comunidade de mangue teria surgido na região do Mar de Tétis, que existia no Paleoceno/Eoceno (de 60 a 50 milhões anos atrás) entre a Laurasia e Gondwana. Além disso, esses dados indicam que as distribuições de espécies atuais seriam consequência majoritariamente dos eventos de vicariância devido aos movimentos tectônicos e

movimento dos continentes (Ellison *et al.* 1999). Por outro lado, utilizando dados genéticos e fósseis, um outro processo foi proposto para explicar essa grande diferença no número de espécies destas regiões. Diferenças nas taxas de surgimento de linhagens evolutivas em um cenário de maiores áreas de manguezais no que hoje é o hemisfério oriental teriam favorecido a migração e posterior adaptação por parte de táxons/populações estritamente terrestres na região IWP, resultando em mais espécies de mangue (Ricklefs *et al.* 2006).

Nestes exemplos, observando a heterogeneidade atual das comunidades de árvores de mangue entre as regiões ACEP e IWP pôde-se propor cenários evolutivos que explicam como elas se originaram (Ellison *et al.* 1999; Ricklefs *et al.* 2006). Além disso, fica claro o papel de variadas fontes de informação, como as paleontológicas e genéticas, para se entender como a variação biológica, neste caso a diversidade de espécies, se distribui ao redor do globo, e também, para se propor mecanismos que geraram esta heterogeneidade. Neste contexto, emergem questões referentes a outros níveis da hierarquia biológica, como por exemplo o das moléculas, permitindo entender de maneira mais holística como essas árvores evoluíram e se distribuíram ao longo do tempo e do espaço.

Desde a metade da década de oitenta, esforços têm sido realizados no sentido de se compreender como a diversidade genética de árvores de mangue está organizada (McMillan 1986). Desde então, o estudo da variação molecular tem proporcionado inúmeros avanços no entendimento da evolução de árvores de mangue. Em níveis supra específicos, verificou-se com o auxílio de dados genéticos que o gênero *Avicennia* L., historicamente incluído na família Verbenaceae ou considerado como único gênero da família Avicenniaceae (Tomlinson 1986), está incluído na família Acanthaceae (Schwarzbach & McDade 2002; McDade *et al.* 2008). Além de resolver questões sistemáticas, análises filogenéticas moleculares foram utilizadas para evidenciar que tanto os mecanismos de exclusão de sal como a viviparidade (o desenvolvimento contínuo de embriões enquanto estes ainda se encontram ligados à árvore genitora

(Tomlinson 1986; Elmqvist & Cox 1996)), surgiram múltiplas vezes e de maneira independente em distintas famílias de árvore de mangue (Shi *et al.* 2005).

Já considerando a variação genética no nível intraespecífico, diversos aspectos sobre a Biologia e história evolutiva de diferentes árvores de mangue foram elucidados. No hemisfério oriental, por meio de uma ampla amostragem que abrangeu quase toda a região IWP, verificou-se que *A. marina* (Forsk.) Vier. apresenta sua diversidade genética estruturada entre distintas populações. Observou-se uma acentuada redução de variabilidade em populações periféricas de sua distribuição geográfica, havendo assim evidências de ocorrência de contração populacional histórica (Arnaud-Haond *et al.* 2006). Tal fato indicaria limitações da dispersão tanto de pólen como de propágulos de *A. marina* (Maguire *et al.* 2000, 2002; Arnaud-Haond *et al.* 2006).

Considerando *A. germinans* L. e *R. mangle*, espécies da região ACEP, também se observou que a diversidade genética está organizada entre distintas populações (Dodd *et al.* 2000, 2002; Pil *et al.* 2011; Cerón-Souza *et al.* 2012; Takayama *et al.* 2013), mesmo em uma escala geográfica de dezenas de quilômetros, dentro de um único estuário (Cerón-Souza *et al.* 2012). Esse baixo nível de fluxo gênico seria, em parte, explicado pelos sistemas mistos de cruzamento destas espécies com relativa frequência elevada de autofecundação e de cruzamento biparental, limitando o fluxo gênico por meio do pólen (Cerón-Souza *et al.* 2012; Nettel *et al.* 2013). Embora isso indique que, em conjunto, pólen e propágulos não se movimentem extensamente, de modo bastante intrigante, há evidências de dispersão a longa distância (LDD da sigla em inglês) para essas espécies (Nettel & Dodd 2007; Takayama *et al.* 2013). No caso de *R. mangle,* além de haver a dispersão transatlântica, como observada para *A. germinans* (Nettel & Dodd 2007), há também evidências de ocorrência do movimento de indivíduos entre as ilhas do sul do Pacífico (Samoa, e Tonga) e a costa americana do Oceano Pacífico (Takayama *et al.* 2013). Estes eventos de LDD são majoritariamente raros, mas têm um papel importante nos processos que ocorrem em uma grande escala geográfica, como expansão populacional, adaptação e colonização de novos locais (Nathan *et al.* 2008;

Kremer *et al.* 2012). Ressalta-se também o papel das correntes marinha superficiais como importante fator que molda a diversidade genética de *R. mangle,* principalmente no litoral brasileiro. Nesta região há a bifurcação da corrente marinha Sul Equatorial no estado do Rio Grande do Norte em corrente do Norte do Brasil (que se direciona às Guianas) e corrente do Brasil (que se dirige ao sul), o que explicaria a diferenciação entre amostras provenientes das costas ao norte e ao sul do país (Pil *et al.* 2011; Takayama *et al.* 2013).

Em termos históricos, para ambas as espécies, foram propostos cenários de extinções locais durante o último período glacial com posterior expansão populacional após o aquecimento progressivo do planeta (Nettel & Dodd 2007; Pil *et al.* 2011). Tais interpretações se baseiam no fato de temperatura e ocorrência de geadas serem importantes componentes que limitam o desenvolvimento e estabelecimento de árvores de mangue (Duke *et al.* 1998; Krauss *et al.* 2008; Quisthoudt *et al.* 2012), bem como no avanço atual dos manguezais em direção aos polos frente às mudanças climáticas globais contemporâneas (Perry & Mendelssohn 2009; Osland *et al.* 2013; Saintilan *et al.* 2013). No caso de *A. germinans*, propôs-se que durante a glaciação do Pleistoceno as populações do limite norte da espécie na América Central tenham se extinguido, de modo que esta região teria sido recolonizada posteriormente por indivíduos provenientes de locais mais próximos do Equador (Nettel & Dodd 2007). Já em relação a *R. mangle,* foi proposto um cenário similar no litoral brasileiro. Durante a glaciação, populações ao sul do Rio Grande do Norte teriam se extinguido e, posteriormente, estas regiões teriam sido recolonizadas por indivíduos da costa norte brasileira (Pil *et al.* 2011).

O estudo da variação genética também permitiu mostrar que tanto *A. germinans* como *R. mangle* são linhagens evolutivas cujos limites não são absolutos, de modo que ambas apresentam evidências de hibridação interespecífica (Nettel *et al.* 2008; Cerón-Souza *et al.* 2010; Takayama *et al.* 2013). No caso da primeira, propõe-se que no litoral pacífico da América Central tenha havido cruzamento entre *A. bicolor* Standl., uma

espécie de distribuição restrita a esta região (Tomlinson 1986; Spalding *et al.* 2010), e *A. germinans* (Nettel *et al.* 2008). Isso seria um dos processos que explicaria a maior diversidade genética encontrada para a última espécie no litoral pacífico desta região quando comparado com populações do litoral atlântico (Nettel & Dodd 2007; Cerón-Souza *et al.* 2012). Em relação a *R. mangle,* as evidências moleculares apontam que esta espécie e *R. racemosa* Meyer são linhagens evolutivas distintas que se intercruzaram no passado, e que este processo continua ocorrendo atualmente (Cerón-Souza *et al.* 2010; Takayama *et al.* 2013). O cruzamento interespecífico não é uma exclusividade destas espécies de *Avicennia* e de *Rhizophora* do Novo Mundo, uma vez que na região IWP há registros similares com relação a distintos táxons: *Rhizophora, Bruguiera* (Rhizophoraceae), *Acrostichum* (Pteridaceae), *Sonneratia* (Lythraceae) and *Lumnitzera* (Combretaceae) (Tomlinson 1986; Parani *et al.* 1997; Qiu *et al.* 2008; Zhou *et al.* 2008; Duke 2010; Guo *et al.* 2011; Sun & Lo 2011; Zhang *et al.* 2013). Apesar da extensa lista de registros de hibridação em distintas famílias de plantas de mangue, não há registro de esforços na compreensão de como esse processo pode influenciar a evolução dos híbridos e das espécies parentais (Barton 2001; Soltis & Soltis 2009; Abbott *et al.* 2013), o que consideramos ser uma linha de pesquisa promissora.

Nota-se por esta breve revisão majoritariamente enfocada em árvores de mangue da região ACEP, o grande potencial do estudo da diversidade genética para o entendimento de distintos aspectos da Biologia dessas espécies. Ilustramos que, por meio de marcadores genético-moleculares, pode-se abordar uma ampla gama de questões, da descrição do sistema reprodutivo e ocorrência de híbridos interespecíficos, passando pela organização da diversidade genética, até o modo com que esses organismos podem ter respondido a processos históricos. Todas estas questões são de interesse da Ecologia Molecular, uma disciplina recente e bastante abrangente da Biologia Evolutiva, que utiliza técnicas moleculares para responder perguntas ecológicas e de história evolutiva (Freeland *et al.* 2011; Andrew *et al.* 2013).

É exatamente neste contexto que se insere este trabalho. Por meio de uma ampla amostragem ao longo do litoral brasileiro e utilizando marcadores microssatélites e baseados em sequências de genoma nuclear (nDNA) e cloroplastidial (cpDNA), buscamos compreender como a variação genética de duas espécies de mangue da região ACEP, *A. germinans* e *A. schaueriana* Moldenke, está distribuída no espaço e entre esses táxons. Posteriormente, buscamos entender os fatores históricos e contemporâneos que influenciaram e continuam influenciando o modo com essa diversidade se distribui e se ela está relacionada a um nível hierárquico superior, o de comunidades de mangue.

Com o objetivo de esclarecer ao máximo a linha de raciocínio desenvolvida e defendida nesta tese, dividimos o trabalho em três grandes "áreas" interconectadas de investigação que correspondem a três distintas etapas desenvolvidas: Genética de Populações, Filogeografia e Genética de Comunidades.

A primeira teve como objetivo o estudo da diversidade genética com base nas frequências alélicas avaliadas por meio dos microssatélites, marcadores de alta taxa de mutação (Sunnucks 2000), para se entender quais processos contemporâneos (ecológicos) afetam a variação genética. Isso, seguindo a ideia de que a Genética de Populações é o diálogo entre as predições dos princípios mendelianos de herança e as frequências genotípicas e alélicas medidas experimentalmente, formando-se assim a base para testes de hipóteses (Hamilton 2011).

A segunda, usando marcadores com taxas menores de mutação, visou à compreensão dos processos históricos que influíram na história evolutiva de *A. germinans* de *A. schaueriana* com uma abrangência geográfica ainda maior devido à disponibilidade de dados previamente publicados. Fazendo a conexão entre Filogenia e Genética de Populações, a Filogeografia se interessa pela interseção dessas disciplinas, onde fatores intrínsecos e extrínsecos aos organismos de interesse impactam a distribuição histórica e contemporânea das espécies, integrando aspectos evolutivos e ecológicos (Avise 1998, 2000, 2009; Hickerson *et al.* 2010).

A terceira etapa objetivou avaliar, de modo preliminar, se diferentes medidas de variabilidade genética, acessadas pelas duas classes de marcadores utilizadas, estariam relacionadas à riqueza de espécies esperada em cada local de amostragem. A questão básica a ser respondida é: como a informação genética avaliada por meio destes marcadores é transmitida a níveis mais abrangentes da hierarquia biológica, neste caso, o de comunidades? Essa pergunta se justifica pelo fato de, em ambientes naturais, em geral, a diversidade genética e a de espécies apresentarem correlação positiva (Vellend 2003, 2005a) sendo ela feita em um contexto de Genética de Comunidades (Vellend 2005a; Whitham *et al.* 2006; Hughes *et al.* 2008; Bailey *et al.* 2009).

A. germinans e *A. schaueriana* – espécies de mangue do gênero *Avicennia* do litoral brasileiro

O gênero *Avicennia* apresenta oito espécies que ocupam os manguezais de todo o mundo (Tomlinson 1986; Duke 1991). Como outras espécies verdadeiras mangue (*sensu* Tomlinson (1986)), elas ocorrem apenas em manguezais, possuem papel majoritário na estrutura da comunidade, apresentam especializações morfológicas e fisiológicas (como mecanismos de exclusão de sal e propágulos dispersos pela água) para a vida no ambiente entremarés, e são isoladas filogeneticamente de grupos terrestres relacionados (Tomlinson 1986). Entretanto, ao contrário do observado para as demais plantas estritas de mangue, *Avicennia* apresenta tolerância a uma grande amplitude de níveis de salinidade (Mehta *et al.* 2005; Suárez & Medina 2005; Krauss & Ball 2012) e de temperatura (Kao *et al.* 2004; Pickens & Hester 2010; Quisthoudt *et al.* 2012), o que explicaria, ao menos em parte, o fato de este ser o gênero de mangue com maior distribuição geográfica (Duke 1991; Spalding *et al.* 2010).

Ao longo desta ampla distribuição, as espécies de *Avicennia* se distribuem de maneira disjunta, de modo que uma mesma espécie não é encontrada em ambas as regiões biogeográficas de mangue (ACEP e na IWP) (Tomlinson 1986; Duke 1991;

Duke *et al.* 1998). Além disso, embora não incluam todas as oito espécies, análises filogenéticas moleculares indicam que além de o gênero ser monofilético, ele está dividido em dois clados distintos os quais correspondem às espécies orientais e ocidentais (Schwarzbach & McDade 2002; McDade *et al.* 2008).

Apesar destas consistentes diferenças no nível do DNA, morfologicamente as espécies de *Avicennia* são árvores ou arbustos, diferenças geralmente causadas pelas distintas condições edáficas e de salinidade (Naidoo 2006; Lovelock *et al.* 2007), que possuem um aspecto relativamente uniforme (Tomlinson 1986). Indivíduos do gênero possuem folhas opostas, simples, com superfície adaxial glabra e abaxial recoberta por tricomas, os quais conferem distintas cores e texturas *in vivo* (Tomlinson 1986). Nas folhas, encontram-se glândulas especializadas na exclusão de sal para controle osmótico, de modo que na face adaxial, não é raro se encontrar cristais de cloreto de sódio (Tomlinson 1986; Krauss & Ball 2012) os quais podem também estar associados a uma proteção contra eventuais insetos herbívoros (Landry 2013)

Já considerando a porção reprodutiva, as inflorescências podem ser consideradas como panículas com órgãos progressivamente reduzidos, em relação tanto ao tamanho como ao número em direção ao ápice. Com relação às flores, embora existam três tipos florais em relação às oito espécies (*germinans*, *officinalis* e *marina*), em todas, observa-se apenas um ovário súpero, unilocular que gera um fruto com apenas uma semente. Existem semelhanças no arranjo das bractéolas e do cálice entre todas as espécies e, em todos os tipos florais, há produção de néctar (Tomlinson 1986) e os órgãos sexuais masculinos se desenvolvem antes dos femininos (Tomlinson 1986; Rathcke *et al.* 1996; Nadia *et al.* 2013) o que favoreceria o fluxo gênico entre indivíduos, reduzindo a autofecundação espontânea e, portanto, a endogamia.

Devido à relativa homogeneidade das estruturas vegetativas do gênero, os caracteres reprodutivos são os principais elementos utilizados para diferenciação precisa de espécies (Tomlinson 1986). Em relação às espécies do litoral brasileiro, *A. germinans* é reconhecida pelas folhas geralmente ovadas com ápice abrupto e pelas

flores com estames longos os quais ultrapassam a linha de inserção das pétalas cujas faces internas são recobertas por tricomas (Figura 2A). Já *A. schaueriana* possui folhas usualmente obovadas, flores com corola glabra ou pobre em tricomas e estames de comprimentos iguais que se encontram no máximo até a linha de inserção das pétalas (Figura 2B) (Tomlinson 1986; Nadia *et al.* 2013).

Apesar dessas diferenças morfológicas florais que são utilizadas para diferenciação destas espécies, as flores das duas espécies são relativamente similares e há evidências de que ambas apresentam um sistema generalista de polinização, com amplo espectro de polinizadores incluindo abelhas, moscas, borboletas e vespas (Rathcke *et al.* 2001; Sánchez-Núñez & Mancera-Pineda 2012; Landry 2013;Nadia *et al.* 2013). Considerando que em determinados locais em que as duas espécies coexistem elas podem florescer simultaneamente (Menezes *et al.* 2008), são plausíveis os eventos de fluxo gênico interespecífico não apenas históricos como relatado na América Central (Nettel *et al.* 2008), mas também contemporâneos.

Os frutos deste gênero podem ser descritos como cápsulas coriáceas com o pericarpo delgado verde ou verde-acinzentado e apresentam apenas uma semente sem endosperma, mas com dois cotilédones. O fruto é considerado incipientemente vivíparo, pois o embrião germina logo após se desprender da planta genitora (Tomlinson 1986). Este embrião, que pode ser considerado a unidade de dispersão (propágulo), apresenta radículas e hipocótilo bem desenvolvidos (Tomlinson 1986), é flutuante e tem uma alta longevidade inclusive em água salgada (Rabinowitz 1978; Clarke *et al.* 2001; Alleman & Hester 2011). Isso permitiria especular que há um alto nível de fluxo gênico entre distintas localidades, havendo relativa homogeneidade entre as populações.

Entretanto, no nível intraespecífico, apresentar expectativas em relação ao movimento gênico entre locais de amostragem em relação a estas espécies é uma tarefa complicada. As características dos propágulos de *A. germinans* e de *A. schaueriana* explicariam a relativa alta frequência de eventos de LDD (Nettel & Dodd 2007; Takayama *et al.* 2013). Por esta perspectiva, seria factível um cenário com a diversidade

genética homogênea e altos níveis de fluxo gênico. Entretanto, como já mencionado, tanto para *A. germinans* como para *R. mangle,* foi encontrada uma estrutura genética dentro de um único estuário, indicando limitação na dispersão tanto de pólen como de propágulos (Cerón-Souza *et al.* 2012). Neste caso, pode-se esperar que essas espécies apresentem sua diversidade genética organizada em *pools* gênicos separados, havendo pouco fluxo genético entre eles mesmo em escalas geográficas menores.

Figura 2. Flores de A) *A. germinans* e de B) *A. schaueriana*. Ficam evidentes as diferenças dos tamanhos relativos dos estamos e da presença/ausência de tricomas nas faces internas das pétalas, caracteres utilizados para diferenciação entre as espécies (Tomlinson 1986; Nadia *et al.* 2013). Fotografias: © Gustavo M. Mori.

Um sistema biológico complexo e repleto de particularidades como as espécies de *Avicennia* encontradas no litoral brasileiro se mostra um modelo bastante interessante para estudos ecológicos, genéticos e evolutivos.

Objetivos

"(...)

I can learn to get along

With all the things I can't explain

I can learn to resist

Anything but frustration

I can learn to persist

With anything but aiming low

(...)

You can fight

Without ever winning

But never ever win

Without a fight"

Geddy Lee, Alex Lifeson & Neil Peart (Rush), 1996, Resist

Objetivos

Geral

Este trabalho tem como objetivo geral compreender como se encontra a variação genética de espécies de árvores de mangue do gênero *Avicennia* no hemisfério ocidental e, em especial no litoral brasileiro. Além disso, buscamos entender como fatores ecológicos, históricos e contemporâneos, influenciam e influenciaram esta diversidade, bem como se esta está relacionada à variação no nível das comunidades.

Específicos

- Desenvolver marcadores microssatélites para *A. germinans* e *A. schaueriana;*

- Avaliar o sistema reprodutivo de *A. schaueriana* por meio de um arranjo de progênie proveniente do litoral norte brasileiro;

- Estudar a variabilidade genética de *A. germinans* e de *A. schaueriana* por meio de microssatélites e de marcadores de genoma nuclear e cloroplastidial;

- Compreender como fatores contemporâneos intrínsecos, como a ocorrência de hibridação, e extrínsecos, como correntes marinhas, influem na variação genética;

- Entender como fatores históricos, tais como expansões demográficas, influenciaram na distribuição da diversidade genética;

- Investigar uma eventual relação entre riqueza de espécies e diversidade genética, medida por microssatélites e por marcadores de genoma nuclear e cloroplastidial.

Capítulo I

Microsatellites for the mangrove tree *Avicennia germinans* (Acanthaceae): Tools for hybridization and mating system studies

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AJB Primer Notes & Protocols in the Plant Sciences

Microsatellites for the mangrove tree *Avicennia germinans* (Acanthaceae): Tools for hybridization and mating system studies[1]

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• *Premise of the study:* We developed a new set of microsatellite markers for the black mangrove *Avicennia germinans*, to provide new informative tools for further studies of the mating system, interspecific hybridization, and population genetics.

• *Methods and Results:* We used the microsatellite-enriched library approach to isolate and characterize 25 new primer pairs. Sixteen of them are polymorphic, showing a variable degree of variation in *A. germinans*, while nine were monomorphic in the samples examined. Eight exhibited private alleles in *A. schaueriana*.

• *Conclusions:* These results indicate that these new microsatellite markers will be useful molecular tools for further studies of *A. germinans* and *A. schaueriana* population genetics, mating systems, and hybridization.

Key words: black mangrove; genetic diversity; hybrids; SSR.

Mangrove forests are found all over the world, essentially within the intertidal zone of tropical latitudes (Tomlinson, 1986). A distinctive feature of mangals is its low species diversity, mainly when compared to other tropical ecosystems, such as the Amazon or many rainforests. *Avicennia germinans* (L.) L. is distributed in the Atlantic, Caribbean, and East Pacific regions, while *A. bicolor* Standl. is restricted to the Pacific coast of Central America. In areas where both species overlap, there is evidence of ancient introgression (Nettel et al., 2008) despite their morphological, ecological, and phenological differences.

A similar scenario is found in the northern South American Atlantic coast, where there is a relatively large area of sympatry between *A. germinans* and *A. schaueriana* Stapf & Leechm. ex Moldenke, an endemic species to the Atlantic coast. However, in contrast to the first case, these species are ecologically and morphologically distinct, but their reproductive phenology is similar at some localities, so that their flowering period may overlap (Menezes et al., 2008). This makes feasible current hybridization between *A. schaueriana* and *A. germinans*. To identify hybridization, study the genetic variation, and analyze the reproductive biology of these species, we developed a new set of microsatellite markers for *A. germinans* and tested its transferability to *A. schaueriana*.

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METHODS AND RESULTS

To identify and characterize microsatellites, we developed a microsatellite-enriched library for *A. germinans*. Genomic DNA was isolated from one individual of *A. germinans*, sampled in northern Brazil (1°2′42.60″S; 46°45′23.31″W) using a modified cetyltrimethyl ammonium bromide (CTAB) protocol. The DNA sample (5 μg) was digested with *Rsa*I and ligated to the double-strand adaptors 5′-CTCTTGCTTACGCGTGGACTA-3′ and 5′-TAGTCCACGCGTAAGCAAGAGCACA-3′. The enrichment was done using a hybridization-based capture with $(GT)_8$ and $(CT)_8$ biotin-linked probes and streptavidin magnetic-coated beads (Streptavidin MagneSphere Paramagnetic Particles, Promega, Madison, WI). Selected fragments were then cloned into a pGEM-T easy (Promega) vector and inserted into *Escherichia coli* XL1-Blue competent cells. Recombinant colonies were selected by white/blue screening. We randomly selected positive clones that were double sequenced on an ABI PRISM 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Every sequence obtained was aligned, edited, and eliminated if redundant in SeqMan (DNASTAR, Madison, WI). MICROSAT software (A.M.Risterucci, CIRAD, personal communication) was used to eliminate adaptors and restriction sites of the sequences. The Simple Sequence Repeat Identification Tool (SSRIT) (Temnykh et al., 2001) was used to identify perfect microsatellites according to the following criteria: we considered only dinucleotides with six or more repeats, trinucleotides with four or more repeats, and tetra-, penta-, and hexanucleotides with three or more repeats.

Primer pairs were designed using the software PrimerSelect (DNASTAR) and Primer3Plus (Untergasser et al., 2007). Polymerase chain reactions (PCRs) were carried out in a final volume of 20 μL containing 2 ng of template DNA, 1× PCR buffer, 1.5 mM magnesium chloride, 0.2 μM of each dNTP, 0.3 μM of each primer, and 1 U *Taq* DNA polymerase. All primers were first evaluated in a gradient temperature from 45° to 65°C to establish the optimal annealing temperature of each primer pair as follows: 94°C for 2 min, 30 cycles of 94°C for 1 min, T_a for 1 min, 72°C for 1 min; and 72°C for 5 min. If needed, *touchdown* PCR was performed according to the following thermocycler parameters: 94°C for 2 min, 2× [10 cycles of 94°C for 1 min, 65°C (–1°C/cycle) for 1 min

TABLE 1. Characteristics of 25 microsatellite markers developed for *Avicennia germinans*. Shown for each primer pair are the forward and reverse sequences, expected size based on the clone fragment, repeat motif, optimal PCR amplification conditions (AC), and GenBank accession number. TD65-55 indicates touchdown PCR with temperatures ranging from 65 to 55°C.

Marker	Primer Sequence (5′ - 3′)	Size	Repeat motif	AC	GenBank
Agerm1-01	F: CAGTTTGGTGAGAAGGATGTT R: TTTGAGGTCGGCTCGTTAAG	127	$(ac)_{15}$	53.4°C	HM470003
Agerm1-02	F: TAACTAGCCGCCCATCCATC R: ACCAGCCCACATCCAACAAT	168	$(ca)_{11}$	53.4°C	HM470004
Agerm1-03	F: CCATGTTTTTGACTTTTTATTTTG R: TTACGATAGGGTGGATTGAGATTTT	161	$(ca)_9$	48.2°C	HM470005
Agerm1-04	F: GCGATCTGGAAGTCACCCTA R: AGGGATTTGGCTGTTGCATA	159	$(atc)_4$	45.5°C	HM470006
Agerm1-05	F: AGAAACGGGAGAATTCAGCA R: ATACTCGCCCTCGTCTCGTA	188	$(caac)_3$	53.4°C	HM470007
Agerm1-06	F: GAATTGGCTGGAATGAGGAA R: GTGTTTTGGAAGGAGCCTGT	175	$(gt)_6$	63.4°C	HM470008
Agerm1-07	F: CCTGACACTCTGGGACATCA R: CCTTTTGACGCATTTGTGG	157	$(gt)_9$	50.5°C	HM470009
Agerm1-08	F: CTGCCGAGCAAAGGCTTA R: GCAAGATCCACAGCTTCACA	183	(tg)9	TD65-55	HM470010
Agerm1-09	F: CCATTTTTGCTCTCCTTTCC R: GTGGCCCAAACGTTGAATAG	204	(tca)5	53.4°C	HM470011
Agerm1-10	F: CCATTTTTGCTCTCCTTTCC R: GTGGCCCAAACGTTGAATAG	230	(ac)7	53.4°C	HM470012
Agerm1-11	F: GCCCTCAGCATGAAACTTGT R: AAATAATAAGGCGCCCGTGT	138	$(tg)_{10}$	53.4°C	HM470013
Agerm1-12	F: CAGTTTGGTGAGAAGGATGTT R: TTTGAGGTCGGCTCGTTAAG	127	$(ac)_{15}$	53.4°C	HM470014
Agerm1-13	F: CACTTGTGCTGTTTTCTCA R: ATTAGCTTCCATTTTTCAG	137	(ca)11	53.4°C	HM470015
Agerm1-14	F: CCAATTGTGTCGTCCTTTTA R: AGCCTTACTTTTCCTTTGT	159	$(ca)_8(at)_6$	59.6°C	HM470016
Agerm1-15	F: ACTTACACACAAAATGCACA R: CTGAGAGTGCCGACTGAATG	248	$(ca)_4...(ac)_{13}$	56.7°C	HM470017
Agerm1-16	F: CCTAATACAAATGACACTAAAA R: TGCATGTCAATTATCAGTCT	176	$(tg)_9$	53.4°C	HM470018
Agerm1-17	F: TTGGACTCTAGAAGGGCATACA R: CGATTGGTTTACTTCCTACTTG	154	(ca)7	64.5°C	HM470019
Agerm1-18	F: CAGCGGGAAAAATCAAACCAA R: CCTGTGCACATCCGCCTCTC	243	$(ag)_{16}$	64.5°C	HM470020
Agerm1-19	F: TTGGCTGGAATGAGGAAC R: AAGATTTGTGTTTTGGAAGGA	179	$(ga)_4...(gt)_6$	59.6°C	HM470021
Agerm1-20	F: TATAACAATGCCCTGACACTCT R: ACAAACATGCCAACACAAATA	203	$(gt)_9$	59.6°C	HM470022
Agerm1-21	F: GGAGCAATTGTCGAAAGGAC R: CGTTGCTGAGACAAGGAACA	150	$(ca)_8$	61.8°C	HM470023
Agerm1-22	F: CACAGGTTCTACTCGGAAGATG R: CGTCCGGGTCTACTCAAAAA	167	$(tttctt)_4$	63.4°C	HM470024
Agerm1-23	F: TTTGCTCTCCTTTCCCATTTA R: GATGAGCTACCAGGGCAACA	137	(tca)5	64.5°C	HM470025
Agerm1-24	F: CTTTCCTCATTTCTGCATTTTG R: TCCTGCCATTTTCTCCACTT	227	(ac)7	59.6°C	HM470026
Agerm1-25	F: GAGCAAAACTGGATACTCAAATG R: AATAATAAGGCGCCCGTGT	237	$(tg)_{10}...(tg)_4$	65.5°C	HM470027

and 72°C for 2 min]; 18 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min; and 72°C for 5 min. Amplification products were visualized in TBE 0.5× 3% agarose gels stained with bromide ethidium prior to vertical electrophoresis in TBE 1× 6% polyacrylamide denaturing gel silver nitrate stained. Product sizes were determined by comparison to a 10 bp DNA ladder (Invitrogen, Carlsbad, CA). Statistical analyses were performed in GENEPOP on the Web (Raymond & Rousset, 1995) and PIC Calculator (Kemp, 2002).

We sequenced 96 positive colonies of 192 that compose this enriched library, and 61 clones possessed 75 microsatellites. Due to the enrichment method using $(GT)_8$ and $(CT)_8$ probes, dinucleotide motifs were the most abundant, followed by tetranucleotide (approximately 70% and 10%, respectively). Other motifs were very low frequency. The number of repetitions of dinucleotide microsatellites varied from 5 to 18, with an average of 9.07. We designed 33 primer pairs so that the product size did not exceed 250 bp for accurate genotyping using vertical electrophoresis in TBE 1× 6% polyacrylamide gels. Twenty-five of these primer pairs generated consistent patterns of amplification with matching expectations based on the size of the sequenced fragment and were used for further characterization (Table 1). The remaining eight loci were discarded due to amplification failure, nonspecific pattern of banding in preliminary tests, or unexpected amplification product size. For polymorphism analysis, 25 individuals of *A. germinans* and five individuals of *A. schaueriana* were sampled from Bragança/PA, Brazil, where these species are sympatric (0°56′20″S; 46°43′17″W). These species were differentiated based on reproductive material in the field: *A. germinans* individuals were recognized by their long exserted stamens and by their conspicuously hairy petals; *A. schaueriana* was identified by its inserted stamens and glabrous corolla (Tomlinson, 1986). Population voucher specimens were deposited in the IAN herbarium (Embrapa Amazônia Oriental herbarium, Belém, Brazil); see Appendix 1.

Sixteen loci were polymorphic within *A. germinans* with a varying degree of diversity (Table 2). The mean number of alleles was 4.375 (2–10

TABLE 2. Results of initial microsatellite marker screening in one population of *A. germinans* ($N = 25$) within a sympatric region with *A. schaueriana* ($N = 5$) in northern Brazil. For each marker, it is shown the number of alleles in *A. germinans* (A), observed heterozygosity (H_O), gene diversity (H_E), polymorphism information content (PIC), P-values for Hardy–Weinberg equilibrium tests, and the number of alleles found in *A. schaueriana* (As). *indicates alleles restricted to *A. schaueriana* (private alleles).

Marker	A	H_O	H_E	PIC	HWE	As
Agerm1-01	2	0.60870	0.51014	0.3745	0.4211	2*
Agerm1-02	5	0.54167	0.75000	0.6874	0.1009	1*
Agerm1-03	2	0.64000	0.50939	0.3746	0.2445	3
Agerm1-04	1	-	-	-	-	1
Agerm1-05	1	-	-	-	-	-
Agerm1-06	2	0.24000	0.27429	0.2327	0.4840	1
Agerm1-07	3	0.40000	0.42204	0.3430	1.0000	1
Agerm1-08	1	–	–	–	–	1*
Agerm1-09	1	–	–	–	–	2*
Agerm1-10	1	–	–	–	–	1
Agerm1-11	9	0.78261	0.83382	0.7949	0.6088	–
Agerm1-12	9	0.91304	0.83478	0.7943	0.9624	–
Agerm1-13	1	–	–	–	–	1*
Agerm1-14	7	0.56522	0.72657	0.6694	0.1222	2*
Agerm1-15	10	0.79167	0.86702	0.8323	0.2124	1*
Agerm1-16	3	0.32000	0.28000	0.2462	1.0000	1
Agerm1-17	1	–	–	–	–	–
Agerm1-18	6	0.77273	0.62368	0.5641	0.2769	1*
Agerm1-19	2	0.24000	0.27429	0.2327	0.4839	1
Agerm1-20	2	0.33333	0.28455	0.2392	1.0000	–
Agerm1-21	4	0.28000	0.61469	0.5309	0.0006	–
Agerm1-22	2	0.04000	0.04000	0.0384	1.0000	2
Agerm1-23	1	–	–	–	–	1
Agerm1-24	1	–	–	–	–	–
Agerm1-25	2	0.56000	0.50286	0.3714	0.6913	2

alleles), the Polymorphism Information Content (PIC) varied from 0.0384 to 0.8323, and significant departure from Hardy–Weinberg equilibrium was found only for Agerm1-21 ($p < 0.001$). Eleven of these loci cross-amplified in *A. schaueriana*, and five showed private alleles for this species. Nine other microsatellite markers were monomorphic in *A. germinans*; five also generated product in *A. schaueriana*; and three loci exhibited alleles restricted to this species (see Table 2).

CONCLUSIONS

When coupled with previously published markers (Nettel et al., 2005; Cerón-Souza et al., 2006), these newly developed polymorphic markers will be useful for mating system studies as they increase the availability of informative loci and, thus, will improve statistically the power of the analysis (Lynch & Ritland, 1999). Besides, these eight markers presenting *A. schaueriana* private alleles would be a valuable tool for detecting ongoing hybridization between these species within their area of sympatry.

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APPENDIX 1. Taxa used in this study, Specimens are deposited in IAN Herbarium (Embrapa Amazônia Oriental herbarium).

Taxon – Voucher Specimens, Collection locale.

Avicennia germinans – IAN 185071, Bragança. IAN 185072, Bragança.

Avicennia schaueriana - IAN 185073, Bragança. IAN 185074, Bragança.

Capítulo II

Elucidating the multiple-geographic-scale genetic structure of two Neotropical mangrove trees: the role of mating system, hybridization, limited dispersal and extrinsic factors

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Running title: Multiple-geographic-scale genetic structure

Author's contributions: GMM conceived and designed this study, collected materials, performed experiments, analyzed the data and wrote the paper with assistance of MIZ and APS. MIZ contributed to data analyses. APS received funding and provided laboratory and molecular biology facilities. All authors read, reviewed and approved the final manuscript.

Abstract

Mangrove plants comprise a unique group of organisms that growths within the intertidal zone of tropical and subtropical regions. We studied the genetic diversity of two neotropical mangrove trees, *Avicennia germinans* and *A. schaueriana,* considering different geographic scales, using microsatellites previously developed for *A. germinans* and the first set of markers characterized for *A. schaueriana*, described herein. We also evaluated intrinsic and extrinsic factors that likely shape the genetic variation of these species. As reported for other sea-dispersed species, there is a strong differentiation between *A. germinans* and *A. schaueriana* populations sampled north and south of the northeastern extremity of South America, likely due to the influence of superficial marine currents. Within each group, a fine-scale genetic structure was observed even when no obvious physical barrier existed, indicating pollen and propagule dispersal limitation. A local genetic structure was observed considering two samples of *A. germinans* separated by few kilometers but under distinct hydrographic regimens, suggesting the role of the tide as another major factor influencing mangrove trees's genetic diversity. The genetic structure on smaller scales is likely favored by the species mixed mating system, the occurrence of crosses between relatives and biparental inbreeding. Although we report the first evidence of ongoing hybridization between *Avicennia* species and that hybrids may be fertile, this extant interspecific cross has not contributed to an increase in the genetic diversity of the populations where this process is taking place.

Key-words: genetic variation, simple sequence repeat, isolation-by-distance, progeny array, introgression

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Introduction

Mangrove plants encompass a polyphyletic and heterogeneous group whose definition is based on ecological and physiological traits that are considered adaptations to life within the intertidal zone of tropical and subtropical regions establish discrete communities, known as mangrove forests (Tomlinson 1986), which are globally distributed, covering approximately 137,700km^2 worldwide (Giri *et al.* 2011). Within this distribution, the mangrove species richness is heterogeneously allocated in such way that the Eastern or Indo-West Pacific (IWP) region is one order of magnitude more diverse than the Western or Atlantic, Caribbean and East Pacific (ACEP) region (Duke *et al.* 1998). Moreover, within each of these biogeographic regions, the number of different species decreases as the latitude increases (Duke *et al.* 1998; Ellison 2002).

These patterns of species diversity are influenced by abiotic factors such as oceanographic, climatic, topographic and edaphic factors (Duke *et al.* 1998). Additionally, biotic factors play important roles in the maintenance of these patterns. For instance, the limited effective dispersal (Duke *et al.* 1998) and establishment of the floating, long-lived, salt-tolerant propagules with variable degrees of "viviparity", the continuous development of the embryo without dormancy (Rabinowitz 1978; Clarke *et al.* 2001), affect the species composition at many scales (Duke *et al.* 1998). From these issues regarding the patterns of distribution of the mangrove species richness emerge further questions regarding other levels of biological hierarchy, such as the organization of genetic diversity.

Since the 1980s, the genetic variation of mangrove plants at the molecular level has been evaluated by different methods in both biogeographic regions (Triest 2008).

More recently, a wide range of questions have been answered such that new patterns have emerged (for example, Pil *et al.*, 2011; Sun & Lo, 2011; Cerón-Souza *et al.*, 2012; Takayama *et al.*, 2013; Zhang *et al.*, 2013). For instance, large land and ocean barriers to propagule dispersal are important evolutionary factors that differentiate populations of mangroves (Takayama *et al.* 2013). However, considering smaller geographic scales, substantial genetic structure is also observed in different genera (Geng *et al.* 2008; Cerón-Souza *et al.* 2012). Another important recent advance is that more ancient and ongoing interspecific hybridization has been recorded (Cerón-Souza *et al.* 2010; Sun & Lo 2011; Takayama *et al.* 2013), including taxa in which no morphologically intermediate individuals are found (Nettel *et al.* 2008).

Using two Neotropical species of *Avicennia* L. (Acanthaceae) as models, we evaluated these extrinsic and intrinsic factors that shape the genetic variation of different species of mangrove plants. *Avicennia* is the most widely distributed genus of mangrove plants (Duke 1991) and is found both in the IWP (five species) and in the ACEP (three species) regions. The ACEP *Avicennia* species with the largest distribution is *A. germinans* L., distributed across the entire region except for the southern part of Brazil, where *A. schaueriana* Stapf and Leechman ex Moldenke is dominant (Fig. 1). These species are partially sympatric from the northeastern coast of Brazil to the northern limit of distribution of *A. schaueriana* in the Lower Lesser Antilles (Tomlinson 1986; Duke 1991). Within their sympatry zone, there is preliminary evidence of chloroplast capture between these species (Nettel *et al.* 2008). This interaction is feasible since they present a generalist pollination system and share similar pollinators (Landry 2013; Nadia *et al.* 2013), and in some localities, their reproductive phenology overlaps (Menezes *et al.* 2008).

We hypothesized that, as independently observed for many true and associate mangrove plants (Takayama *et al.* 2006, 2013; Nettel & Dodd 2007; Pil *et al.* 2011; Cerón-Souza *et al.* 2012), there are multiple geographic scales of genetic structure of *A.*

germinans and *A. schaueriana*. To evaluate this hypothesis and possible intrinsic and extrinsic factors that are currently influencing these genetic structures, we studied the population genetics of these species considering three geographic scales, the *A. schaueriana* mating system and the hybridization between them using microsatellite markers.

Materials and Methods

Plant material and sampling strategy

From June 2008 to December 2010, we sampled 400 individuals of *A.schaueriana* from 11 localities and 181 individuals of *A.germinans* from seven locales along the Brazilian Coast covering more than 4900km of coastline to evaluate local (thousands of meters – microscale), regional (hundreds of kilometers, mesoscale) and continental geographic scales (thousands of kilometers, macroscale) (Table1). Latitude and longitude were recorded using a global positioning system (Garmin 76CSx, considering the WGS84 standard). Each sample of each species was denoted as table 1, with Ag and As indicating *A. germinans* and *A. schaueriana,* respectively, and three letters indicating the locality where the sample was obtained. For *A. germinans*, in Bragança, Pará, Brazil we collected individuals from two geographically close localities under different tidal influences: one area where the inundation frequency was reduced due to changes in the hydrographic regime caused by the construction of a highway (AgPAa) (Menezes *et al.* 2008), and another under a regular tidal pattern (AgPAb) (Fig. 1, Table 1). We distinguished the species in the field based both on vegetative and reproductive traits to minimize misidentification. We recognized *A. germinans* individuals by the ovate leaves that usually present a blunt apex, by their long, exserted stamens and by their conspicuously hairy petals. We identified *A. schaueriana* by its obovate leaves, its inserted stamens and its glabrous inner-face corolla (Tomlinson 1986). Voucher specimens from every location, except for samples of AsALC and AgALC, were deposited and cataloged in the University of Campinas (UEC) and

Embrapa Amazônia Oriental (IAN) herbaria, both located in Brazil. From each flowering tree, which was at least 20 m from anyother, we sampled leaves and kept them in zip lock bags containing silica-gel. This leaf material was lyophilized and stored at -20°C prior to DNA isolation. Despite our sampling effort, we collected five individuals of *A. germinans* presenting flowers at AgPRC (Table 1).

Table 1. Sampled populations of *A. germinans* (Ag) and *A. schaueriana* (As) are indicated by following three capital letters. Sample sizes are indicated between parentheses. City and state in Brazil, geographic coordinate and numbers correspondent to Fig. 1 are denoted for each site.

As	Ag	Locality (City, state)	Geographic Coordinate	Location in Figure 1
	AgMRJ(31)	Soure, Pará	0° 43' 26"S, 48° 29' 24"W	1
AsSAL(22)		Salinópolis, Pará	0°36'36"S, 47°22'41"W	2*
AsAJU(46)$		Bragança, Pará	0° 49' 12"S, 46° 36' 56"W	2*$
AsPRM(47)		Bragança, Pará	0° 57' 42"S, 46° 37' 5 "W	2*
	AgPAa(28)*	Bragança, Pará	0° 54' 17"S, 46° 41' 15"W	2*
	AgPAb(27)	Bragança, Pará	0° 56' 21"S, 46° 43'17"W	3#
AsALC(30)	AgALC(29)	Alcântara, Maranhão	2° 24' 37"S, 44° 24' 22"W	4
	AgPNB(29)	Parnaíba, Piauí	2° 46' 42"S, 41° 49' 20"W	5
AsPRC(31)	AgPRC(5)	Paracuru, Ceará	3° 24' 47"S, 39° 3' 23"W	6
	AgTMD(32)	Tamandaré, Pernambuco	8° 31' 35"S, 35° 0' 48"W	7
AsVER(31)		Vera Cruz, Bahia	12°59'1"S, 38°41'5"W	8
AsGPM(35)		Guapimirim, Rio de Janeiro	22°42'5"S, 43°0'26"W	9
AsUBA(32)		Ubatuba, São Paulo	23° 29' 22"S, 45° 9' 52"W	10
AsCNN(32)		Cananéia, São Paulo	25° 1'12"S, 47°55'5"W	11
AsPPR(28)		Pontal do Paraná, Paraná	25°34'30"S, 48°21'9"W	12
AsFLN(66)		Florianópolis, Santa Catarina	27°34'37"S, 48°31'8"W	13

* indicates a sample under a reduced inundation frequency (Menezes *et al.,* 2008), $ indicates the locality where the progeny array was sampled.

For *A. schaueriana* mating system analyses, in June 2008 we arbitrarily chose 24 mother trees separated by at least 30m from AsAJU (Fig. 1, Table 1). From each of these trees, young leaves and healthy propagules that were still attached to the tree were sampled, with a mean of 12 propagules per parental plant (ranging from 8 to 14). Each

progeny was stored in an individualized zip-lock bag in the field, and when brought to the laboratory, every fruit was kept in distilled water to isolate the pericarp from the embryo, which was stored at -80°C until DNA extraction.

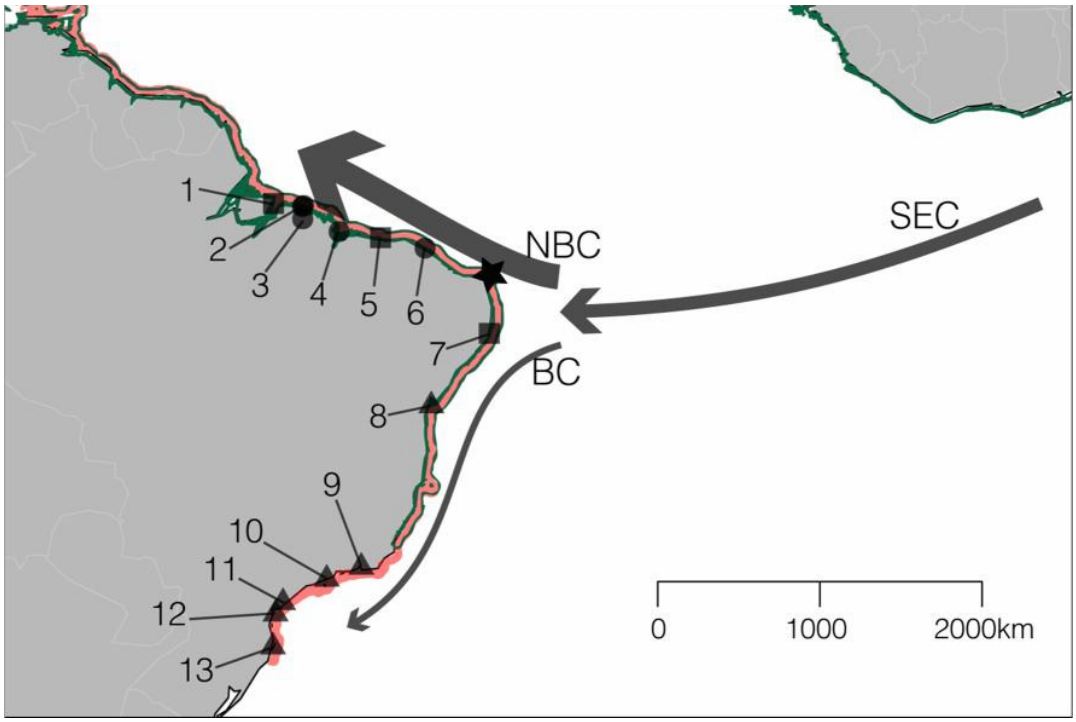

Figure 1. Map of the South Atlantic displaying the geographic distribution of *A. germinans* (green) and *A. schaueriana* (red) and their sympatric region (green and red). Squares and triangles respectively indicate locations where only *A. germinans* and *A. schaueriana* presented reproductive branches during sampling. Circles represent locales where both species presented flowers. The star indicates the northeastern extremity of South America. Sampling locations are displayed according to Table 1. Arrows denote the near surface ocean currents that influence the sampling range: South-Equatorial (SEC), North Brazil (NBC) and Brazil currents (BC). Arrow size and line widths illustrate the mean current speed (Lumpkin & Johnson 2013).

A. schaueriana *microsatellites development and molecular biology procedures*

We ground leaf samples and whole embryos into fine powder in liquid nitrogen for total genomic DNA isolation according to a cetyltrimethyl ammonium bromide protocol. The genetic diversity of each *Avicennia* species was analyzed using previously published microsatellite markers for *A. germinans* (Nettel *et al.* 2005; Cerón-Souza *et al.* 2006; Mori *et al.* 2010) and markers developed for *A. schaueriana,* described herein, which were isolated to achieve more reliable results using a larger number of loci for both species. The *A. schaueriana* microsatellites were developed by using the method that successfully permitted the isolation of *A. germinans* markers (Mori *et al.* 2010). Monomorphic markers for *A. germinans* (Mori *et al.* 2010) were tested in a subsample of eight individuals from three and five locales for *A. germinans* and *A. schaueriana*, respectively. Those markers presenting intra- or interspecific polymorphism were considered for subsequent examinations (Table S2, Supporting information).

Polymerase chain reactions (PCRs) were carried out as previously specified (Mori *et al.* 2010) or with modifications (Nettel *et al.* 2005; Cerón-Souza *et al.* 2006) (Table S2, Supporting information). Amplification products were visualized by vertical electrophoresis in 1x TBE 6% polyacrylamide denaturing gels that were stained with silver nitrate. Product sizes were determined by comparison to a 10 bp DNA ladder (Invitrogen, Carlsbad, CA, USA). We re-genotyped 48 individuals to evaluate scoring genotyping errors. To evaluate *A. schaueriana* mating systems, only markers that presented polymorphism at AsSAL, AsAJU and AsPRM were employed, except for the loci Agerm6 and Agerm8, out of a total of 13 markers.

Intraspecific genetic diversity analyses

After characterizing the *A. schaueriana* markers, we tested the occurrence of linkage disequilibrium (LD) for all pairs of markers per sample using FSTAT 2.9.3 (Goudet 2002). Because we found consistent evidence of LD for *A. germinans* and *A.*

schaueriana, of the total of 27 and 25 loci we evaluated, 22 and 17 of them were considered for further analyses, respectively (Table S2, Supporting information).

Using MICRO-CHECKER (Van Oosterhout *et al.* 2004) we observed evidence of null alleles and stuttering (Table S2, Supporting information). However, in spite of these indications, we found no substantial differences between the global and pairwise G_{ST} of samples corrected and not corrected for null alleles using the "excluding null alleles" method implemented in FreeNA (Chapuis & Estoup 2007) (Tables S3 and S4, Supporting information); thus, we used the original dataset for further analyses. We evaluated intraspecific genetic variation by the average effective number of alleles (N_e), the expected (H_E) and observed heterozygozities (H_O) and the fixation index (f) for each sample using GenAlEx6.5 (Peakall & Smouse 2012). The apparent outcrossing rate (t_a) was determined as $\frac{(1-f)}{(1+f)}$ (Fyfe & Bailey 1951) (Table 2). We tested each sample for Hardy-Weinberg equilibrium (HWE) using heterozygote deficiency as the alternative hypothesis in Genepop 4.0 (Rousset 2008).

Population structure within species

Considering different sets of microsatellite markers for each species, we used different approaches to evaluate how the genetic diversity within each species is organized. We assessed the population structure using the summary statistics D (Jost 2008) and G_{ST} (Nei 1973) using diveRsity (Keenan 2013). The reliability of these statistics was verified using 10^5 permutations. We also verified the pairwise relations of these statistics between every pair of localities. We also evaluated the isolation by distance (IBD) by performing a Mantel test in ade4 (Chessel *et al.* 2004) considering the approximate linear distance between the sample locales along the coastline and G_{ST} (Nei 1973) and D (Jost 2008). The Mantel correlograms where calculated in vegan (Oksanen *et al.* 2013), considering 10 classes of geographic distance for *A. germinans* and 15 for *A. schaueriana* using 10^5 permutations were performed to test the significante correlations.

To further comprehend how the genetic diversity is structured within each species, a multivariate method, discriminant analysis of principal components (DAPC) (Jombart *et al.* 2010) implemented in adegenet 1.3.7 (Jombart & Ahmed 2011), was used considering the samples from each locale as a different group. We applied this model-free approach which provides principal components (PCs) that only rely on the inter-population variability (Jombart *et al.* 2010) retaining seven and six PCs that represented 49.9% and 60.4% of the total genetic information of *A. germinans* and *A. schaueriana* samples, respectively, with the number of clusters (k) varying from 1 to 50. The number of PCs was chosen based on the *optim.a.score* function to avoid over-fitting during discrimination. The choice of optimal k was based on the Bayesian information criterion provided for each k tested. We also used the model-based clustering method implemented in Structure 2.3.4, assuming correlated allele frequencies and admixture (Pritchard *et al.* 2000; Falush *et al.* 2003) and disregarding any *a priori* information. For each k, ranging from 1 to 10 for *A. germinans* and from 1 to 15 for *A. schaueriana,* we carried out 50 independent Markov chain Monte Carlo (MCMC) runs with 5.10^5 iterations following a burn-in period of 5.10^5 iterations. The k that best explained our data was determined using both the maximization of the logarithm likelihood of the data, lnL (Pritchard *et al.* 2000) and the *ad hoc* statistic ΔK (Evanno *et al.* 2005). We used CLUMPP (Jakobsson & Rosenberg 2007) to adress label switching and multimodality issues using the *Greedy* algorithm with 10^6 random input orders. For *A. schaueriana,* to determine finer scale population structure we used the same strategy described above for each of the two clusters obtained (see below), with k ranging from 1 to 10 for each group. This approach was justified by the results of pairwise G_{ST} and D (Fig. 2 and Supplementary Material 1, Supporting information) and DAPC analyses (Fig. 5). We used an extended model-based approach implemented in Instruct (Gao *et al.* 2007) to consider inbreeding coefficients, which is a likely violated condition in these species (see below), without prior information on spatial location or sample membership. We performed five independent runs of 10^6 MCMC repetitions

and a 5.10^5 burn-in period for each. We also carried out hierarchical analyses of molecular variance (AMOVA) using permutation procedures (10^5 iterations) in Arlequin3.5 (Excoffier & Lischer 2010) considering the clusters found using both multivariate and Bayesian methods. For this purpose, we considered the F_{ST} analog estimator under the infinite alleles mutation model (IAM) (Weir & Cockerham 1984).

Ongoing hybridization between A. germinans *and* A. schaueriana

To evaluate ongoing hybridization between these *Avicennia* species, we used a different set of markers because only microsatellites that presented PCR products for both species were considered to reduce marker development bias. In these analyses, we evaluated 20 microsatellites including intraspecific monomorphic markers that presented variation between species (Table S2, Supporting information).

The same methods to study the genetic diversity and the population structure described above, except for Instruct, were used in this investigation. For DAPC analysis, we used seven PCs, which explained 67.7% of the variance using every species samples as *a priori* clusters. In addition to using the same model-based clustering approach described above (Pritchard *et al.* 2000; Falush *et al.* 2003) with k ranging from 1 to 10, we arbitrarily defined a threshold of 0.15 for membership probability to consider any sign of historical hybridization. We also evaluated the existence and the categories (F_1, F_2 and backcrosses between a "pure species" and a F_1) of eventual two-generation hybrids between each "pure species" employing a different model-based method implemented in *NewHybrids* 1.1beta (Anderson & Thompson 2002). For this purpose, we evaluated the posterior distributions using five independent chains of 10^6 MCMC after 5.10^5 burn-in steps without prior allele frequency information, considering Jeffrey-type and uniform distribution priors for θ and π. We also evaluated the groups found by these approaches using hierarchical AMOVA to evaluate how the genetic diversity is organized between and within *A. germinans* and *A. schaueriana*.

A. schaueriana *mating system*

Using the 24 open-pollinated progeny arrays, we evaluated the mating system of *A. schaueriana* under the mixed mating model for unlinked markers (Ritland & Jain 1981) using MLTR v3.4 (Ritland 2002). With this method, considering both Newton-Raphson (NR) and expectation-maximization (EM) methods regarding the absence and presence of null alleles, we estimated the multilocus (t_m), single-locus (t_s) and between related individuals outcrossing rates (t_m-t_s), the average single locus inbreeding coefficient of maternal parents (F_m) and the multilocus paternity correlation ($r_{p(m)}$). The standard errors were determined based on 10^4 bootstraps among families.

Results

A. schaueriana *microsatellite development*

Of the 192 sequenced clones that constitute the library we constructed, 52 presented a total of 60 microsatellites. Based on these loci, we designed 43 primer pairs, 16 of which were excluded from further analyses due to amplification failure, unexpected fragment size or nonspecific products. Of the remaining markers, 18 were monomorphic for *A. germinans* and *A. schaueriana,* one did not present amplification products for the former and the other eight were polymorphic for both species (Tables S1 and S2, Supporting information) and were thus used for genotyping both species.

Intraspecific genetic diversity

We found a varying degree of polymorphism among markers within each and among samples of each species in terms of N_e, H_E and H_O (Table 2). For both species, we found significant a departure from HWE for every sample. We also observed values of f ranging from 0.112 to 0.481 for *A. schaueriana* and -0.002 to 0.662 for *A. germinans,* with averages of 0.242 and 0.174, respectively. Therefore, there is evidence that these species exhibit mixed mating systems as the average t_a estimated for the former was 0.610 and for the latter 0.704 (Table 2).

Table 2. Intraspecific genetic diversity regarding each sample, denoted as table 1, of *A. germinans* and *A. schaueriana*. Average effective number of alleles (N_e), expected (H_E) and observed (H_O) heterozygozities and fixation index (f), with respective standard errors between parentheses, and outcrossing apparent rate (t_a) are denoted.

A. germinans samples	N_e		H_o		H_e		f		t_a
AgMRJ	3.461	(0.559)	0.541	(0.056)	0.587	(0.05)	0.066	(0.049)	0.876
AgALC	2.731	(0.336)	0.452	(0.045)	0.540	(0.05)	0.132	(0.032)	0.767
AgPAa	2.356	(0.304)	0.437	(0.054)	0.452	(0.056)	-0.002	(0.029)	1.005
AgPAb	3.259	(0.506)	0.522	(0.056)	0.569	(0.053)	0.083	(0.048)	0.846
AgPNB	1.788	(0.170)	0.294	(0.046)	0.350	(0.053)	0.131	(0.036)	0.768
AgPRC	2.397	(0.179)	0.310	(0.049)	0.598	(0.048)	0.452	(0.079)	0.377
AgTMD	1.174	(0.064)	0.025	(0.012)	0.110	(0.037)	0.662	(0.083)	0.203
Average	2.452	(0.142)	0.369	(0.022)	0.458	(0.023)	0.174	(0.024)	0.704
A. schaueriana samples	N_e		H_o		H_e		f		t_a
AsSAL	1.816	(0.247)	0.242	(0.050)	0.333	(0.066)	0.209	(0.056)	0.654
AsAJU	2.002	(0.349)	0.245	(0.051)	0.346	(0.066)	0.308	(0.075)	0.529
AsPRM	2.168	(0.443)	0.21	(0.043)	0.358	(0.071)	0.378	(0.040)	0.452
AsALC	1.879	(0.273)	0.255	(0.051)	0.336	(0.068)	0.207	(0.061)	0.656
AsPRC	1.279	(0.079)	0.136	(0.036)	0.178	(0.046)	0.217	(0.052)	0.644
AsVER	1.455	(0.170)	0.144	(0.036)	0.213	(0.058)	0.187	(0.073)	0.685
AsGUA	1.638	(0.319)	0.146	(0.041)	0.216	(0.065)	0.195	(0.055)	0.674
AsUBA	1.174	(0.080)	0.05	(0.022)	0.104	(0.044)	0.481	(0.095)	0.351
AsCNN	1.529	(0.202)	0.193	(0.060)	0.215	(0.067)	0.112	(0.077)	0.799
AsPPR	1.626	(0.321)	0.155	(0.049)	0.213	(0.067)	0.185	(0.049)	0.688
AsFLN	1.477	(0.211)	0.148	(0.052)	0.188	(0.064)	0.248	(0.072)	0.603
Average	1.640	(0.081)	0.175	(0.014)	0.246	(0.019)	0.242	(0.020)	0.61

A. schaueriana *mating system*

Regardless of the method used (NR or EM, considering or not the presence of null alleles), the results were similar and differed only when thousandths were considered, thus only the EM disregarding null alleles outcomes are shown. For this progeny array, we observed a predominantly outcrossing mixed mating system (t_m = 0.542 ± 0.062, t_s= 0.232 ± 0.065) which was in accordance with the t_a (0.529) estimated for AsAJU, composed only by reproductive individuals, with F_m = 0.232 ± 0.065, assuming Wright equilibrium. We also observed that biparental inbreeding (t_m - t_s =

0.151 ± 0.026) contributed to the apparent selfing rate of the progeny, which presented a fraction composed of full-sibs ($r_{p(m)}$ = 0.178 ± 0.057).

Population structure within species

We found substantial evidence of differentiation among samples with global G_{ST} and D values of, respectively, 0.241 and 0.263 for *A. germinans* and 0.480 and 0.127 for *A. schaueriana.* The pairwise G_{ST} and D for both species were highly correlated, showing a varying degree of differentiation among different pairs of samples for both species ($r \geq 9.11$ for all cases Fig. 2 and Supplementary Material 1, Supporting information) despite the lower values of D compared to G_{ST} for *A. schaueriana.* These results indicate that there is a substantial genetic structure for both species mainly when samples north and south of the northeastern extremity of South America (NEESA – Fig. 1) are considered (Fig. 2). For the sake of simplicity, we will consider "Ag" and "As" as acronyms for *A. germinans* and *A. schaueriana,* respectively, and "N" or "S" as codes for samples north and south of the NEESA.

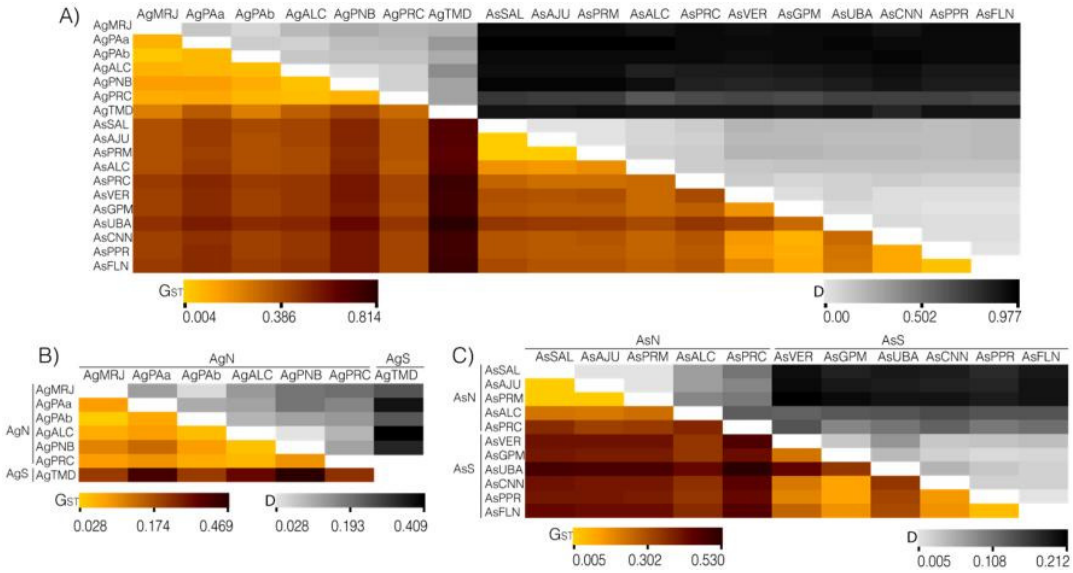

Figure 2. Pairwise measurements of the genetic variation between samples in terms of Nei's G_{ST} (1973 - below diagonal) and D (Jost 2008 - above diagonal) in A) both *A. germinans* and *A. schaueriana;* B) *A. germinans* and C) *A. schaueriana* samples. AgN and AgS, and AsN and AsS, respectively refer to samples of *A. germinans* and *A. schaueriana* taken from locales north and south of the NEESA. Every measure was significant ($p < 0.05$).

There was a significant association between genetic and geographic distances as indicated by D and G_{ST} for *A. germinans* and *A. schaueriana* ($p<0.01$ for both species, with $r_M = 0.777$ and $r_M = 0.696$ for the former considering G_{ST} and D, and $r_M = 0.755$ and $r_M = 0.830$ for the latter). The IBD observed was even more evident when we evaluated the correlograms of r_M and classes of geographic distance (Fig. 3), which indicate significant positive spatial structure. This pattern of IBD was highlighted when the multivariate DAPC analyses were considered. Using this method, for *A. germinans,* we found k = 4 with a substantial differentiation between TMD and the remaining samples (Fig. 4A). For *A. schaueriana*, the inferred most likely k was 10, and similarly, a clear pattern of divergence between AsN and AsS was observed (Fig. 5A).

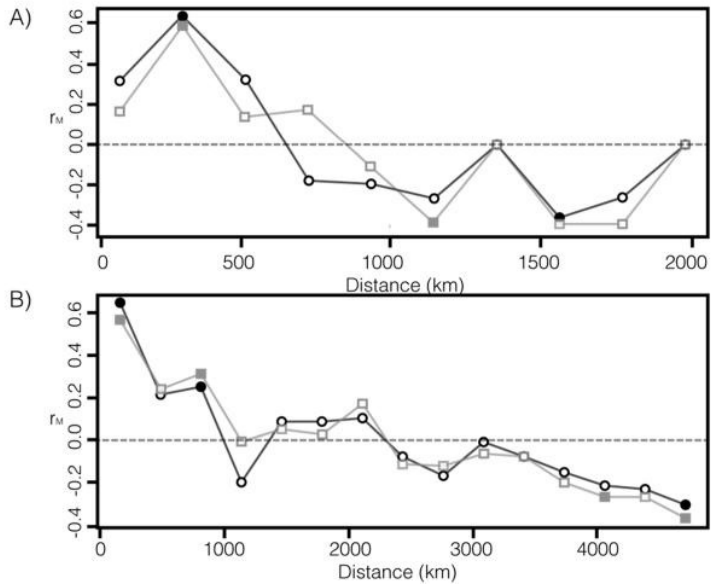

Figure 3. Mantel correlograms performed on approximated distances between each pair of sample locales along the coastline for A) *A. germinans* and B) *A. schaueriana.* Black interconnected circles refer to D (Jost 2008) and grey interconnected squares point to Nei's genetic distance (Nei 1972). Filled squares and circles indicate significant correlations ($p < 0.05$) after 10000 permutations.

Using the model-based method implemented in Structure software we observed two possible scenarios for *A. germinans*: k = 2 (AgN and AgS) as the most likely number of populations according to the ΔK approach, and k = 8 as an alternative

scenario of finer genetic structure according to lnL and to a smaller peak of the ΔK value (Fig. 4B). This scenario highlights the existence of a multiple-scale genetic structure. In the finer-scale scenario, suggesting the divergence of the samples across every sampling site we evaluated, including AgPAa and AgPAb, which are separated by thousands of meters. For *A. schaueriana*, the most likely k was 2 (AsN and AsS), according only to ΔK (Fig. 4C). Each of these groups was evaluated separately due to the results of pairwise G_{ST}, D (Fig. 2), and DAPC (Fig. 5A). The evidences of finer scale genetic structure arose with AsN presenting k = 4, according to ΔK values, with AsS showing k = 2, taking into account the ΔK value, and with AsS showing k = 6, according to lnL and to a smaller peak of the ΔK value (Fig. 4D and 4E). When inbreeding was considered, despite the different inferred k for both species (10 for *A. germinans* and 11 for *A. schaueriana*), similar patterns of genetic structure were observed (Figs. S1 and S2). Therefore, it is clear that for both species there is genetic structure on different geographic scales, which is partially in accordance with the IBD results, as we found two inferred groups (AgPAa and AgPAb) that despite their geographic proximity were clearly distinguished genetically. For all of the cases, there was correspondence between samples from each locality and the clusters inferred, which also allowed for the inference of admixed individuals and migrants (Figs. 5 and 6).

The analysis of hierarchical AMOVA showed that indeed, the major amount of variation arose between AsN and AsS (45.88%). For this species, when finer-scale scenarios were considered (k = 4 for AsN, and k = 5 or k = 8 for AsS), there were significant values of among group fixation indexes, but a lower gain in terms of proportion of variation (Table 3). Concerning *A. germinans,* the variation between AgN and AgS, although substantial, was not supported by permutation tests. However, when the seven samples were considered, a significant and substantial proportion of variation was explained by this level of the hierarchy (15.37%) (table 3).

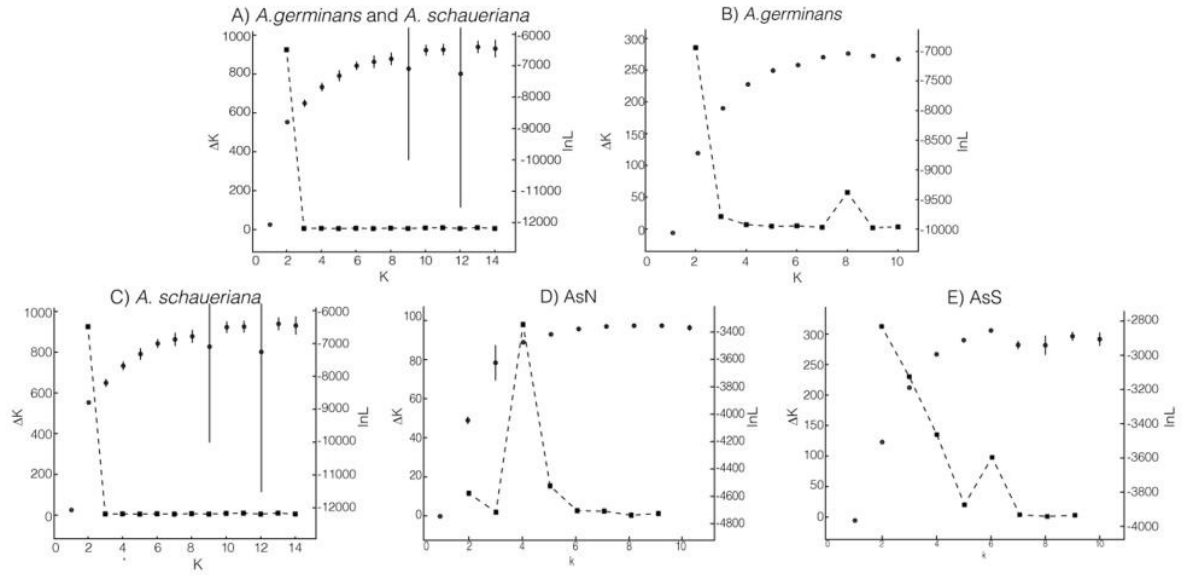

Figure 4. Bayesian inference of the number of clusters (k) considering the mean values of log posterior probability of data (lnL - isolated grey circles (Pritchard *et al.* 2000)) and the ΔK *ad hoc* statistic (interconnected squares, (Evanno *et al.* 2005)) considering A) samples of both *A. germinans* and *A. schaueriana*, B) samples of *A. germinans*, C) individuals of *A. schaueriana* from all locales sampled, D) individuals of *A. schaueriana* from the northern cluster (AsN) and E) samples of *A. schaueriana* from the southern cluster (AsS).

Table 3. Results of hierarchical analysis of molecular variance. AMOVA considering A) both *A. germinans* (Ag) and *A. schaueriana* (As) as groups*;* B) northern (AgN) and southern (AgS) to the northeast extreme of South America (NEESA) of *A. germinans* as groups*;* and C) samples of *A. schaueriana* north (AsN) and south (AsS) to the NEESA; samples of AgN with k = 4 and AsN with k = 2 (D), k = 5 (E), according to Fig. 5. % indicates total variance; F-FI: fixation indexes considering infinite allele model (IAM). All results are significant ($p<0.005$) except for those presenting ^{NS}.

A)	As and Ag		
	%	F-FI	
Between As and Ag	58.92	F_{GT}	0.589
Among populations within As and Ag	17.60	F_{SG}	0.428
Among individuals within populations	3.54	F_{IS}	0.150
Within individuals	19.94	F_{IT}	0.800

B)	AgN, AgS		
	%	F-FI	
Between AgN and AgS	27.52	F_{GT}	0.275^{NS}
Among populations within AgN and AgS	15.37	F_{SG}	0.212
Among individuals within populations	7.07	F_{IS}	0.123
Within individuals	50.04	F_{IT}	0.499

C)	AsN, AsS			D)	Ag k = 4, As k = 5		E)	Ag k = 4, As k = 8	
	%	F-FI		%	F-FI		%	F-FI	
Among groups	45.88	F_{GT}	0.458	49.22	F_{GT}	0.492	51.23	F_{GT}	0.512
Among populations within groups	14.83	F_{SG}	0.274	5.36	F_{SG}	0.105	1.33	F_{SG}	0.027
Among individuals within populations	9.85	F_{IS}	0.250	11.38	F_{IS}	0.250	11.89	F_{IS}	0.250
Within individuals	29.44	F_{IT}	0.705	34.04	F_{IT}	0.659	35.55	F_{IT}	0.644

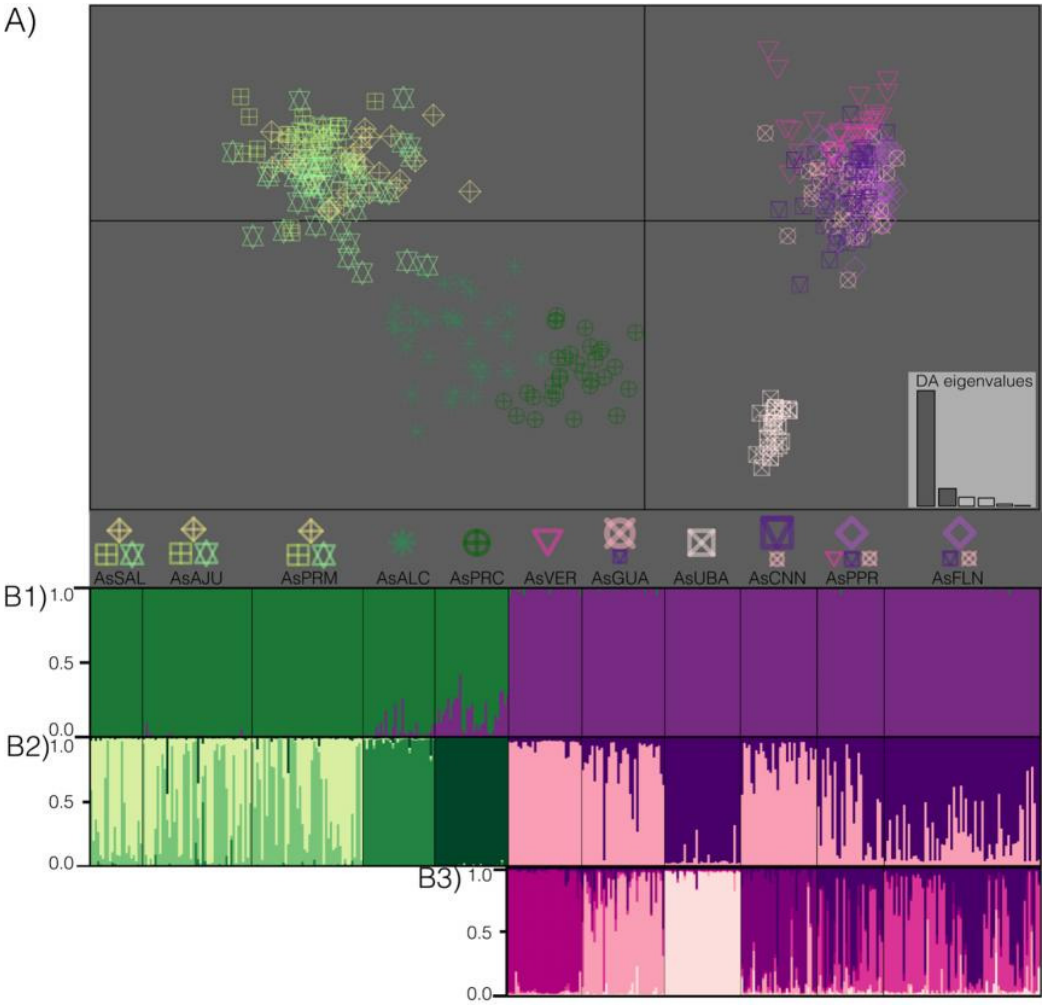

Figure 5. *A. schaueriana* population structure along the Brazilian coast. A) Scatterplot of the first two principal components of the multivariate analysis of DAPC (Jombart *et al.* 2010). Each symbol indicates the group to which each individual was assigned. The geographic origin of each individual is denoted for each locality sample, such that more than 10% of the total number of individuals wass composed of the inferred cluster. A larger symbol indicates that a cluster was predominant in the locality sample (pairwise clusters ratio larger than 1:5) whereas equal symbol sizes indicate similar cluster contributions to the total individuals of each sample. B1) Model-based clustering analyses (Pritchard *et al.* 2000) considering k = 2, where each individual is represented by a vertical line, each color refers to one inferred cluster and the posterior probability of group membership is indicated. These Bayesian analyses were extended to the inferred groups observed in B1, which correspond to samples north (green) and south (violet) of the NEESA. The posterior probabilities of group membership of this finer-scale analysis of AsN (k = 4) and AsS (k = 2) are shown in B2, and further evaluations of AsS (k = 6) are displayed in B3.

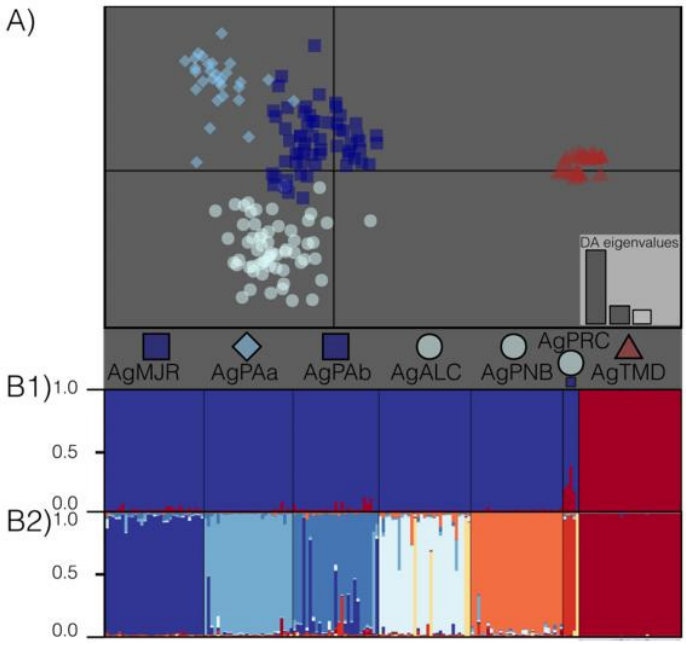

Figure 6. *A. germinans* population structure along the Brazilian coast. A) Scatterplot of the first two principal components of the multivariate analysis of DAPC (Jombart *et al.* 2010), where each symbol indicates the group to which each individual was assigned. The geographic origin of each individual is denoted for each locality sample such that more than 10% of the total number of individuals was composed of the inferred cluster. A larger symbol indicates that a cluster was predominant in the locality sample (pairwise clusters ratio larger than 1:5) whereas equal symbol sizes indicate similar cluster contributions to the total individuals of each sample. B1) Model-based clustering analyses (Pritchard *et al.* 2000) considering k = 2 and B2) k = 8, where each individual is represented by a vertical line, each color refers to one inferred cluster and the posterior probability of group membership is indicated.

Ongoing hybridization between A. germinans *and* A. schaueriana

We observed that, as expected, the differentiation between the species was greater than the observed differentiation among samples within each species. The values of global G_{ST} and D were 0.583 and 0.549, respectively, considerably higher than those observed for individual species. Pairwise comparisons between samples within and between species also indicated that the interspecific differences were much more pronounced than between samples in each species (Fig. 2). These results also agree with the hierarchical AMOVA approach which revealed that the majority of the variation

(58.92%) was explained between the species while there was still considerable and significant genetic variation between populations within each species (17.6%) (Table 2).

Regarding the DAPC results, we observed an optimum of 11 clusters in such a way that the difference between *A. germinans* and *A. schaueriana* was evident (Fig. 7A). The pattern of genetic structure within each species agreed with the multi-scale results presented above, when each taxon was evaluated separately including the clear divergence between northern and southern samples for *A. schaueriana* and, less evidently for *A. germinans*. Using DAPC, despite the individual assignment, there was evidence of one F_1 interspecific hybrid from AgPRC (Fig. 7A) which was graphically placed between the two main clusters. Based on two different model-based approaches, we found more evidence that these species may be hybridizing and that these hybrids are fertile. Considering the model implemented in Structure (Pritchard *et al.* 2000), the most likely k was 2 according only to ΔK (Fig. 4). The differentiation between *A. germinans* and *A. schaueriana* remained obvious, but compared to the DAPC results, there was more evidence for ongoing hybridization between these species. Considering the arbitrary threshold of the assignment probability of 0.15, we found four individuals that were most likely the results of mating between *A. germinans* and *A. schaueriana*. The same individual placed between the two main clusters in the multivariate analysis (Fig. 7A) presented assignment probabilities of 0.566 and 0.434 for each inferred group, a reliable indication of an F_1 hybrid (Fig. 7B). Considering each species as a "pure category" we used another model-based approach to verify the presence of different classes of up to two generation hybrids. Because the use of Jeffrey-like and uniform priors yielded slightly different results with the latter providing more conservative outcomes in terms of the numbers and likelihood of hybrids, we only considered the uniform distribution as prior. The individual inferred as a likely hybrid from PRC considering the Structure and DAPC analyses was unequivocally assigned as an F_1 hybrid with posterior probability of 1.0. We also observed that two plants from AgALC were likely descendants of a cross between an F_1 individual and an *A. germinans* genitor

(unidirectional backcross - probability >0.80) (Fig. 7C).. There was no sign of hybridization considering individuals identified as *A. schaueriana* indicating a unidirectional hybridization process (Fig. 7).

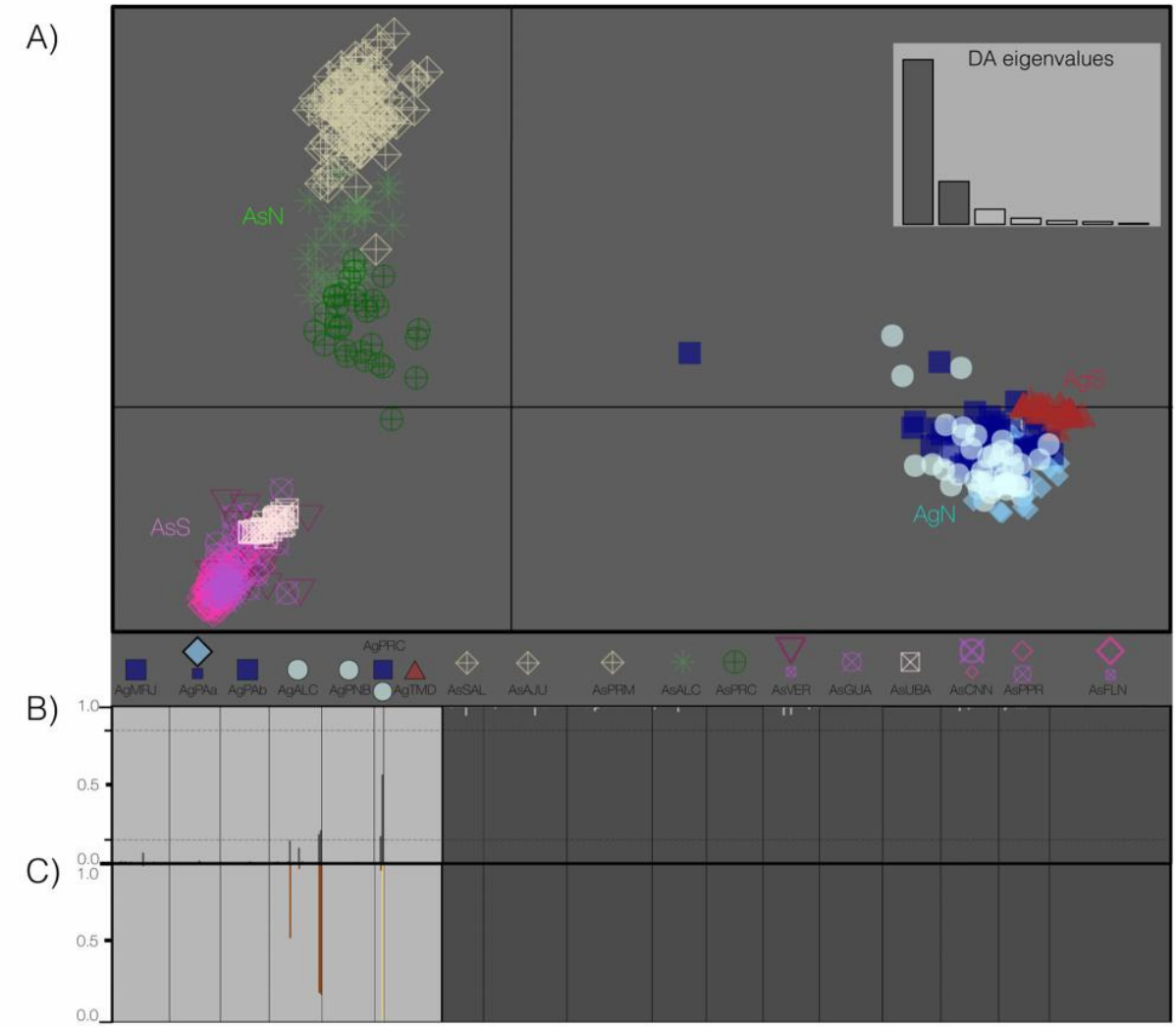

Figure 7. Analyses of ongoing hybridization between *A. germinans* and *A. schaueriana.* A) Scatterplot of the first two principal components of the multivariate analysis of DAPC (Jombart *et al.* 2010). Each symbol indicates the group to which each individual was assigned. The geographic origin of each individual is denoted for each locality sample such that more than 10% of the total of individuals is composed of the inferred cluster. A larger symbol indicates that a cluster was predominant in the locality sample (pairwise clusters ratio larger than 1:5) whereas equal symbol sizes indicate similar cluster contributions to the total individuals of each sample. B) Model-based clustering analyses (Pritchard *et al.* 2000) considering k = 2, where each individual is represented by a vertical line, each color refers to one cluster and the posterior probability of group membership is indicated. The dashed horizontal lines denote the arbitrary threshold of 0.15 used as a sign of possible interspecific hybridization. C) Posterior probability

of the model-based approach to identify species hybrids (Anderson & Thompson 2002). Each vertical line also refers to an individual and different colors indicate distinct classes of individuals: light gray: "pure" *A. germinans;* dark gray: "pure" *A. schaueriana*; dark orange: *A. germinans* second generation backcrossed individuals; and yellow: - F1 interspecific hybrid.

Discussion

A. schaueriana *microsatellite markers*

To complement the molecular markers developed for *A. germinans* (Nettel *et al.* 2005; Cerón-Souza *et al.* 2006; Mori *et al.* 2010) we characterized the first set of microsatellites for *A. schaueriana*. We found a variable degree of polymorphism among loci and a high transferability rate between these species (Tables S2, Supporting information). These markers, as shown herein, are valuable molecular tools to address a wide range of questions regarding these species.

Mating system, instraspecific genetic diversity and structure in A. germinans *and* A. schaueriana

Considering the progeny array of *A. schaueriana,* we found an intermediate outcrossing rate, which significantly departed from 0 and 1, indicating that this population displays a mixed mating system (Goodwillie *et al.* 2005). Comparing the t_a estimated for *A. schaueriana* from AsAJU (0.529) we observed that this variable closely approximated the t_m estimated by the progeny array (0.542 ± 0.062), indicating that inbreeding is a consistent feature of this population across generations. These results are in accordance with the species' reproductive biology as it is a self-compatible tree with a pollinator-dependent generalist pollination system (Nadia *et al.* 2013), similar to the gray mangrove *A. marina* (Clarke & Myerscough 1991), a widespread species from the IWP region. Regarding three *A.germinans* progeny arrays from the Pacific coast, comparable values of t_m (0.583 ± 0.09, 0.774 ± 0.09 and 0.770 ± 0.12 for each array) and proportions of the progeny that shared the same male parent for two of the three

progenies were found, indicating that this species may also exhibit moderate levels of self-fertilization. Considering the *A. germinans* mating system evaluations, there was evidence of much lower biparental inbreeding (Nettel *et al.* 2013). Unfortunately, these analyses are not readily comparable to ours, because we used more loci (13) than Nettel et al. (2013) (six), and the difference between t_m and t_s is sensitive to the number of loci considered in that more markers give values closer to the true difference between these parameters (Ritland 2002). However Cerón-Souza and colleagues (2012), using a different method, suggested that biparental inbreeding could indeed be relevant regarding the *A. germinans* mating system, indicating that inbreeding in these species might be impacted by both selfing and mating among relatives. Together, this evidence suggests that, generally, *A. germinans,* whose flowers may also be visited by a wide range of insects (Landry 2013), and *A. schaueriana* present similar mixed mating systems.

For both species, considering each species throughout the sampling range, we also found evidence of a mixed mating system (average t_a of 0.704 for *A. germinans* and 0.610 for *A. schaueriana*) and a varying degree of inbreeding for most of the evaluated samples (Table 2). Previous studies regarding *A. germinans* (Nettel *et al.* 2008; Cerón-Souza *et al.* 2012), *A. marina* (Maguire *et al.* 2000; Arnaud-Haond *et al.* 2006; but see Duke *et al.* 1998;) and *A. bicolor* (Nettel *et al.* 2008) agree with these findings. We did not observe, however, evidence of high inbreeding in the southernmost sample of *A. germinans* or the southernmost or northernmost limit samples of *A. schaueriana,* as reported for *A. marina* (Arnaud-Haond *et al.* 2006), that may be due to ecological, geographical and/or historical differences between these species. This mixed mating system involving a broad range of pollinators, may represent an adaptation (Vallejo-Marín & Uyenoyama 2004) that may be especially important to mangrove trees as colonizers (Burns & Ogden 1985; Tomlinson 1986) in terms of reproductive assurance (Goodwillie *et al.* 2005). These findings suggest that self-fertilization (and likely mating between relatives) is a frequent feature throughout the genus and has substantial

influence on the species' genetic structure due to pollen dispersal restrictions and, consequently, limited gene flow.

The pattern of genetic structure we observed for both species support these reproduction-related findings. We argue that this mixed mating system presenting biparental inbreeding influences the substantial genetic variation among the samples despite the different global measures of population differentiation. D was considerably lower for *A. schaueriana*, which may be explained by its low diversity within populations, likely due to the high levels of inbreeding and/or mating between relatives (Jost 2008). These summary values are comparable to previously reported for *A. germinans* (Nettel *et al.* 2008; Cerón-Souza *et al.* 2012), *A. bicolor* (Nettel *et al.* 2008) and *A. marina* (Maguire *et al.* 2000). Pairwise comparisons considering the different population differentiation measurements were correlated and significant for every pair of samples (Fig. 2). This finding indicates that, despite some low values of G_{ST} and D, even for geographically close samples there are significant genetic differences, which is in accordance with multivariate and model-based assignment analyses, whose results are quite similar.

Considering the fine-scale genetic structure from the DAPC and Bayesian population assignment method, with k = 8 for *A. germinans* k = 4 for AsN and k = 6 for AsS, we observed well-defined groups at the local and regional scales. Even where there is no clear physical barrier to pollen and propagule dispersal, for instance between AsSAL, AsAJU, AsPRM and AsALC or between AgPAa, AgPAb or AgALC, samples within the world's largest continuous area covered with mangrove forests (Nascimento *et al.* 2013), there is substantial genetic structure (Figs. 5 and 6). AMOVA also supported this fine scale genetic structure pattern as both species had significant values of the fixation indexes when the variation among population within groups was considered (Table 3). For instance, the clear differentiation between AgPAa and AgPAb was remarkable. These populations are geographically close, few kilometers distant from each other, but are under distinct hydrographic regimen (Menezes *et al.* 2008). In

this case, assuming microsatellites as neutral genetic markers, we argue that the tide plays an important role in shaping the genetic diversity of *Avicennia.* We also recognize that, despite the putative neutrality of the microsatellites, this phenomenon might be enhanced by selective pressures that could be acting on these neutral markers by the hitchhiking effect (Kaplan *et al.* 1989) leading to even more differentiated populations. Thus, hydrographic patterns with a low-frequency tide play an important role as a barrier to dispersal, as expected from the water-based dispersal of mangrove propagules. There was only one likely admixed individual within the AgPAa sample indicating that there is also a limited pollen dispersal constraint, which may be enhanced by the species' mating system. In this sense, despite the evidence of LDD for *A. germinans* (Nettel & Dodd 2007), our results indicate that, regardless of its significant evolutionary consequences (Duke *et al.* 2002; Nathan *et al.* 2008), these phenomena are sufficiently rare that *A. germinans* and *A. schaueriana* populations present divergent gene pools even at the local and regional geographic scales.

Therefore, current and previous evidence indicates that the mating system of these species pollen and propagule dispersal constraints influence their genetic structure on the small geographic scale. However, despite the genetic divergence of these local and regional spatial scales, there is a positive relationship between genetic and geographic distances, leading to a pattern of IBD (Fig. 3). As reported for *R. mangle* (Pil *et al.* 2011; Takayama *et al.* 2013) and for *Hibiscus pernambucensis* (Takayama *et al.* 2008), there is substantial divergence between samples from locales north and south of the NEESA within both *A. germinans* and *A. schaueriana*. For most of the approaches we considered, the most pronounced indications of genetic structure emerged when this divergence was taken into account. Pairwise values of G_{ST} and D were the highest for pairs of samples between these regions (Fig. 2). The DAPC analyses suggest that the greatest differentiation was found between these northern and southern groups, compared to samples within each cluster (Fig. 5A and 6A). The ΔK quantities were also highest for k = 2 for both *A. germinans* and *A. schaueriana* (Fig. 4).

For the latter species, the amount of variation between these groups was 45.88% by AMOVA, and lower variation, although significant, was added when we contemplated more levels of the hierarchy. Considering the former species however, regardless of the mutation model assumed, the between-group fixation indexes were not significant (Table 3) probably because AgTMD was the only sample of AgS and this species exhibits substantial local and regional genetic structure.

As previously discussed (Takayama *et al.* 2008, 2013; Pil *et al.* 2011), the bifurcation of the southern branch of the South Equatorial Current (SEC) into the Brazil Current (BC) and the cross-equatorial North Brazil Current (NBC - northwestward) (Fig. 1 - Lumpkin & Johnson, 2013) constrains the movement of propagules between the northern and southern groups. Ind addition acting as a barrier, the branching of the SEC and the high velocity of the NBC compared to the BC (Lumpkin & Johnson 2013) favor the migration of individuals from the southern to the northern regions, leading to a higher frequency of admixture events in the latter region. This pattern is readily observed when the Structure results are taken into account (Figs. 5 and 6). For instance, considering *A. germinans,* for k = 2, the absence of admixed individuals in the TMD sample and their presence in AgPRC follow the direction of the NBC marine current. Additionally, for *A. schaueriana,* the same pattern is found: for k = 2 there is little evidences for admixture in the southern group while there is substantial evidence admixed individual in AsALC and AsPRC samples. Moreover, regarding AsN, there are indications that the propagule flow follows the NBC direction. In contrast, AsS presents a more complicated pattern in which there is no single direction of admixed individuals for k = 2 and k = 6 (Fig. 5B). This result might be related to the slower mean velocity of the BC and its seasonal variation in terms of velocity and direction (Lumpkin & Johnson 2013) in southern and southeastern Brazil, where AsGPM, AsUBA and AsCNN were sampled. In other words, not only the direction but also the speed and seasonal variance of the marine currents play an important role in the genetic diversity of these sea-dispersed plants in multiple geographic scales.

Ongoing hybridization between A. germinans *and* A. schaueriana

There is evidence of ancient introgression between *A. germinans* and *A. bicolor,* a species with a limited distribution on the Pacific coast of Central America (Tomlinson 1986), and suggestions of chloroplast capture between *A. germinans* and *A. schaueriana* in the Atlantic basin (Nettel *et al.* 2008). Although we were not able to evaluate any chloroplast genome marker, we found evidences that ongoing hybridization between these species is indeed occurring within the *A. germinans* and *A. schaueriana* sympatry zone. To the best of our knowledge, this is the first report of ongoing hybridization in *Avicennia.*

These species present different gene pools which is clear when they are compared using the pairwise G_{ST} and D between samples, or by the DAPC approach in which the genetic diversity between each species is much greater compared to the variation within each species (Fig. 2 and 7), which is in accordance with the AMOVA at the interspecific level of the hierarchy (Table 3). These analyses in addition to differentiating the two species, also recover the previously discussed genetic structure within each species. The multivariate assignment approach suggests a likely interspecific hybrid identified as *A. germinans* from AgPRC, graphically located between each cluster of individuals representing each species (Fig. 7A). Further Bayesian evaluations not only corroborated the likely hybrid identified with DAPC as an F_1 hybrid but also assigned other *A. germinans* individuals from AgALC as likely two-generation backcrosses between F_1 hybrids and this species (Fig. 7C), indicating that eventual hybrids are fertile, at least considering the mating with *A. germinans.* The Bayesian methods also revealed that the interspecific hybridization is likely unidirectional because only individuals identified as *A. germinans* presented evidences of ongoing admixture between the species. These species share similar pollinators (Landry 2013; Nadia *et al.* 2013) and flower traits (Tomlinson 1986; Nadia *et al.* 2013) and there is a reported reproductive phenological overlap (Menezes *et al.* 2008) indicating that hybridization is possible between these species. Additionally, *A.*

germinans is more commonly found in Alcântara, Maranhão, Brazil (Menezes *et al.* 2008), but was much less abundant in Paracuru, Ceará, throughout our sampling. These findings indicate that post-zygotic mechanisms are related to this asymmetric hybridization, as previously observed for other mangrove genera (Zhou *et al.* 2008; Zhang *et al.* 2013). We consider this matter worthy of further investigation in terms of the mechanisms that generate and maintain this unidirectional hybridization.

The ancient introgression between *A. bicolor* and *A. germinans* observed on the Pacific coast of Central America (Nettel *et al.* 2008) was related to a higher diversity in that region compared to the Atlantic coast (Nettel *et al.* 2008; Cerón-Souza *et al.* 2012). Considering the South American Atlantic coast, this association is not possible because AgAJU and AgPRC do not show higher genetic diversity in terms of the number of alleles or expected heterozygosity compared to locales where we found no evidence of hybridization (Table 2). Thus, we argue that ongoing unidirectional hybridization does not increase the genetic diversity of *A. germinans* across the samples we evaluated. This possibility may indicate that the interspecific mating is recent and too little time has passed for *A. schaueriana* alleles to spread among the *A. germinans* populations or even that natural selection acts against the hybrid reducing the spread of its alleles. Once again, although we may hypothesize about the consequences of the observed hybridization, we encourage further investigations to better explore the impacts of this interspecific hybridization not only on the genetic diversity of populations but also on the individual phenotype to evaluate the hybrid's fitness, for instance.

Concluding remarks

Using the first set of microsatellites developed for *A. schaueriana* and markers previously developed for *A. germinans,* we studied three aspects of these species biology: the existence of hybridization between them, their mating system, and how the neutral genetic variation is organized on different geographic scales. Our results suggests that an interplay between intrinsic (mating system, limited pollen and

propagule dispersal, but not hybridization) and extrinsic factors (marine currents and tide) shape the genetic diversity of *A. germinans* and *A. schaueriana* leading to a genetic diversity structured on micro-, meso- and macro-scales for both of the *Avicennia* species.

Similar patterns of the organization of neutral genetic variation have been observed in different taxa of mangrove species. For instance, a large-scale genetic structure was reported for *Rhizophora mangle* (Pil *et al.* 2011; Takayama *et al.* 2013), but its genetic diversity is also locally and regionally organized on smaller scales (Cerón-Souza *et al.* 2012). This multiple-scale genetic structure is observed in these phylogenetically distant species regardless of, for instance, the different propagule features of each genus (Rabinowitz 1978; Clarke *et al.* 2001). The generality or specificity of these findings and the mechanisms that generate these similar patterns remain to be evaluated in unstudied mangrove species or using a landscape genetics approach (Storfer *et al.* 2010), but our investigation of three geographic scales indicates that for two Neotropical *Avicennia* species this multiple-geographic-scale mangrove species genetic structure pattern is consistent. Moreover, we are aware that these different spatial scales imply distinct temporal scales of evolutionary response. In this sense, our current effort is to answer questions regarding the processes that maintain the patterns we observed and the processes that generated them.

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Supporting information

Table S1. Characteristics of nine polymorphic microsatellite markers developed for *Avicennia schaueriana.* The primer pair sequences, expected size based on the clone fragment, repeat motif, optimal PCR amplification conditions (AC) and GenBank accession number are shown for each marker. TD65-55 indicates touchdown PCR with temperatures ranging from 65 to 55°C.

Marker	Primer sequence (5'-3')	Size (bp)	Repeat motif	AC(ºC)	GenBank
Aschau1-01	F: AACGACAAACCATTAGAAACCAA R: CAATTGAATTTTCTGATTCCCTAA	219	$(tg)_{21}...(at)_6$	46.7	KC783259
Aschau1-02	F: ACACTACACCCTTCAGCTCAATAA R: ACCCCCAATGGTAGGACAT	150	$(ac)_{15}$	60	KC783260
Aschau1-03	F: GCGGTATCTCCCGTGATTT R: TAGAGGGGAGATTGGTGTGG	227	$(ca)_9gc(ca)_{14}$	60	KC783261
Aschau1-04	F: ACGTAAGCTGTGGACGAAGG R: AAGGGATGGGAAGTGGATTC	218	$(ct)_6(ac)_6gc(ac)_5$	60	KC783262
Aschau1-05	F: TCTAATTGGACGATGGCAGA R: TGTAGCTGAAATTCCCCTTTTT	179	$(gt)_4gc(gt)_5tt(gt)_4$	60	KC783263
Aschau1-06	F: AACGTTTTGCCTACACCCTCT R: GCAAGAACTATCGTTCCATCA	176	$(ca)_{14}$	60	KC783264
Aschau1-07	F: TGGCAGATGTGTCTTCCTGA R: CCTCAGACTTGAATCAGCAGTG	209	$(tg)_{11}$	56.7	KC783265
Aschau1-08	F: AATAATTAAGCATCCACTCG R: TTTAACTTGATGAGGAACTTG	174	$(gt)_{14}…(gt)_6$	TD 65-55	KC783266
Aschau1-09	F: TATCCCTTTGCATTGTTTGAGT R: TTTCAACTCAACTTCATCCT	202	$(ca)_{21}$	60	KC783267

Table S2. Characteristics of the microsatellite markers developed for *Avicennia germinans* previously developed (Nettel *et al.* 2005; Cerón-Souza *et al.* 2006; Mori *et al.* 2010) and for *A. schaueriana,* described herein. The expected size based on the clone fragment, repeat motif, optimal PCR amplification conditions (AC) are shown for each marker. TD65-55 indicates touchdown PCR with temperatures ranging from 65 to 55°C. P indicates polymorphic and M denotes monomorphic marker for *A. germinans* (Ag) and *A. schaueriana* (As). * indicates evidence of null alleles for three or more samples.

Marker	Marca	Repeat motif	AC	Reference	Ag	As	Both
Agerm1-02	Ag1A6	$(ca)_{11}$	53.4ºC	(Mori *et al.* 2010)	P	P	P
Agerm1-03	Ag1A9	$(ca)_{9}$	48.2ºC	(Mori *et al.* 2010)	P	P	P
Agerm1-06	Ag1B9	$(gt)_{6}$	63.4ºC	(Mori *et al.* 2010)	P	P	P
Agerm1-07	Ag1C1	$(gt)_{9}$	50.5ºC	(Mori *et al.* 2010)	P	P	P
Agerm1-08	Ag1D1	$(tg)_{9}$	TD65-55	(Mori *et al.* 2010)	M	P	P
Agerm1-12	Ag1E5	$(ac)_{15}$	53.4°C	(Mori *et al.* 2010)	P	M	P
Agerm1-14	Ag1E11	$(ca)_{8}(at)_{6}$	59.6°C	(Mori *et al.* 2010)	P	M	P
Agerm1-15	Ag1E12	$(ca)_{4}...(ac)_{13}$	56.7°C	(Mori *et al.* 2010)	P	M	P
Agerm1-16	Ag1F1	$(tg)_{9}$	53.4°C	(Mori *et al.* 2010)	P	-	-
Agerm1-18*	Ag1F8	$(ag)_{16}$	63ºC	(Mori *et al.* 2010)	P	P	P
Agerm1-21*	Ag1G4	$(ca)_{8}$	61.8°C	(Mori *et al.* 2010)	P	-	-
Agerm1-22	Ag1H3	$(tttctt)_{4}$	63.4°C	(Mori *et al.* 2010)	P	M	P
Agerm1-25	Ag1H12	$(tg)_{10}...(tg)_{4}$	65.5°C	(Mori *et al.* 2010)	P	-	-
CTT01		$(ctt)_{8}$	TD65-55	(Cerón-Souza *et al.* 2006)	P	P	P
T7*		$(cat)_{2}(at)_{3}(gtat)_{5}$	48.2	(Nettel *et al.* 2005)	P	P	P
T8		$(tgta)_{6}$	59.6	(Nettel *et al.* 2005)	P	-	-
Aschau1-01		$(tg)_{21}...(at)_{6}$	46.7	This work	P	P	P
Aschau1-02		$(ac)_{15}$	60	This work	P	P	P
Aschau1-03		$(ca)_{9}gc(ca)_{14}$	60	This work	P	P	P
Aschau1-04		$(ct)_{6(}ac)_{6}gc(ac)_{5}$	60	This work	P	P	P
Aschau1-05		$(gt)_{4}gc(gt)_{5}tt(gt)_{4}$	60	This work	P	P	P
Aschau1-06		$(ca)_{14}$	60	This work	P	P	P
Aschau1-07		$(tg)_{11}$	56.7	This work	-	P	-
Aschau1-08		$(gt)_{14}…(gt)_{6}$	TD 65-55	This work	P	P	P
Aschau1-09		$(ca)_{21}$	60	This work	M	P	P

Table S3. Pairwise G_{ST} regarding *A. germinans* samples considering (above diagonal) and not considering null alleles (below diagonal) using the method implemented in FreeNA (Chapuis & Estoup 2007).

	AgALC	AgMRJ	AgPAa	AgPAb	AgTMD	AgPNB	AgPRC
AgALC		0.15248	0.194459	0.105602	0.52152	0.08938	0.116662
AgMRJ	0.154614		0.197007	0.056699	0.422091	0.263621	0.166099
AgPAa	0.19817	0.199035		0.16896	0.58455	0.330845	0.234802
AgPAb	0.111851	0.055144	0.174333		0.422717	0.19299	0.128432
AgTMD	0.544967	0.44249	0.601351	0.448288		0.626313	0.606566
AgPNB	0.090494	0.267037	0.339511	0.200083	0.649346		0.247768
AgPRC	0.131166	0.194667	0.248797	0.149596	0.639252	0.261579	

Table S4. Pairwise G_{ST} regarding *A. schaueriana* samples considering (above diagonal) and not considering null alleles (below diagonal) using the method implemented in FreeNA (Chapuis & Estoup 2007).

	AsAJU	AsPRM	AsSAL	AsGPM	AsVER	AsALC	AsPPR	AsFLN	AsCNN	AsUBA	AsPRC
AsAJU		0.013171	0.016007	0.511992	0.522756	0.283788	0.513524	0.561715	0.519784	0.595578	0.436597
AsPRM	0.006418		0.01708	0.517269	0.526272	0.310654	0.515317	0.561235	0.523999	0.597374	0.449071
AsSAL	0.006666	0.007095		0.553491	0.567828	0.304543	0.55792	0.602303	0.563873	0.665052	0.510289
AsGPM	0.531818	0.531387	0.55842		0.266205	0.465435	0.147969	0.174467	0.159345	0.451664	0.556271
AsVER	0.541768	0.539382	0.573117	0.293845		0.457831	0.242601	0.318235	0.279517	0.569099	0.609898
AsALC	0.30497	0.323484	0.309449	0.48163	0.477107		0.47137	0.513226	0.480749	0.570962	0.500903
AsPPR	0.529528	0.523881	0.558169	0.169841	0.264906	0.482366		0.070697	0.179505	0.428488	0.579456
AsFLN	0.585392	0.579374	0.615636	0.206552	0.35121	0.537977	0.075175		0.203604	0.36852	0.595592
AsCNN	0.53547	0.53481	0.567241	0.172109	0.29008	0.495385	0.198356	0.228261		0.437516	0.588892
AsUBA	0.612865	0.60732	0.67098	0.478236	0.598174	0.587784	0.454288	0.416079	0.484246		0.68663
AsPRC	0.456768	0.459293	0.51787	0.575367	0.629219	0.518806	0.596456	0.625324	0.613709	0.712541	

Suplementary material. Pairwise measuremnts of the genetic variation among samples in terms of G_{ST} (Nei 1973), D (Jost 2008) regarding A) *A. germinans* and *A. schaueriana;* B) *A. germinans* and C) *A. schaueriana.*

A) *A. germinans* and *A. schaueriana* - G_{ST} (above diagona), D (below diagonal)

	AgMRJ	AgPAa	AgPAb	AgALC	AgPNB	AgPRC	AgTMD	AsSAL	AsAJU	AsPRM	AsALC	AsPRC	AsVER	AsGUA	AsUBA	AsCNN	AsPPR	AsFLN
AgMRJ		0.0923	0.03	0.0952	0.1646	0.1118	0.2528	0.4078	0.4008	0.3959	0.3979	0.4629	0.4482	0.4481	0.499	0.4447	0.4466	0.4583
AgPAa	0.1353		0.0697	0.079	0.1563	0.1234	0.3741	0.4677	0.4605	0.455	0.4614	0.5236	0.51	0.5077	0.5627	0.5061	0.5078	0.519
AgPAb	0.0601	0.1116		0.0646	0.1129	0.0824	0.2589	0.414	0.407	0.402	0.4077	0.4708	0.4576	0.4562	0.5075	0.4568	0.456	0.4669
AgALC	0.1923	0.0961	0.1222		0.0481	0.062	0.3597	0.436	0.4296	0.4239	0.4237	0.4891	0.4794	0.478	0.533	0.4822	0.4796	0.4902
AgPNB	0.2517	0.1713	0.1487	0.0285		0.0958	0.4387	0.5334	0.5251	0.5188	0.5228	0.5861	0.5729	0.57	0.6321	0.5745	0.572	0.5832
AgPRC	0.2123	0.1774	0.1403	0.0856	0.0909		0.3231	0.3913	0.3813	0.3797	0.3628	0.4299	0.4271	0.4225	0.4826	0.4284	0.4283	0.4354
AgTMD	0.22	0.3176	0.2364	0.3743	0.2898	0.2826		0.6823	0.6685	0.6648	0.67	0.7633	0.745	0.7386	0.8137	0.7352	0.7407	0.7541
AsSAL	0.9324	0.9723	0.9308	0.9053	0.9456	0.7277	0.8949		0.0045	0.0074	0.1918	0.3351	0.407	0.385	0.4838	0.3919	0.387	0.4121
AsAJU	0.9356	0.9769	0.9357	0.9117	0.9533	0.7122	0.8825	0.0003		0.0054	0.1809	0.3059	0.3883	0.3678	0.4604	0.3702	0.3695	0.3922
AsPRM	0.9334	0.9753	0.9315	0.9075	0.9489	0.7203	0.9044	0.0014	0.0017		0.2025	0.308	0.3935	0.3746	0.4615	0.3776	0.3721	0.3919
AsALC	0.8922	0.9681	0.9194	0.8399	0.9034	0.6012	0.8877	0.0656	0.0612	0.0852		0.3206	0.3278	0.3247	0.4201	0.3359	0.3285	0.3465
AsPRC	0.9348	0.9369	0.9314	0.8483	0.8235	0.6516	0.8975	0.0996	0.0884	0.1016	0.1082		0.4197	0.3466	0.4826	0.385	0.3663	0.3879
AsVER	0.9119	0.9244	0.9178	0.8474	0.8111	0.6726	0.8866	0.2002	0.1932	0.2102	0.1345	0.1123		0.1914	0.4328	0.1815	0.1644	0.221
AsGUA	0.9196	0.9318	0.925	0.8602	0.8244	0.6613	0.8679	0.1804	0.179	0.1968	0.1388	0.0689	0.0291		0.3244	0.1001	0.102	0.1223
AsUBA	0.9348	0.9429	0.9305	0.8814	0.8476	0.7029	0.8813	0.1873	0.1721	0.1858	0.1438	0.0843	0.0831	0.0457		0.3228	0.2917	0.2749
AsCNN	0.8975	0.9243	0.9396	0.9124	0.8868	0.7014	0.8167	0.1805	0.167	0.1864	0.1551	0.1018	0.0311	0.0221	0.0494		0.1179	0.1312
AsPPR	0.9204	0.9335	0.9222	0.8771	0.8458	0.693	0.8823	0.1678	0.1649	0.1752	0.1423	0.0813	0.0359	0.013	0.0301	0.021		0.0389
AsFLN	0.9263	0.9377	0.9235	0.874	0.8406	0.6834	0.8891	0.188	0.1814	0.1831	0.1422	0.0866	0.0386	0.0173	0.0215	0.0214	0.0031	

B) *A. germinans* - G_{ST} (above diagona), D (below diagonal)

	AgMRJ	AgPAa	AgPAb	AgALC	AgPNB	AgPRC	AgTMD
AgMRJ		0.111	0.0279	0.0855	0.1556	0.1124	0.283
AgPAa	0.1491		0.0949	0.1111	0.2029	0.1292	0.4216
AgPAb	0.0494	0.1252		0.0606	0.1103	0.0856	0.2812
AgALC	0.1563	0.137	0.1047		0.0483	0.0709	0.3637
AgPNB	0.2163	0.216	0.1353	0.0277		0.1266	0.4691
AgPRC	0.221	0.1872	0.1516	0.1094	0.1406		0.3012
AgTMD	0.268	0.3743	0.2575	0.4091	0.3425	0.2711	

C) *A. schaueriana* - G_{ST} (above diagona), D (below diagonal)

	AsSAL	AsAJU	AsPRM	AsALC	AsPRC	AsVER	AsGUA	AsUBA	AsCNN	AsPPR	AsFLN
AsSAL		0.0058	0.0067	0.1842	0.3313	0.3924	0.377	0.475	0.3843	0.3797	0.4075
AsAJU	0.0005		0.0049	0.1827	0.2991	0.3835	0.3706	0.4631	0.3738	0.3734	0.3989
AsPRM	0.0013	0.0016		0.1989	0.3036	0.3846	0.3726	0.4593	0.3759	0.3711	0.394
AsALC	0.0652	0.068	0.0882		0.3413	0.3145	0.3151	0.4104	0.3262	0.3196	0.3399
AsPRC	0.1036	0.0855	0.1012	0.1251		0.4469	0.3937	0.5303	0.428	0.413	0.4401
AsVER	0.1958	0.2006	0.2116	0.1224	0.1316		0.1843	0.4236	0.1755	0.1588	0.2163
AsGUA	0.1807	0.1901	0.2025	0.1277	0.0875	0.0265		0.32	0.0969	0.0991	0.1211
AsUBA	0.1895	0.1848	0.1939	0.1334	0.1045	0.0761	0.0416		0.3178	0.288	0.2746
AsCNN	0.1823	0.1798	0.1941	0.1433	0.1229	0.0285	0.0201	0.045		0.1148	0.1292
AsPPR	0.1701	0.1776	0.1832	0.1315	0.1013	0.0329	0.0118	0.0274	0.0191		0.0391
AsFLN	0.1903	0.1942	0.1914	0.132	0.1068	0.0355	0.0157	0.0195	0.0196	0.0029	

Capítulo III

Phylogeographic patterns explain the species distribution and reveal ongoing introgressive hybridization and trans-Atlantic dispersal in western *Avicennia* species

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Running title: Phylogeographic patterns of Western Mangroves

Author's contributions: GMM conceived and designed this study, collected materials, performed experiments, analyzed the data and wrote the paper with assistance of MIZ, IS and APS. MIZ contributed to data analyses. IS supported the conception of the project and provided field support. APS received funding and provided laboratory and molecular biology facilities. All authors read, reviewed and approved the final manuscript.

Abstract

Mangrove plants grow within the intertidal zone of tropical and subtropical zones worldwide. Their global latitudinal distribution is mainly influenced by climatic and oceanographic features such that, because of current climate changes, poleward range expansions have been reported in both major biogeographic regions of mangrove forests, the Western and Eastern Hemispheres. In the past, there is evidence that mangrove forests have also responded similarly after the last glaciation expanding their ranges to warmer regions. In this context, the use of genetic tools is an informative approach to understand how historical processes and factors impact the distribution of mangrove species. We investigated the phylogeographic patterns of Neotropical *Avicennia* species using nuclear and chloroplast genomes markers. Our results indicate that although *A. bicolor, A. germinans* and *A. schaueriana* are independent lineages, introgressive hybridization between *A. bicolor* and *A. germinans* and *A. schaueriana* and *A. germinans* is an relevant evolutionary process. They also reinforce the role of long-distance dispersal in widespread mangrove species, such as *A. germinans*, for which we observed signs of transatlantic dispersal, a process that have likely contributed to the large extent of the *A. germinans* distribution. However, in the southern coast of South America, *A. schaueriana* is the only representative of the genus. This pattern of distribution of *A. germinans* and *A. schaueriana* is explained by different responses to the past climate changes and by their unequal historical effectiveness of relative gene flow by propagules and pollen.

Key-words: Phylogeography; introgression, cpDNA, nDNA, transoceanic dispersal, demographic expansion

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Introduction

Mangrove forests are unique tree communities that occupy narrow elevation ranges within the intertidal zones of tropical and subtropical regions. The few species that occupy these forests are characterized by physiological and ecological traits that make them highly adapted to the coastal environment (Tomlinson 1986). The latitudinal distribution of these organisms is mainly determined by both climatic and oceanographic features, including the occurrence of frosts, air and sea surface temperature, precipitation and a suitable intertidal habitat (Duke *et al.* 1998; Pickens & Hester 2010; Mckee *et al.* 2012; Quisthoudt *et al.* 2012; Saintilan *et al.* 2013). In the context of the recent global climate changes, there is evidence that these species are currently expanding their geographic distributions poleward within the two major mangrove biogeographic regions, the Atlantic Caribbean East-Pacific region (ACEP) (Perry & Mendelssohn 2009; Comeaux *et al.* 2012; Soares *et al.* 2012; Osland *et al.* 2013; Saintilan *et al.* 2013) and the Indo West-Pacific region (IWP) (Saintilan & Williams 1999; Adams *et al.* 2004; Lovelock *et al.* 2007; Stokes *et al.* 2010). As would be expected from this evidence of current expansion, palynological and stratigraphic data indicate that in the recent past (from the late Holocene and Pleistocene), these climatic alterations have also influenced the worldwide distribution of mangroves (Ellison 1996, 2008; Mckee *et al.* 2012). The use of genetic data is an interesting approach to complement the palynologic and stratigraphic methods and shed light on how the distribution of mangrove trees has changed over time and space.

In the ACEP region, for instance, it is reported that *Rhizophora mangle* L. (Rhizophoraceae) has expanded its distribution southward along the Brazilian coast since the Last Glacial Maximum (LGM) (Pil *et al.* 2011). Furthermore, in the northern

part of the ACEP biogeographic region, *Avicennia germinans* L. (Acanthaceae) populations have expanded their ranges northward since the last glaciation (Nettel & Dodd 2007). For both species, there is evidence of long-distance dispersal (LDD) (Nettel & Dodd 2007; Takayama *et al.* 2013), reinforcing the key role of dispersal as an important biogeographic mechanism in the process of population extinction and posterior recolonization (Nettel & Dodd 2007). To expand on these efforts and better understand how mangrove forests have been changing in response to historical factors and processes, we studied the phylogeographic patterns of two Neotropical *Avicennia* species: *A. germinans* and *A. schaueriana* Moldenke. The former is a widespread species, found throughout most of the ACEP region, whereas the latter is restricted to the Atlantic coast of South America and the Southern Caribbean (Tomlinson 1986; Schaeffer-Novelli *et al.* 1990; Spalding *et al.* 2010) (Fig. 1).

The genetic structure of these species is influenced by both intrinsic factors such as mixed mating system, biparental inbreeding, ongoing hybridization, limited pollen and propagule dispersal and, intriguingly, LDD (Nettel & Dodd 2007; Nettel *et al.* 2008, 2013, Mori *et al.,*submitted) and extrinsic factors such as marine currents and tide patterns (Mori *et al.,*submitted). The combination of this complex set of ecological features that shape the genetic diversity of *A. germinans* and *A. schaueriana* and historical processes, such as global climate changes after the LGM may shed light on possible explanations for the current distribution of mangrove species in South America (Tomlinson 1986; Schaeffer-Novelli *et al.* 1990; Spalding *et al.* 2010).

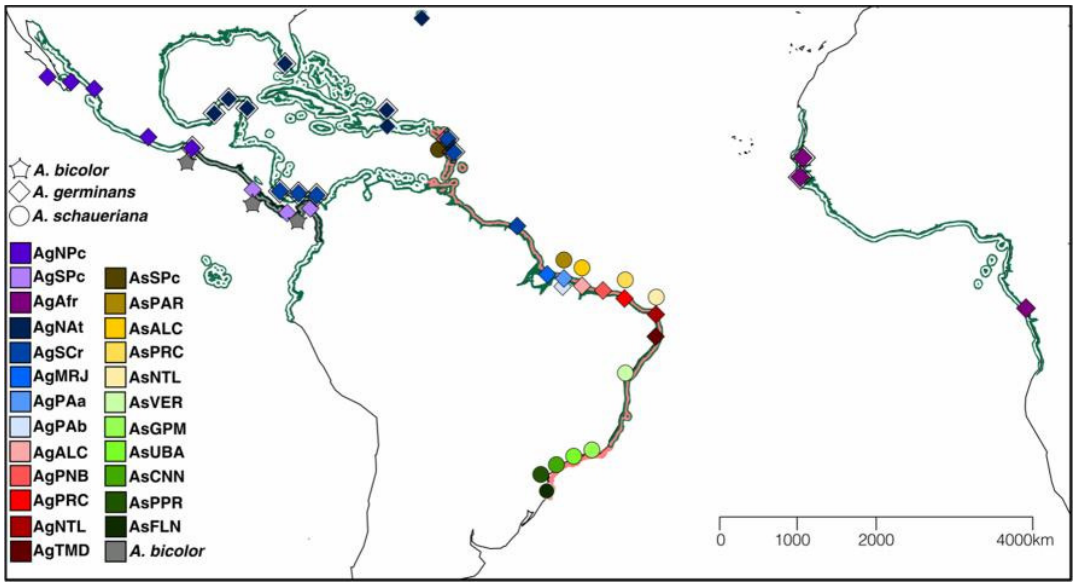


Figure 1. Locations of the samples of *Avicennia bicolor, A. germinans* and *A. schaueriana* (represented by the shape of the polygons) across the Western Hemisphere. The color of each polygon refers to the geographic region where the sample was obtained according to Table S1. The current geographic distribution of *A. bicolor, A. germinans* and *A. schaueriana* are shown along the coastlines of the continents in gray, green and red, respectively; the sympatry zones between the species are denoted by overlapping colors.

Through an intensive and extensive sampling along the Brazilian coastline coupled with samples from the Pacific coastline areas of Central America, the Caribbean and West Africa (Nettel & Dodd 2007; Nettel *et al.* 2008) and the sequencing of cpDNA and nDNA markers, we provide a large-scale assessment of the genetic variation of *Avicennia,* covering nearly the entire ACEP region. This strategy enabled us to gain a broad molecular perspective on the evolutionary history of the genus, including the three species found in this biogeographic region, *A. germinans, A. schaueriana* and *A. bicolor* Standl.; the distribution of *A. bicolor* is restricted to the Pacific coast of Central America (Fig. 1). This distribution of species is particularly interesting because there is evidence to suggest that there has been of hybridization between *A. germinans* and *A. bicolor* within their sympatry zone (Nettel *et al.* 2008) and between the *A. germinans* and *A. schaueriana* on the northern coast of South America (Nettel *et al.* 2008; Mori *et al.,* submitted). We also studied the geographic distribution

of the genetic diversity across the ACEP region to better comprehend the previously described complex interplay between the intrinsic and extrinsic factors that are influencing the species neutral genetic variation (Mori *et al.* submitted). We then evaluated the evidence for historical demographic fluctuations of *A. germinans* and *A. schaueriana* in response to past climate changes and historic ecological differences between them to explain the current pattern of species distribution along the South American continent.

Materials and methods

Plant material

We sampled 138 individuals of *A. germinans* and 193 of *A. schaueriana* from 11 localities along the Brazilian coastline; the samples were georeferenced using a global positioning system (Garmin 76CSx, considering the WGS84 standard) (Fig.1 and Table S1, Supporting material). For simplicity purposes, each sample is henceforth denoted as in Table S1, with Ag and As indicating *A. germinans* and *A. schaueriana*, respectively, followed by a three letter abbreviation corresponding to the site where the individuals were obtained. These species were identified in the field based both on their floral structures and vegetative branches (Tomlinson 1986) to minimize the chances of misidentification. Voucher specimens from every site, except for Alcântara, Maranhão were deposited in the EMBRAPA Amazônia Oriental (IAN) and University of Campinas (UEC) herbaria.

From each individual plant, we selected young and visually healthy leaves and maintained them in sealed bags containing silica gel; the samples were kept in these until they were lyophilized and stored at -20ºC. The desiccated material was then ground into a fine powder using liquid nitrogen, and the resulting powder was used to isolate total DNA via a cetyltrimethyl ammonium bromide protocol (as described in Mori *et al*. submitted).

Genetic analyses

To evaluate the distribution of the genetic variation, we sequenced two intergenic spacers of the chloroplastidial genome and one region of the nuclear ribosomal internal transcribed spacer (ITS). The *trn*D-*trn*T and *trn*H-*trn*K spacers of the cpDNA were amplified using the previously described DT and HK primer sets (Demesure *et al.* 1995), and the polymerase chain reaction (PCR) amplification of the ITS region was carried out using the previously described LEU1 and ITS4 primers (White *et al.* 1990). The sequencing reactions were performed using two primers specific to the *trn*D-*trn*T and ITS markers and the *trn*H-*trn*K locus was partially sequenced with primer H. The sequences were deposited in the DNA Data bank of Japan (Table 1). To augment the geographic distribution of our study and to include samples of *A. bicolor* and two species from the IWP, *A. alba* Blume and *A. marina* (Forssk.) Vierh., we also included previously analyzed sequences (Nettel & Dodd 2007; Nettel *et al.* 2008). We only considered data from *A. alba* and *A. marina* in the phylogenetic analyses and samples with more than eight individuals for population-level studies. Due to the differences in the publicly available sequences of previous studies (Nettel & Dodd 2007; Nettel *et al.* 2008), we considered different numbers of individuals for the cpDNA and nDNA markers (Table S1, Supporting material).

We assembled and manually verified the chromatograms using CLC Genomics Workbench 4.9 software (CLC Bio). When we detected evidence of heterozygotes, three new amplifications and sequencing reactions were carried out: only consistent double peaks were considered to be an indicator of a heterozygous site. The alignment and phasing of the whole dataset was performed using MUSCLE (Edgar 2004) and PHASE (Stephens *et al.* 2001), respectively, and the haplotypes were unambiguously reconstructed. Due to the assumed maternal heritance of the cpDNA with low recombination rate, the *trn*D-*trn*T and *trn*H-*trn*K spacers were concatenated, and will

henceforth be jointly referred to as DTHK. The nucleotide substitution models that best fit the data were selected using the method implemented in the jModelTest2 program (Darriba *et al.* 2012) given the Akaike Information Criterion(Akaike 1974); this method resulted in JC and GTR+I+G models for the cpDNA and nDNA markers, respectively. The former refers to the simplest substitution model (Jukes & Cantor 1969), and the latter refers to the general time-reversible model (Tavaré 1986) with invariant sites (I) and gamma-distributed rates (G), these models were used for phylogenetic analyses using PhyML 3.0 (Guindon *et al.* 2010) under a maximum-likelihood approach. The starting tree used for heuristic search was inferred using the BioNJ algorithm (Gascuel 1997), and we used the nearest neighbor interchanges (NNI) as the tree topology search operation. The reliability of the reconstructed genealogy was assessed by bootstrapping with 1,000 replicates. The sequence of *A. alba,* a mangrove tree from the IWP region was used as an outgroup. To further study the genealogical relationships among the ACEP region samples, we also applied a statistical parsimony implemented in the TCS v1.21 software (Clement *et al.* 2000) using default settings to consider multifurcations and/or reticulations in a phylogenetic network approach.

We next determined the haplotype frequencies of each sample and calculated the haplotype diversity (h), nucleotide diversity (π), and estimates of groups pairwise Φ_{ST} values, considering the haplotype frequency using Arlequin 3.5(Excoffier & Lischer 2010). The pairwise Φ_{ST} matrix was then dimensionally represented using a multidimensional scaling (MDS) in R software (R Core Team 2013). The global values of G_{ST} were inferred using DnaSp5.1 (Librado & Rozas 2009) considering gaps as the fifth state and haplotype data information (Nei 1973). Then, we estimated the pollen-to-seed migration ratio as ($r = m_p/m_s = \{(1/G_{STbipar} - 1)(1 + F_{IS})\} - 2\ (1/G_{STmat} - 1)/(1/G_{STmat} - 1)$) (Ennos 1994) given the global G_{ST} of each marker ($G_{STbipar}$ for ITS and G_{STmat} for DTHK) and the previous average values of F_{IS} estimated for *A. germinans* (0.174) and *A. schaueriana* (0.242) using microsatellites (Mori *et al.,* submitted).

Table 1. Analysis of molecular variance for different grouping models based on previous hypotheses regarding the genetic structure based on microsatellite markers, on the current distribution of mangrove forest (*a priori* hypotheses) and on the genealogical relationships of the haplotypes studied (*a posteriori* models). The acronyms refer to the geographic region where samples were obtained and are identical to those used in Table S1. The samples labeled "North Brazil" were obtained from the states of Pará, Maranhão, Piauí and Ceará, and the samples labeled "South Brazil" were the remaining samples from the Brazilian coastline regions. AMMC designates samples from the Amazon Macrotidal Mangrove Coast from Pará and Maranhão States.

A .germinans

Hypothesis		Hypothesized grouping	DTHK					ITS				
			Φ_{SC}	Φ_{ST}	Φ_{GT}	% Among groups	P Φ_{GT}	Φ_{SC}	Φ_{ST}	Φ_{GT}	% Among groups	P Φ_{GT}
Ag1	*a priori*	[Atlantic][Pacific]	0.631	0.222	-1.112	-111.210	0.17822+-0.00343	0.438	-0.115	-0.983	-98.340	0.10891+-0.00318
Ag2	*a priori*	[Atlantic][AgNPc][AgAgSPc]	0.668	0.385	-0.854	-85.440	0.85297+-0.00321	0.668	0.385	-0.854	-85.440	0.85297+-0.00321
Ag3	*a priori*	[North Brazil][South Brazil][Pacific]	0.722	0.313	-1.470	-147.010	0.83010+-0.00390	0.534	-0.331	-1.853	-185.350	0.52743+-0.00479
Ag4	*a priori*	[AgNAt][AgSAt][AgSPc][AgNPc]	0.837	0.802	-0.218	-21.780	0.35881+-0.00483	0.786	0.775	-0.052	-5.240	0.27693+-0.00469
Ag5	*a priori*	[AgSPc][AgNPc][AMMC][AgPNB][AgTMD]	-1.697	1.162	1.060	106.020	0.25505+-0.00461	2.015	1.082	0.919	91.920	0.15188+-0.00399
Ag6	*a posteriori*	[AgSPc][AgNPc][AgPAa][AgTMD] [AgPAb+AgTMD+AgPNB]	-0.107	1.117	1.106	110.560	0.05733+-0.00228	-2.430	1.149	1.043	104.340	0.32436+-0.00428
Ag7	*a posteriori*	[AgSPc][AgNPc][AMMC][AgPAa] [AgTMD][AgPNB]	-0.019	0.978	0.978	97.820	0.30198+-0.00477	-0.211	0.949	0.958	95.810	0.38851+-0.00487
Ag8	*a posteriori*	[AgSPc][AgNPc][AgPAa+AgTMD] [AgPab+AgMRJ+AgALC][AgPNB]	0.262	0.830	0.770	77.010	0.03465+-0.00186	7.792	1.028	0.996	99.590	0.77861+-0.00409

Table 1. continued

A. schaueriana

Hypothesis		Hypothesized grouping	DTHK Φ_{SC}	DTHK Φ_{ST}	DTHK Φ_{GT}	DTHK % Among groups	DTHK P Φ_{GT}	ITS Φ_{SC}	ITS Φ_{ST}	ITS Φ_{GT}	ITS % Among groups	ITS P Φ_{GT}
As1	*a priori*	[North Brazil][South Brazil]	0.491	-1.432	-3.775	-377.530	0.70446+-0.00405	0.255	4.543	5.756	575.630	0.99000+-0.00098
As2	*a priori*	[AMMC][AsPRC][South Brazil]	0.532	-1.321	-3.958	-395.810	0.67614+-0.00471	0.291	3.318	4.270	426.970	0.96515+-0.00183
As3	*a posteriori*	[AsPAR][AsALC+AsPRC][South Brazil]	-0.044	1.685	1.656	165.650	0.90881+-0.00277	0.333	3.617	4.922	492.150	0.97257+-0.00164
As4	*a posteriori*	[AsPAR][AsALC+AsPRC+ AsGPM+ AsPPR+AsVER+AsUBA+AsFLN+AsCNN]	-0.030	5.706	5.569	556.910	1.000	0.442	-0.056	-0.893	-89.280	1.00000+-0.00000
As5	*a posteriori*	[AsPAR][AsALC+AsPRC][AsGPM+AsPPR] [AsVER+AsUBA+AsFLN+AsCNN]	-0.145	1.080	1.070	106.990	0.96525+-0.00180	0.583	1.193	1.462	146.150	0.99436+-0.00081
As6	*a posteriori*	[AsPAR]{ AsALC+AsPRC][AsVER] [AsGPM+AsPPR+AsUBA+AsCNN][AsFLN]	-0.021	0.458	0.470	46.970	0.01406+-0.00122	3.619	1.065	0.975	97.510	0.01436+-0.00109
As7	*a posteriori*	[AsPAR][AsALC+AsPRC][AsVER][AsGPM] [AsUBA+AsCNN][AsPPR][AsFLN]	0.018	0.869	0.867	86.650	0.85396+-0.00306	-0.168	0.858	0.878	87.810	0.81059+-0.00375
As8	*a priori*	[AsPAR][AsALC][AsPRC][AsGPM] [AsPPR+AsVER+AsUBA][AsCNN+AsFLN]	-0.016	0.418	0.427	42.720	0.53287+-0.00505	0.001	0.882	0.882	88.220	0.89436+-0.00248
As9	*a priori*	[AsPAR][AsALC][AsPRC][AsGPM][AsVER] [AsUBA][AsPPR+AsCNN+AsFLN]	0.001	0.919	0.919	91.870	0.33327+-0.00491	-0.012	0.911	0.912	91.150	0.35238+-0.00448

To better understand the phylogeographic patterns of the observed genetic variation, and because the previously analyzed sequences were obtained from few samples from each location (Nettel & Dodd 2007; Nettel *et al.* 2008) , we arbitrarily grouped them into "geographic regions" according to previous studies (Nettel & Dodd 2007; Nettel *et al.* 2008, Mori *et al.* submitted) (Table S1, Supporting material). Employing the same software, Arlequin 3.5(Excoffier & Lischer 2010), we studied the geographic distribution of the genetic diversity using a hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) considering different hypotheses for cpDNA and nDNA for each species. We created different *a priori* hypotheses regarding *A. germinans* and *A. schaueriana* based on several factors: a)the geographic influences of the American continent, b) the effects of contemporary near-surface marine currents on the genetic diversity of ACEP mangrove species (Dodd *et al.* 2002; Nettel & Dodd 2007; Pil *et al.* 2011; Cerón-Souza *et al.* 2012; Takayama *et al.* 2013, Mori *et al.,* submitted), and c) on the forest continuum of the Amazon Macrotidal Mangrove Coast (AMMC) (Nascimento *et al.* 2013), which includes samples from Pará and Maranhão States, (Table 1). We also tested *a posteriori* groups regarding the genealogical analysis and the geographic distribution of haplotypes. The criteria to determine the best hypothesized arrangement were the significant departure from random distribution and the maximum variance among groups (Φ_{GT}).

We evaluated the demographic fluctuations using several summary statistics and considering the groups that best met the maximum significant Φ_{GT} criterion and also sample arrangements previously inferred using other genetic markers, such as microsatellites (Nettel & Dodd 2007; Nettel *et al.* 2008; Pil *et al.* 2011, Mori *et al.* submitted). This approach of evaluating two distinct scenarios is justified by the differences between the sets of markers that were previously used to study the genetic diversity and the markers we used herein. We evaluated different neutrality tests: Tajima's D (Tajima 1989) and Fu's F_S (Fu 1997) using Arlequin 3.5 (Excoffier & Lischer 2010) and D* and F* (Fu & Li 1993) computed with DnaSP 5.1 (Librado &

Rozas 2009). Assuming the loci to be selectively neutral, we justify the use of these statistics by their different statistical power and sensitivity to recombination (Ramírez-Soriano *et al.* 2008). We then considered Fu's F_S (Fu 1997) for DTHK marker and Tajima's D (Tajima 1989), D* and F* (Fu & Li 1993) for the ITS marker because, as expected, the latter presents more evidences of recombination than the former (data not shown). We then used Arlequin 3.5 to calculate the mismatch distribution of the observed number of differences between haplotype pairs to evaluate demographic expansions by analyzing the raggedness index (Rogers & Harpending 1992). These analyses of the distribution of pairwise differences were considered to be complementary evidence of demographic expansions when neutrality tests significantly departed from random distributions due to their conservativeness (Ramos-Onsins & Rozas 2002). For ITS marker, when only D* and F* (Fu & Li 1993) are significant, it indicates background selection it the mechanism underlying the polymorphism and the reverse suggests population growth (Fu 1997).

Results

To evaluate the distribution of the genetic diversity of three *Avicennia* species considering the Western-Hemisphere scale, we obtained samples of *A. germinans* and *A. schaueriana* from northeastern and southern South America and studied them together with previously evaluated samples using cpDNA and nDNA markers (Nettel & Dodd 2007; Nettel *et al.* 2008). The total number of individuals per sample and the descriptive statistics regarding the genetic diversity are shown in Table 1. The number of polymorphic sites we observed was 91 and 129 totaling 28 and 72 haplotypes for the DTHK and ITS loci, respectively; the haplotype (h) and nucleotide (π) diversities (Table 2) varied among the populations. As expected, each species has unique haplotypes for both markers, but we observed shared haplotypes between individuals identified as *A. germinans* and *A. schaueriana* along the northeastern coast of South

America. One of these shared ITS haplotypes was also observed in African *A. germinans* samples (Figs. 2 and 3).

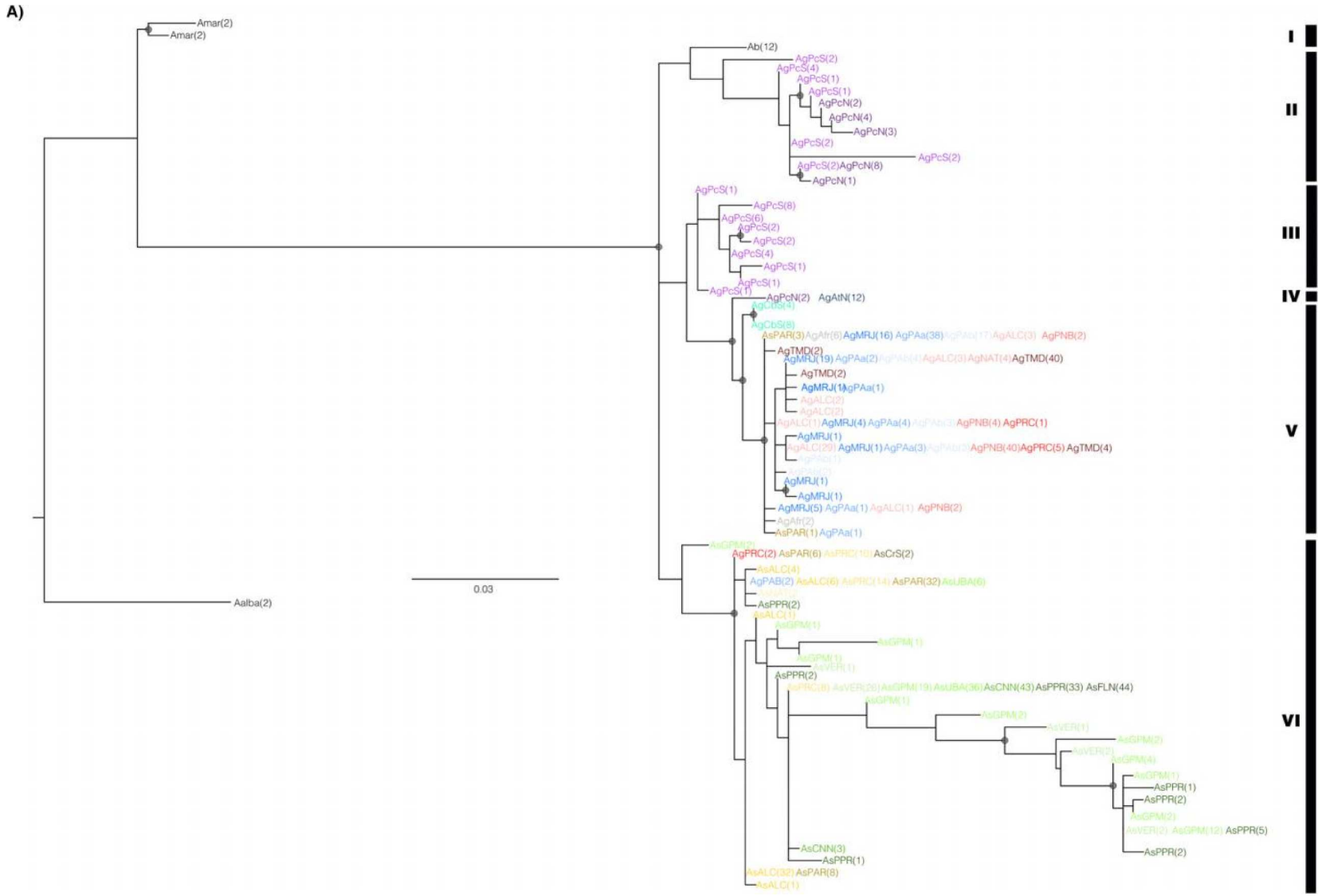
Genealogical relationships

The haplotype phylogenies of each markers indicate a deep divergence between IWP and ACEP species and an intricate relationship among the samples of the latter (Fig. 2 and 3). The most likely ITS phylogeny resolved six main clades that were mostly supported by bootstrap analyses (Fig. 2A). Clade I contains the *A. bicolor* haplotype, while clades II and III include samples of *A. germinans* from the Pacific basin, and clade IV contains samples of *A. germinans* from both the Northern Pacific and Atlantic regions. Samples of *A. germinans* from West Africa and from the southern Caribbean and from northeastern and southern coasts of South America comprise clade V, and *A. schaueriana* individuals constitute clade VI. Within clade VI, there is a highly divergent subgroup composed of the AsPPR, AsGPM and AsVER samples that is partially supported by the bootstrapping method.

Table 2. Descriptive statistics for the *Avicennia* samples evaluated.

	ITS						DTHK					
Statistics	n	N_{subst}	π	*h*	(SD)	N_{hap}	n	N_{subst}	π	*h*	(SD)	N_{hap}
A. alba	2	0	0	0	(0)	1	2	0	0	0	(0)	1
A. bicolor	12	0	0	0	(0)	1	12	0	7.091	0	(0)	1
A. marina	4	1	0.667	0.667	(0.204)	2	4	1	399.333	0.667	(0.204)	2
AgPacNorte	20	5	0.947	0.795	(0.065)	6	16	0	0	0.433	(0.138)	3
AgPacSul	40	10	3.358	0.922	(0.022)	16	36	1	0.203	0.298	(0.093)	3
AgAfrica	8	1	0.429	0.429	(0.169)	2	2	0	0	0	(0)	1
AgAtlanNorte	12	0	0	0	(0)	1	4	0	0	0	(0)	1
AgCaribeSul	14	1	2.901	0.264	(0.136)	2	2	0	0	0	(0)	1
AgMRJ	48	2	0.083	0.728	(0.041)	8	48	1	0.223	0.223	(0.072)	2
AgPAa	50	1	0.115	0.418	(0.086)	7	50	4	0.601	0.353	(0.083)	4
AgPAb	32	5	0.71	0.698	(0.08)	7	32	9	1.427	0.389	(0.106)	5
AgALC	42	1	0.418	0.519	(0.091)	8	42	8	2.499	0.4	(0.085)	3
AgPNB	48	1	0.284	0.301	(0.083)	4	48	0	0	0	(0)	1
AgPRC	8	4	1.821	0.607	(0.164)	3	8	8	3.857	0.429	(0.169)	2
AgNAT	4	0	0	0	(0)	1						
AgTMD	48	3	0.319	0.301	(0.083)	4	48	1	0.383	0.401	(0.072)	3
AsCaribeSul	2	0	0	0	(0)	1	2	0	0	1	(0.5)	2
AsPAR	52	4	0.51	0.59	(0.069)	6	52	11	1.988	0.793	(0.05)	12
AsALC	44	0	0	0.453	(0.085)	5	44	0	0	0	(0)	1
AsPRC	32	0	0	0.669	(0.035)	3	32	0	0	0	(0)	1
AsMAC	2	0	0	0	(0)	1	2	0	0	0	(0)	1
AsVER	32	8	1.196	0.341	(0.105)	5	32	2	0.242	0.234	(0.095)	3
AsGPM	48	5	2.097	0.781	(0.045)	12	48	1	0.082	0.082	(0.053)	2
AsUBA	42	0	0	0.251	(0.078)	2	42	0	0	0	(0)	1
AsCNN	46	0	0	0.125	(0.063)	2	46	0	0	0	(0)	1
AsPPR	46	4	1.337	0.477	(0.087)	7	46	1	0.085	0.085	(0.055)	2
AsFLN	44	0	0	0	(0)	1	44	2	0.178	0.254	(0.085)	4

The species names and abbreviation of samples are identical to those used in Table S1 and Figure 1. n, sample size; N_{subst}, number of substitutions; π, nucleotide diversity; *h* (SD), haplotype diversity and (standard deviation of haplotype diversity); N_{hap}, number of haplotypes.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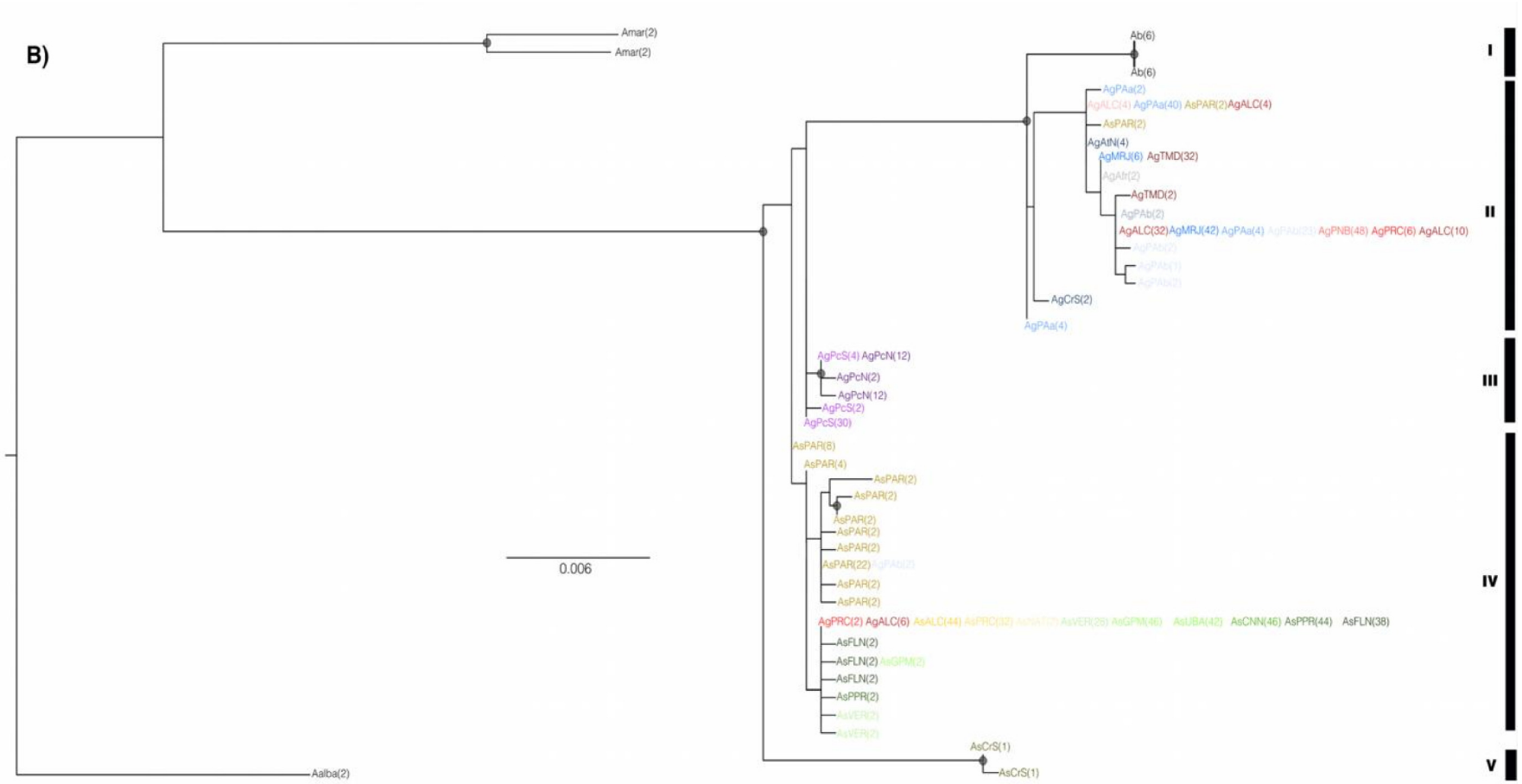

Figure 2. Maximum likelihood genealogy of *Avicennia* 72 and 28 haplotypes considering the A) ITS and B) DTHK markers, respectively. *Avicennia alba* is used as the outgroup. The bootstrap values greater than 60% are indicated with a circle. The clade labels used throughout the text are shown in vertical black rectangles. The samples are listed and colored according to the abbreviations and colors used in Table S1 and Figure 1.

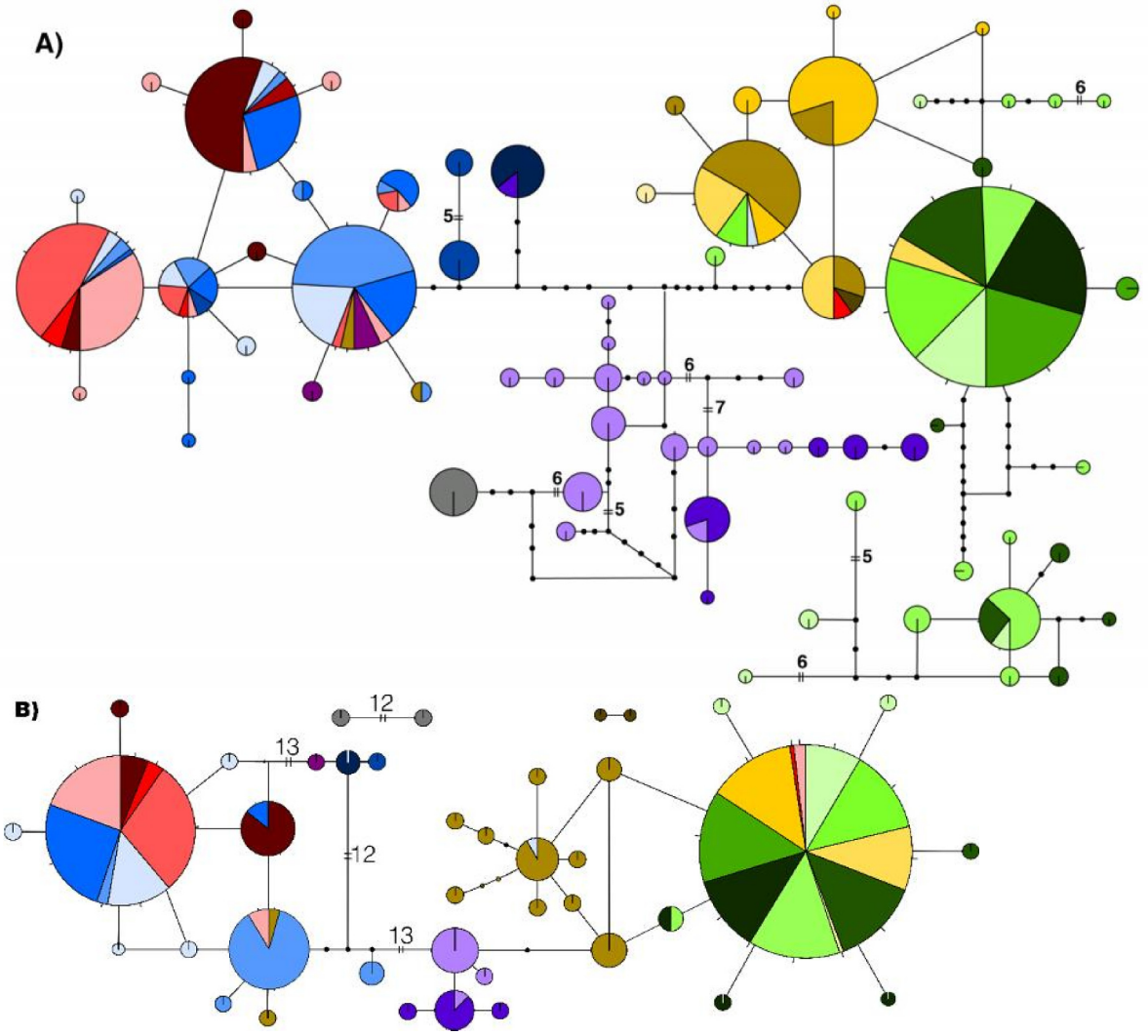

Figure 3. The haplotype networks of the A) ITS and B) DTHK markers considering the Western Hemisphere *Avicennia* species *A. bicolor, A. germinans* and *A. schaueriana.* Each line in the network refers to a single-nucleotide mutation, the double bars combined with numbers indicate the number of mutations between haplotypes, and the black dots indicate missing haplotypes in the samples. The circles denote unique haplotypes and are proportional to the number of sequences, with colors representing the samples according to Table S1 and Figure 1.

Regarding the phylogeny resulted from the DTHK marker (Fig. 2B), we observed five different main clades. Clade I contains the samples from *A. bicolor,* indicating that these samples are closely to clade II, which is composed of the Atlantic American and African samples of *A. germinans.* The samples of this species from the Pacific basin of Central America were grouped with little bootstrap support into clade III. *A. schaueriana* individuals were included into two clades: clade IV, which includes samples from Brazil, and clade V which contains the haplotypes from the southern Caribbean. The latter clade is consistently basally positioned when considering the ACEP *Avicennia* samples.

To better understand the genealogy of *A. bicolor, A. germinans* and *A. schaueriana* and among individuals within each of these species, we evaluated the parsimony networks of both the nDNA and cpDNA markers (Fig. 3). At the species level, as presumed, the haplotypes are mostly congruent with each taxon indicating the complete lineage sorting for both ITS and DTHK. However, the geographic distribution of the haplotypes was slightly different when each of these markers is considered, as was previously observed for the inferred phylogenies. Regarding the nDNA sequences (Fig. 3A), there is a strong relationship between geographic origin and haplotype for some samples, such as the Pacific, southern Caribbean and North Atlantic samples of *A. germinans.* However, mainly regarding the *A. germinans* and *A. schaueriana* samples from the Brazilian coast, there is no obvious pattern of genetic structure due to haplotype sharing between samples from different geographic origins. A highly divergent group composed of the AsPPR, AsGPM and AsVER samples was also observed, supporting the ITS phylogenetic tree (Fig. 2A). Given the cpDNA sequences (Fig. 3B), the geographic structure of the genetic diversity is similar to that observed for the nDNA marker: *A. germinans* samples from West Africa, the North Atlantic and the

southern Caribbean compose a distinct group, and the Pacific haplotypes embrace another clear cluster. As was observed for the ITS marker (Fig. 3A), the Brazilian samples of *A. germinans* present a more complex phylogeographic pattern. In the *A. schaueriana* samples, there is a dominant haplotype that is shared by most of the individuals, whereas the AsPAR samples presents a group of closely related haplotypes.

Regardless of the genealogical approach (network or tree inference) and the marker considered, there is evidence for star-like genealogies (Slatkin & Hudson 1991) which are preliminary signals of recent demographic expansions (Rannala 1997; Rosenberg & Hirsh 2003). In both strategies, the inferred genealogies of the haplotypes that are shared by individuals identified as *A. germinans* and *A. schaueriana* within their sympatry zone are direct indications of hybridization between these species.

Population-level analyses of A. germinans *and* A. schaueriana

Population differentiation analyses indicate that, generally, there is intraspecific genetic divergence between the evaluated samples of *A. germinans* and *A. schaueriana* (global G_{ST} values (Nei 1973) of 0.568 and 0.340 for the former, and 0.397 and 0.386 for the latter, for the DTHK and ITS markers, respectively). The differences between inferences of G_{ST} by means of these markers indicate that *A. germinans* has a pollen-to-seed ratio of r = 0.999, whereas the pollen-to-seed ratio of *A. schaueriana* is negative, or practically zero (r = -0.696). This difference suggests that *A. germinans* gene flow through its propagules is similar to the gene flow by pollen, but in *A. schaueriana,* the movement of genes by seed is one to two times higher than that by pollen.

These G_{ST} values indicate that there is substantial genetic structure, which is more readily observed when one considers the pairwise Φ_{ST} values for each species,

which are mostly significant, except the cpDNA marker and *A. schaueriana* samples (Table S2, Supporting material). The overall organization of the genetic diversity is complex, as shown by the graphical representation of the MDS analyses (Fig. 4), which results in relatively reliable models; the lowest measure of goodness of fit, considering two dimensions, is 0.7893.

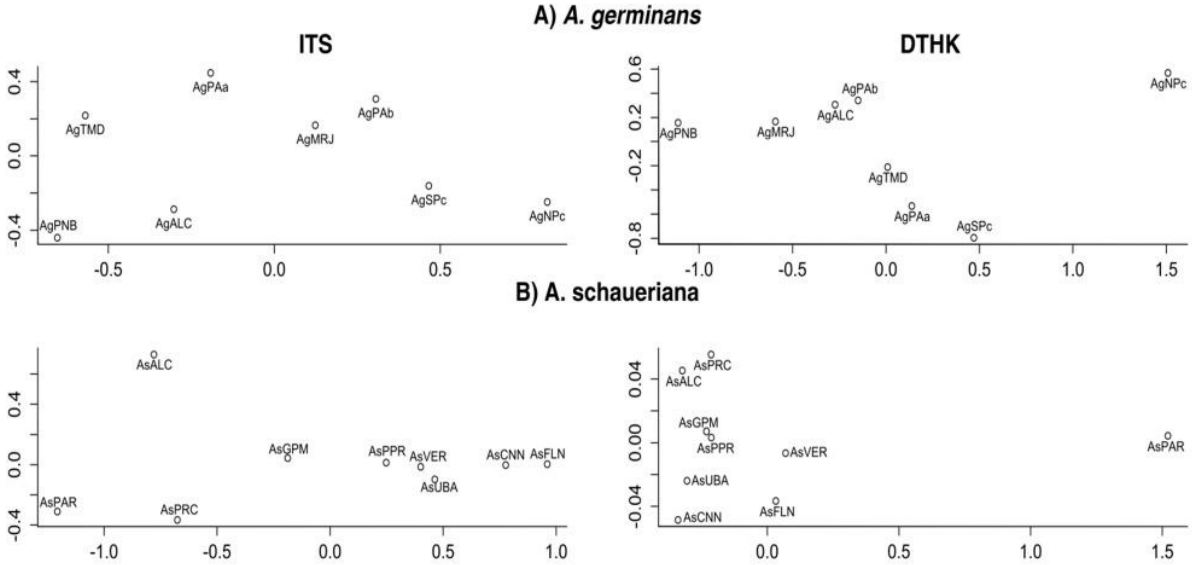

Figure 4. Multi-dimensional scaling (MDS) of pairwise Φ_{ST} among samples of A) *A. germinans* and B) *A. schaueriana* based on the ITS and DTHK markers (Table S2). The sample abbreviations are the same as those used in Table S1 and Figure 1.

To better understand this intricate pattern of genetic structure, we explicitly tested for different geographic grouping hypotheses (Table 1). Despite the previous studies finding evidence of genetic structure in three ACEP region mangrove species (*A. germinans A. schaueriana* and *R. mangle*) (Pil *et al.* 2011; Takayama *et al.* 2013, Mori *et al.,* submitted), AMOVA results considering these cpDNA and nDNA markers of both *A. germinans* and *A. schaueriana,* indicate that *a posteriori* hypotheses better explain the observed molecular variation. The *a priori* models considered different combinations of expected effects of the AMMC, the American continent and/or prominent marine currents on the genetic diversity. The hypothesized groupings of Ag1,

Ag4, As1, As8 and As9 were based on previous studies that used molecular markers to evaluate the genetic variation (Nettel & Dodd 2007; Nettel *et al.* 2008; Cerón-Souza *et al.* 2010; Pil *et al.* 2011; Takayama *et al.* 2013). In general, these hypotheses performed worse than our *a posteriori* models because the Φ_{GT} values were not significantly different from random distributions and due to the highly negative values of among-groups variance, failing to reasonably explain the genetic diversity (Table 1). For *A. germinans,* no hypothesis was consistently supported by the AMOVA outcomes; coupled with the high and significant pairwise Φ_{ST} values, this finding indicates that the genetic variation is likely to be organized among samples with relatively limited gene flow among them. Conversely, for *A. schaueriana,* model As6 was consistently supported by both the DTHK and ITS markers despite the considerably low variation among groups (Φ_{ST} =0.46969) when the former is analyzed (Table 1).

Considering both the genetic structure identified herein (model As6 and considering each samples separately for *A. germinans*) and the *a priori* scenarios (models Ag1 and As1 - Dodd *et al.* 2002; Nettel & Dodd 2007; Pil *et al.* 2011; Cerón-Souza *et al.* 2012; Takayama *et al.* 2013, Mori *et al.,* submitted), we observed different indications of recent demographic expansions in both species from both the *a priori* and *a posteriori* hypotheses. When *A. germinans* model Ag1was taken into account, we found signs of expansion in the AgNPc and AgNAtl groupings, whereas AgSPc showed indications of background selection. When each population was considered, only AgALC, AgPNB and AgTMD did not present signs of demographic changes (Table 3). These results are only supported by the ITS marker, whereas both nDNA and cpDNA loci suggest that recent population growth has also occurred in *A. schaueriana.* Regarding both hypothesized models, there are robust signs of expansion in every group, except for AsFLN (Table 3).

Table 3. Results of tests for neutrality and population expansion given two different evolutionary scenarios for A) *A. germinans* and B) *A. schaueriana* based on microsatellite, cpDNA and nDNA markers. The values of Tajima's D (Tajima, 1989), Fu and Li's D* and F*(Fu & Li, 1993); the raggedness index (Rogers & Harpending, 1992); and Fu's FS (Fu, 1997) are shown. The bold underlined values indicate P<0.02 for FS and P<0.05 for the remaining statistics.

A) *A. germinans*

ITS	model Ag1				*A. germinans* populations							
Statistics	AgNPc	AgSPc	AgNBr	AgTMD	AgNPc	AgSPc	AgMRJ	AgPAa	AgPAb	AgALC	AgPNB	AgTMD
Tajima's D	-3.350	-1.606	-2.412	-1.478	-3.350	-1.606	-2.408	-2.173	-2.988	-1.848	-1.210	-1.478
D*	1.406	1.506	0.659	1.008	1.406	1.506	1.654	-0.829	1.368	1.188	0.895	1.008
F*	1.035	1.762	-0.390	0.491	1.035	1.762	0.656	-1.067	0.536	0.796	0.562	0.491
Raggedness index	0.728	0.166	0.205	0.243	0.728	0.166	0.705	0.605	0.167	0.201	0.267	0.243
DTHK												
FS	$3.4*10^{38}$	-1.502	-1.984	-0.183	$3.4*10^{38}$	-1.502	0.468	-0.402	-0.513	5.718	0	-0.183
Raggedness index	0	0.393	0.306	0.201	0	0.393	0.355	0.658	0.165	0.505	0	0.201

B) *A. schaueriana*

ITS	model As1		model As6				
Statistics	AsNBr	AsSBr	PAR	ALC_PRC	VER	GPM_PPR_UBA_CNN	FLN
Tajima's D	-2.721	-2.398	-2.804	-2.233	-3.094	-2.345	0
D*	0.543	0.477	1.315	-0.990	0.755	1.751	0
F*	-0.264	0.511	0.643	-0.650	0.362	1.839	0
Raggedness index	0.780	0.534	0.498	0	0	0.467	0
DTHK							
FS	-8.218	-11.061	-3.382	0	-3.642	-3.637	-1.250
Raggedness index	0.253	0.680	0.043	0	0.339	0.835	0.459

Discussion

Using a broad-scale sampling strategy that coupled the analysis of publicly available sequences that had been previously evaluated (Nettel & Dodd 2007; Nettel *et al.* 2008) and individuals that had been previously studied by means of microsatellites (Mori *et al.,* submitted), we studied the genetic diversity of the three Neotropical *Avicennia* species. In addition to studying the relationships between *A. bicolor, A. germinans* and *A. schaueriana,* we evaluated different population-level processes that influence the variation of *A. germinans* and *A. schaueriana*.

There is interspecific hybridization among Western Hemisphere Avicennia *species*

By means of both network and tree analyses, we observed that the three *Avicennia* species from the ACEP region may be considered to have different evolutionary lineages (Figs. 2 and 3). However, the isolation of these species is not absolute. Although there is no evidence of ongoing hybridization between *A. bicolor* and *A. germinans,* an ancient introgression between them has already been reported (Nettel *et al.* 2008). The evidence for this historic contact between these species (in the form of the incongruent phylogenetic relationship between them) was revealed in the present study examining additional Atlantic samples of *A. germinans* and *A. schaueriana* (Fig. 2).

On the other side of the American continent, conversely, we found new evidence of current hybridization between *A. germinans* and *A. schaueriana.* Using microsatellites, we have previously observed that these two species may interbreed and, furthermore, that this hybridization is asymmetrical because only individuals identified as *A. germinans* presented signals of interspecific breeding (Mori *et al.,* submitted).

Herein, we find additional evidence of this hybridization but this new data do not support its asymmetry. By means of DTHK and ITS haplotype sharing, we found additional evidence of interbreeding between *A. schaueriana* and *A. germinans* from several locales within their sympatry zone, indicating that this biological process may be more common than previously believed. We favor hybridization/introgression rather than an ancestral polymorphism as the likely mechanism generating this haplotype sharing due to the shared haplotypes position in the phylogenetic trees and because of their relative frequencies. The branches where these haplotypes were placed are not basal, as would be as expected by this biological process (Fig. 2), and two of the four haplotypes in common between these species are rare (less than 3%), whereas ancestral haplotypes are presumed to be more frequent. However, because there are individuals that were identified as *A. germinans* and *A. schaueriana* sharing reciprocal haplotypes, cpDNA and nDNA data no longer support the asymmetrical hybridization between these species, indicating that gene flow may indeed occur bi-directionally.

These observations suggest that introgressive hybridization is a widespread process on both coasts of the American continent for *Avicennia* than previously believed, adding a relevant report to the large list examples of hybridization in mangrove species. Based on morphological and molecular data, interspecific gene flow has been described for the genera *Rhizophora, Bruguiera* (Rhizophoraceae)*, Sonneratia* (Lythraceae)*, Lumnitzera* (Combretaceae) and *Avicennia* (Tomlinson 1986; Nettel *et al.* 2008; Qiu *et al.* 2008; Zhou *et al.* 2008; Cerón-Souza *et al.* 2010; Duke 2010; Sun & Lo 2011; Guo *et al.* 2011). Although we can speculate on the mechanisms that maintain the widespread breeding between related mangrove species and on the evolutionary consequences of this process, we prefer to encourage further genetic and ecological studies regarding these intriguing questions.

Geographic distribution of the intraspecific genetic diversity

At the species level, *A. bicolor, A. germinans* and *A. schaueriana* present clear genetic differentiation despite the evidence of introgression previously discussed here and elsewhere (Nettel *et al.* 2008). Conversely, at the intraspecific level, the organization of the genetic variation in *A. germinans* and *A. schaueriana* is not obvious.

The Pacific and Atlantic samples of *A. germinans* can be clearly clustered into two different groups. The samples from the west coast of Central America are mostly phylogenetically separate from the remaining haplotypes, which can be visualized in clades III in the DTHK based phylogeny and in clades II and III in the ITS marker tree. Clade IV of the ITS marker marker includes haplotypes from Chautengo, on the Pacific coast of Mexico, and from the Atlantic coast of Central and North America. This distribution is likely explained by ancestral polymorphism because this clade is positioned relatively basally to the others near the Atlantic samples (Fig. 2A) and the Panama Isthmus is a strong barrier to the pollen flow for this insect-pollinated species (Cerón-Souza *et al.* 2012). Another explanation for this sharing is the past sea-level fluctuations that may have facilitated pollen gene flow, as has been proposed for *Hibiscus pernambucensis* Arruda (Malvaceae) (Takayama *et al.* 2008), whose pollination is also based on insects. In aggregate, despite this evident Pacific-Atlantic differentiation (Dodd *et al.* 2002; Nettel & Dodd 2007; Nettel *et al.* 2008; Cerón-Souza *et al.* 2012) the evolutionary scenario in the Atlantic basin, where our sampling size is larger, is complex.

Individuals from both sides of the Atlantic Ocean share haplotypes, and those that are different are closely related (Figs. 2 and 3); this has already been reported using

the same set of markers (Nettel *et al.* 2008) and PCR- restriction fragment length polymorphism coupled with chloroplast microsatellites (Nettel & Dodd 2007). This finding supports and extends the role of transatlantic dispersal as a relevant evolutionary process for *A. germinans* and for other sea-dispersed plants (Takayama *et al.* 2006, 2008, 2013; Nettel & Dodd 2007); transatlantic dispersal connects populations from West Africa and East Atlantic and contributes to the widespread distribution of *A. germinans* (Nettel & Dodd 2007) and *R. mangle* (Takayama *et al.* 2013). Interestingly, despite the high longevity and the buoyancy of the propagules (Rabinowitz 1978) that make the LDD relatively frequent among these species, this mechanism is likely to be rare enough that there is no generalized homogenization of the genetic diversity of these species (Takayama *et al.* 2006, 2008, 2013; Nettel & Dodd 2007, Mori *et al.,* submitted). For instance, we previously reported that there is genetic structure on different geographic scales along the Brazilian coast, with significant genetic differentiation between samples separated by distances from thousands of kilometers to hundreds of meter, regarding microsatellites analyses in both *A. germinans* and *A. schaueriana* (Mori *et al.,* submitted). The study of the DTHK and ITS markers supports these results; in this study we observed that although there is considerable haplotype sharing among the *A. germinans* samples, there is also generally substantial and significant genetic differentiation, as measured by global G_{ST} and pairwise Φ_{ST} (Table S2, Supporting material) with a complex pattern in the MDS plot (Fig. 4A). The most robust hypothesis of genetic organization by the hierarchical AMOVA corroborates these results because the most reliable hypothesis was generated by considering each of the samples separately (Table 1), supporting the pattern that accounts for small geographic scale structure using microsatellites (Mori *et al.,* submitted). This result indicates that the historical and current propagule dispersal of *A. germinans* is limited

and usually occurrs locally, as in a forest continuum such as the AMMC or within a single estuary, as observed in Central America (Cerón-Souza *et al.* 2012).

In *A. schaueriana,* there is also a complex relationship between the genealogical inferences and the geographic distribution of haplotypes. Many of the haplotypes are shared by different and geographically distant samples (Figs. 2 and 3), and we similarly observed a high level of genetic structure, as revealed by global G_{ST} measures. Despite the notable differences between the DTHK and ITS results regarding the pairwise Φ_{ST} (Table S2, Supporting material) which are easily observed in the MDS plot (Figure 4B), one *a posteriori* grouping was consistently supported by both markers considering the hierarchical AMOVA outcomes (Table 1). The As6 model slightly differs from the models that examine small-scale genetic structure using microsatellites and the tested *a priori* groupings (models As8 and As9 – Table 1). The relatively low variance among groups (46.97%) for the DTHK marker may be explained by the remarkable genetic diversity of this marker that was observed in AsPAR compared to other *A. schaueriana* samples (Table 2); this diversity led to a large proportion of the molecular variability within the samples (54.16%).

As a whole, the most likely groupings hypothesized herein disagree with the most feasible evolutionary scenarios inferred by means of microsatellite data in *A. germinans* and *A. schaueriana* (Mori *et al.,* submitted) and other sea-dispersed plants, including *R. mangle* (Pil *et al.* 2011; Takayama *et al.* 2013) and *H. pernambucensis* (Takayama *et al.* 2008). For all these species, a similar pattern of genetic structure was observed with a clear distinction between the samples that were collected from sites north and south of the northeastern extremity of Brazil (Fig. 1). Models Ag4 and As1 rely on this pattern of genetic structure and poorly explained the molecular variation we observed (Table 1). This finding indicates that there are different historical and ongoing

processes influencing the genetic diversity of these *Avicennia* species, due to the differences in the mutation rate between these sequence-based markers and microsatellites (Sunnucks 2000; Anderson *et al.* 2010; Wang 2010).

Historical and ecological processes shape the genetic diversity of A. germinans *and* A. schaueriana

This line of reasoning supports the hypothesis that *Avicennia* has been affected by historical demographic changes in the ACEP region, (or, more precisely, in the Pacific basin of Central America (Nettel & Dodd 2007)), and more broadly in the Eastern Hemisphere (Arnaud-Haond *et al.* 2006). During glacial periods, high-latitude edge populations would have gone extinct and been subsequently recolonized by individuals from core regions near the Equator (Arnaud-Haond *et al.* 2006; Nettel & Dodd 2007). The *A. germinans* and *A. schaueriana* samples in this study did not indicate a higher genetic diversity poleward neither by means of the DTHK and ITS markers (Table 2) or microsatellites (Mori *et al.*, submitted). However, the disagreement between the most likely scenarios considering high (Ag4 and As1) and low mutation rate indicates that different processes have shaped and continue to influence the species genetic diversity. We argue that along the Atlantic coast of South America, a similar process likely occurred.

After extinction events due to Quaternary environmental changes, such as high intensity frosts, aridity or physiographic shifts, populations would have become more isolated (Woodroffe & Grindrod 1991). This disjointed distribution coupled with the limited gene flow caused by relatively restricted pollen and propagule dispersal (Mori *et al.*, submitted) would have enabled the evolution of distinct independent lineages that

would later expand their geographic distribution after the glaciation. This evolutionary scenario explains the shared haplotypes between out studied samples and also the genealogical relationships, including the isolated branches (Fig. 2) and network (Fig.3) of the ITS marker, which embraces samples from three sites separated by hundreds of kilometers. This scenario is also consistent with the partial incongruence between the sequence-based and microsatellites genetic structure, as the population inferences using the latter could have retained the signs of these lineages that might have been obscured by contemporary micro-evolutionary processes. To further test this hypothesis, we studied the eventual demographic expansion signals. If this evolutionary history is consistent, we expected to observe significant evidence of demographic expansion across the inferred populations.

For both species, we tested whether the groupings that yielded the most likely genetic structure pattern considering microsatellite and sequence-based marker results (regarding models As1 and As6 for *A. schaueriana* and model Ag1 and taking into account each sample separately for *A. germinans*) present recent demographic changes signs. For both species, in aggregate, we found signs of population growth for different evolutionary scenarios across the samples studied (Table 3). Contrary to our expectations, we found no signs of demographic expansion in the samples from the Pacific basin of southern Central America, which was presumably a refugium during the last glaciation (Nettel & Dodd 2007); instead, we observed preliminary indications of background selection in these samples, due to the mend in the South American Atlantic basin, we found that *A. germinans* and *A. schaueriana* likely responded differently to the post-glacial period. Whereas *A. germinans* showed evidences of population growth on the northern coast of Brazil (model Ag1, and in AgMRJ, AgPAa and AgPAb, when each sample was evaluated), there were consistent indications that recent demographic

expansion occurred along the whole *A. schaueriana* distribution regardless the model assumed (Table 2).

The differences between the patterns of recent population growth explains the current geographic distribution of these species along the Atlantic Coast of South America (Fig. 1) because we found more substantial signs of demographic expansion (with evidence from both DTHK and ITS) in a broader geographic extension for *A. schaueriana* than for *A. germinans*. We argue that because southern limit of *A. schaueriana* distribution presents temperatures within the range of variation of *A. germinans* (Quisthoudt *et al.* 2012) and this climatic factor is regarded as a major driver that influences mangrove latitudinal limits (Duke *et al.* 1998), additional major traits must influence these species distribute in South America. This pattern of geographic distribution may have been originated by an ecological difference between these species; the unequal historical effectiveness of relative gene flow may have resulted from pollen and propagule dispersal. Our results indicate that whereas *A. germinans* pollen and propagules contributed similarly to the gene flow, in *A. schaueriana,* seeds were one or two times more efficacious than pollen at providing the movement of genes along the Brazilian coast; this difference may imply a more efficient dispersal that could have enabled *A. schaueriana* to colonize the southern and southeastern coast of Brazil. These inferences of past pollen to seed gene flow are similar to those observed for *A. germinans* from the Pacific and Atlantic basins of Panama, with r = -0.64 (Cerón-Souza *et al.* 2012), but because different sets of molecular markers were used, direct comparisons between these studies are not possible.

Conclusion

The *Avicennia* species from the ACEP region present genetic structure at different levels of organization. *A. bicolor, A. germinans* and *A. schaueriana* are distinct evolutionary lineages whose boundaries are not complete because there is evidence for past (Nettel *et al.* 2008) and undergoing introgressive hybridization process on the American continent. Given the intraspecific level, in addition to finding new evidences of transatlantic LDD of *A. germinans* which may contribute to its widespread distribution, within the South America Atlantic basin, we observed partially discordant molecular variation patterns between high (microsatellites) and low (DTHK and ITS) mutation rate markers for both *A. germinans* and *A. schaueriana*. We argue that this discordance is likely due to a recent demographic expansion of both species, whose pattern diverge between these species. This disagreement, coupled with a larger proportion of a gene flow brought by propagule seed rather than pollen in *A. schaueriana* but not in *A. germinans* explains the current distribution of these species in South America.

In addition to these retrospective conclusions, the novel details that our findings revealed about the evolutionary history of the Neotropical *Avicennia* species can also provide valuable information about the responses of these plants to the current global climate change. For instance, despite their close phylogenetic relationship, *A. germinans* and *A. schaueriana* have responded differently since the last glaciation and, thus, it is likely that their distinct ecological features may also influence their future in face of the current changing world. Considering this information about the past, our current endeavor is to understand potential impacts of the current climate changes on the neutral genetic variation of *A. germinans* and *A. schaueriana.*

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Supporting material

Table S1. Taxa, collection code with sample sizes within parenthesis for samples we collected, location (degrees decimal, and geopolitical units) and geographic region (with abbreviation between parentheses) of the samples evaluated using ITS (nDNA), *trn*D-*trn*T and *trn*H (cpDNA) markers.

taxon	collection code	ITS	*trn*D-*trn*T	*trn*H	longitude	latitude	Locality	State	Country	Geographic region
A. marina (Forssk.) Vierh.										
A. marina	RSD-MaPe1	EF540978	EF540944	EU352163	-	-	Perth		Australia	
A. marina	RSD-MaMgI	DQ469861	EF540943	EU352164	-	-				Madagascar
A. alba Blume										
A. alba	RSD-alb1	EF540977	EF540951	EU352162	-	-				Indonesia
A. bicolor Standl.										
A. bicolor	RSD-Pb1	EF540988	EF540948	EU352159	10.566	-85.666	Playa Panama		Costa Rica	
A. bicolor	RSD-Pb10	EU352149	EU352149	EU352161	10.566	-85.666	Playa Panama		Costa Rica	
A. bicolor	RSD-Tm4	EF540989	EF540949	EU352167	9.85	-84.683	Tivives		Costa Rica	
A. bicolor	RSD-Tb1	EF540987	EF540950	EU352160	9.85	-84.683	Tivives		Costa Rica	
A. bicolor	RSD-Abt1	EU352150	EU352153	EU443251	15.933	-93.8	Tonalá	Chiapas	Mexico	
A. bicolor	RSD-Abt9	EU352151	EU352152	EU352158	15.933	-93.8	Tonalá	Chiapas	Mexico	
A. germinans (L.) L.										
A. germinans	RSD-8-15	DQ469844	EF540954	EU352182	8.4	-81.583	Chiriqui		Panama	South Pacific (AgSPc)
A. germinans	RSD-8-11	DQ469845	EF540955	EU352183	8.4	-81.583	Chiriqui		Panama	South Pacific (AgSPc)
A. germinans	RSD-8-6	DQ469846	EF540956	EU352184	8.4	-81.583	Chiriqui		Panama	South Pacific (AgSPc)
A. germinans	RSD-6-1	DQ469847	EF540957	EU352175	8.833	-79.566	Aguadulce		Panama	South Pacific (AgSPc)
A. germinans	RSD-5-9	DQ469849	EF540946	EU352176	8.833	-79.566	Aguadulce		Panama	South Pacific (AgSPc)
A. germinans	RSD-2-15	EF540984	EF540958	EU352177	8.645	-79.703	Punta chame		Panama	South Pacific (AgSPc)
A. germinans	RSD-TL11	EF540982	EF540959	EU352171	9.85	-84.683	Tivives		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-Tm17	EF540985	EF540960	EU352174	9.85	-84.683	Tivives		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-Tm6	EF540981	EF540961	EU352173	9.85	-84.683	Tivives		Costa Rica	South Pacific (AgSPc)

Table S1 continued

A. germinans	RSD-Pm8	EF540980	EF540972	EU352168	10.566	-85.666	Playa Panama		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-Pge8	EF540979	EF540973	EU352172	10.566	-85.666	Playa Panama		Costa Rica	South Pacific (AgSPc)
A. germinans	6_10	DQ469848			8.833	-79.566	Aguadulce		Panama	South Pacific (AgSPc)
A. germinans	11_5	DQ469843			10.066	-84.966	Punta Morales		Costa Rica	South Pacific (AgSPc)
A. germinans	Pg17	DQ469863			9.609	-80.665	Galeta		Panama	South Pacific (AgSPc)
A. germinans	RSD-14-3	DQ469837	EF540963	EU352186	10.3	-85.25	Tempisque		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-14-2	DQ469838	EF540962	EU352187	10.3	-85.25	Tempisque		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-13-8	DQ469840	EF540964	EU352188	10.3	-85.25	Tempisque		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-13-6	DQ469839	EF540965	EU352189	10.3	-85.25	Tempisque		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-13-4	DQ469841	EF540966	EU352190	10.3	-85.25	Tempisque		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-11-9	DQ469842	EF540953	EU352191	10.066	-84.966	Punta Morales		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-11-10	EF540990	EF540967	EU352185	10.066	-84.966	Punta Morales		Costa Rica	South Pacific (AgSPc)
A. germinans	RSD-20-2	DQ469852	EF540968	EU352192	9.55	-79.65	Rio Claro		Panama	South Pacific (AgSPc)
A. germinans	RSD-ALI-1	DQ469835	EF540974	EU352181	24.133	-110.433	La Paz	Baja California	Mexico	North Pacific (AgNPc)
A. germinans	RSD-Ct2	DQ469836	EF540975	EU352180	16.683	-99.95	Chautengo	Guerrero	Mexico	North Pacific (AgNPc)
A. germinans	RSD-PO1T	EU352145	EU352156	EU352169	15.933	-93.8	Tonalá	Chiapas	Mexico	North Pacific (AgNPc)
A. germinans	RSD-PO9T	EU352146	EU352157	EU352170	15.933	-93.8	Tonalá	Chiapas	Mexico	North Pacific (AgNPc)
A. germinans	RSD-Agt1	EU352147	EU352154	EU443253	15.933	-93.8	Tonalá	Chiapas	Mexico	North Pacific (AgNPc)
A. germinans	RSD-Agt10	EU352148	EU352155	EU443252	15.933	-93.8	Tonalá	Chiapas	Mexico	North Pacific (AgNPc)
A. germinans	RSD-C21T	EF540983	EF540947	EU352178	24.916	-112.217	La Cigueña	Chiapas	Mexico	North Pacific (AgNPc)
A. germinans	RSD-APC1-4	DQ469834	EF540976	EU352179	24.916	-112.217	Bahia Magdalena		Mexico	North Pacific (AgNPc)
A. germinans	Mex1	EF136920			23.216	-106.416	Mazatlan		Mexico	North Pacific (AgNPc)
A. germinans	Mex2	EF136921			16.683	-99.95	Chautengo		Mexico	North Pacific (AgNPc)
A. germinans	RSD-Be1	DQ469853	EF540970	EU352195	32.333	-64.75			Bermuda	North Atlantic (AgNAt)
A. germinans	RSD-Db3	DQ469854	EF540971	EU352194	19.316	-69.5			Dominican Republic	North Atlantic (AgNAt)
A. germinans	AgCe6	DQ469850			20.86	-90.4	Celestum		Mexico	North Atlantic (AgNAt)
A. germinans	AgDOM	EF136923			19.96	-69.56			Dominican Republic	North Atlantic (AgNAt)

Table S1 continued

A. germinans	AgUSA	EF136922			26.266	-82.3		Florida	USA	North Atlantic (AgNAt)
A. germinans	T11	DQ469850			20.2166	-87.4666	Tulum		Mexico	North Atlantic (AgNAt)
A. germinans	RSD-An2-7	DQ469860	EF540969	EU352193	12.36	-5.116	Soyo		Angola	West Africa (AgAfr)
A. germinans	AgB7	DQ469859			12.28	-16.15			Guinea-Bissau	West Africa (AgAfr)
A. germinans	AgGMB	EF136927			13.05	-15.516			Gambia	West Africa (AgAfr)
A. germinans	AgSEN	EF136928			12.583	-16.26	Zinguichor		Senegal	West Africa (AgAfr)
A. germinans	AgGUA1	EF136924			16.35	-62.03			Guadeloupe	South Caribe (AgSCr)
A. germinans	AgGUA2	EF136925			16.33	-61.733			Guadeloupe	South Caribe (AgSCr)
A. germinans	AgMt1	DQ469855			14.066	-61			Martinique	South Caribe (AgSCr)
A. germinans	AgMt2	DQ469856			14.066	-61			Martinique	South Caribe (AgSCr)
A. germinans	AgA5	DQ469857			5.383	-52.833			French Guiana	South Caribe (AgSCr)
A. germinans	N4	DQ469858			5.766	-35.25	Natal	Rio Grande do Norte	Brazil	Natal (AgNTL)
A. germinans	NAT	EF136926			5.7	-35.3	Natal	Rio Grande do Norte	Brazil	Natal (AgNTL)
A. germinans	AgALC (21)	AB860420-B860440	AB860751-AB860771	AB861082-AB861102	-2.40971	-44.4057	Alcântara	Maranhão	Brazil	Alcântara (AgALC)
A. germinans	AgMRJ (24)	AB860441-AB860464	AB860772-AB860795	AB861103-AB861126	-0.70565	-48.4863	Soure	Pará	Brazil	Marajó (AgMRJ)
A. germinans	AgPAa (25)	AB860465AB860489	AB860796-AB860820	AB861127-AB861151	-0.89277	-46.687	Bragança	Pará	Brazil	Pará* (AgPAa)
A. germinans	AgPAb (16)	AB860490-AB860505	AB860821-AB860836	AB861152-AB861167	-1.93916	-46.7214	Bragança	Pará	Brazil	Pará (AgPAb)
A. germinans	AgPNB (24)	AB860506-AB860529	AB860837-AB860860	AB861168-AB861191	-1.93916	-46.7214	Bragança	Pará	Brazil	Pará (AgPAb)
A. germinans	AgPRC (4)	AB860530-AB860533	AB860861-AB860864	AB861192-AB861195	-2.78051	-41.8236	Parnaíba	Piauí	Brazil	Parnaíba (AgPNB)
A. germinans	AgTMD (24)	AB860534-AB860557	AB860865-AB860888	AB861196-AB861219	-3.41269	-39.0571	Paracuru	Ceará	Brazil	Paracuru (AgPRC)
A. germinans	AgALC (21)	AB860420-B860440	AB860751-AB860771	AB861082-AB861102	-8.58974	-35.0645	Tamandaré	Pernambuco	Brazil	Tamandaré (AgTMD)
A. schaueriana Stapf and Leechm. Ex Moldenke										
A. schaueriana	RSD-Sh1	DQ469862	EF540952	EU352166			Macao		Brazil	Natal (AsNTL)
A. schaueriana	RSD-ShG1	EF540986	EF540945	EU352165					Guadeloupe	South Caribe (AsSCr)

Table S1 continued

A. schaueriana	AsPAR (26)	AB860558-AB860583	AB860889-AB860914	AB861220-AB861245	-2.40971	-44.4057	Alcântara	Maranhão	Brazil	Pará (AsPAR)
A. schaueriana	AsALC (22)	AB860584-AB860605	AB860915-AB860936	AB861246-AB861267	-24.8971	-47.8472	Cananéia	São Paulo	Brazil	Alcântara (AsALC)
A. schaueriana	AsCNN (23)	AB860606-AB860628	AB860937-AB860959	AB861268-AB861290	-27.5678	-48.5189	Florianópolis	Santa Catarina	Brazil	Cananéia (AsCNN)
A. schaueriana	AsFLN (22)	AB860629-AB860650	AB860960-AB860981	AB861291-AB861312	-22.6989	-43.0015	Guapimirim	Rio de Janeiro	Brazil	Florianópolis (AsFLN)
A. schaueriana	AsGPM (24)	AB860651-AB860674	AB860982-AB861005	AB861313-AB861336	-0.82	-46.615	Bragança	Pará	Brazil	Guapimirim (AsGPM)
A. schaueriana	AsPPR (23)	AB860675-AB860697	AB861006-AB861028	AB861337-AB861359	-25.623	-48.355	Ponta do Paraná	Paraná	Brazil	Pontal do Paraná (AsPPR)
A. schaueriana	AsPRC (16)	AB860698-AB860713	AB861029-AB861044	AB861360-AB861375	-3.41269	-39.0571	Paracuru	Ceará	Brazil	Paracuru (AsPRC)
A. schaueriana	AsUBA (21)	AB860714-AB860734	AB861045-AB861065	AB861376-AB861396	-23.49	-45.163	Ubatuba	São Paulo	Brazil	Ubatuba (AsUBA)
A. schaueriana	AsVER (16)	AB860735-AB860750	AB861066-AB861066	AB861397-AB861397	-12.934	-38.6742	Vera Cruz	Bahia	Brazil	Vera Cruz (AsVER)

Table S2. Pairwise Φ_{ST} between samples of A) *A. germinans* and B) *A. schaueriana.* The values below the diagonal were obtained using the ITS marker, and the values above the diagonal were obtained using the DTHK marker. The bold and underlined numbers indicate the non-significant pairwise Φ_{ST} values after 10,000 bootstraps.

A)	AgNorthPac	AgSouthPac	AgMRJ	AgPAa	AgPAb	AgALC	AgPNB	AgTMD
AgNorthPac	0	0.62011	0.71146	0.62097	0.53751	0.58846	0.88317	0.58822
AgSouthPac	0.12008	0	0.74324	0.67136	0.6134	0.64837	0.86992	0.64548
AgMRJ	0.24291	0.17712	0	0.68928	0.05731	0.06755	0.10638	0.56896
AgPAa	0.43288	0.34072	0.20583	0	0.56636	0.56554	0.80509	0.61726
AgPAb	0.25753	0.18733	0.06627	0.04003	0	0.02684	0.18289	0.48602
AgALC	0.36376	0.28111	0.32617	0.48135	0.31844	0	0.17296	0.52436
AgPNB	0.50875	0.40074	0.46251	0.60427	0.4623	0.02111	0	0.74692
AgTMD	0.50875	0.40074	0.23006	0.62517	0.45982	0.54002	0.67609	0

B)	AsPAR	AsALC	AsPRC	AsVER	AsGPM	AsUBA	AsCNN	AsPPR	AsFLN
AsPAR	0	0.58305	0.54811	0.45358	0.55423	0.57764	0.58832	0.54826	0.46527
AsALC	0.34819	0	0	0.08477	0.01863	0	0	0.0208	0.06977
AsPRC	0.0952	0.4112	0	0.06452	0.0092	0	0	0.01093	0.05402
AsVER	0.51788	0.59724	0.36622	0	0.031	0.0816	0.08788	0.02874	0.00182
AsGPM	0.31557	0.37978	0.19219	0.1411	0	0.01723	0.01997	0.00051	0.01792
AsUBA	0.52768	0.63921	0.38043	0.02725	0.21234	0	0	0.01933	0.06733
AsCNN	0.633	0.71365	0.51747	0.03993	0.27771	0.05683	0	0.02222	0.07216
AsPPR	0.46438	0.53446	0.30882	0.00156	0.08606	0.05325	0.08631	0	0.02694
AsFLN	0.68716	0.77326	0.59863	0.11533	0.34297	0.12541	0.0426	0.15192	0

Capítulo IV

A diversidade genética de espécies dominantes de árvores de mangue está associada à riqueza de espécies nos manguezais brasileiros?

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Resumo

Uma questão fundamental para se compreender a biota é entender como a informação é transmitida entre diferentes níveis da hierarquia biológica. Este é um dos objetivos do campo de investigação denominado "Genética de Comunidades" que pretende avaliar o papel da variação genética nas interações entre espécies e na estrutura da comunidade. Neste contexto, tendo as florestas de mangue do litoral brasileiro como sistema de estudo, pretendemos avaliar de modo preliminar como a diversidade genética neutra de *Avicennia germinans* (Acanthaceae) e de *A. schaueriana* se relaciona com a riqueza de espécies de árvores de mangue. Utilizando distintos marcadores, microssatélites e baseados em genoma nuclear e cloroplastidial, observamos que apenas os primeiros apresentam correlação positiva com a riqueza de espécies, o que pode estar relacionado à sua maior taxa de mutação e, portanto, maior sensibilidade a processos evolutivos recentes. Entretanto, considerando apenas os microssatélites, os resultados indicam que os fatores "espécies" (*A. germinans* ou *A. schaueriana*) e posição geográfica explicam a variação observada, enquanto que riqueza de espécie não o faz. Uma das potenciais razões para isso são as expansões demográficas pós-glaciais que estas espécies sofreram e este processo levaria à incongruência entre diversidade genética e de espécies. A inclusão de dados obtidos para outra espécie de mangue, *Rhizophora mangle*, pode, no futuro, auxiliar a esclarecer estes resultados preliminares e a lançar luz sobre as relações entre diversidade genética e a riqueza de espécies de árvores de mangue.

Palavras-chave: Manguezais, Brasil, níveis hierárquicos, variação genética, diversidade de espécies

Introdução

Um número crescente de esforços vem sendo realizados para entender como diferentes níveis da hierarquia biológica se relacionam com um dos estratos mais fundamentais, o genético (Neuhauser *et al.* 2003; Whitham *et al.* 2003, 2006; Johnson & Stinchcombe 2007; Hughes *et al.* 2008; Bailey *et al.* 2009; Bailey 2011). No entanto, estas conexões têm sua importância reconhecida desde muito antes dos anos 2000. No ano de 1992, Antonovics já discutia a diversidade biológica como central nas interações de espécies quando apresentou pela primeira vez o campo da "Genética de Comunidades" (Antonovics 1992), como uma síntese entre Ecologia de Comunidades e Genética Evolutiva (Neuhauser *et al.* 2003; Vellend 2005; Vellend & Geber 2005; Johnson & Stinchcombe 2007). O objetivo deste campo de investigação é entender o "papel da variação genética como influência nas interações entre espécies e como determinante da estrutura da comunidade" (Antonovics 1992).

Curiosamente, neste mesmo ano, um dos mais influentes trabalhos em Ecologia (Chave 2013) chamava a atenção para a importância de se compreender de modo integrado os distintos níveis de organização (Levin 1992). Nele, o autor declara que: *"Yet unless we can find ways to relate detailed information at the molecular level to patterns of change at the level of the individual, the population, and the community, we will not have advanced our understanding of the evolution of the biosphere"* (Levin 1992).

Considerando a diversidade genética e a diversidade de espécies, há basicamente três tipos de processos não mutuamente excludentes que as relacionam. Eles podem ser divididos em A) processos paralelos que resultam em uma correlação positiva entre diversidade genética e de espécies; B) processos em que a diversidade genética influencia unidirecionalmente a diversidade de espécies e; C) processos em que a diversidade de espécies afeta a diversidade genética (Vellend & Geber 2005). Um exemplo do primeiro caso (A) é o processo de migração em que novos alelos ou

espécies são incluídos em uma dada população ou comunidade (Vellend & Geber 2005; Vellend 2005). Já com relação ao segundo tipo de processo (B), a diversidade genética não-neutra em relação à seleção natural pode ter efeitos na riqueza de espécies, como por exemplo, em comunidades com alta competição em que uma maior diversidade genética permitiria uma cobertura mais ampla de nichos, promovendo, desta maneira a coexistência de espécies (Vellend & Geber 2005; Vellend 2006; Hughes *et al.* 2008). Um exemplo de processo em que o reverso ocorre, ou seja, a diversidade de espécies influencia a diversidade genética, tipo de processo (C) é quando a riqueza de espécies imprime uma seleção diversificadora ao favorecer diferentes genótipos de uma espécie competidora (Vellend & Geber 2005).

Distintos estudos indicam que, de fato, a diversidade de espécies e genética não estão aleatoriamente relacionadas, havendo uma tendência de covariação positiva entre elas (Vellend & Geber 2005). Por meio de outra meta-análise, observou-se que, em plantas, a resposta dos efeitos genéticos pode ser bastante intensa em outros níveis de organização, embora, geralmente, ela diminui à medida que estratos mais elevados são considerados (Bailey *et al.* 2009). Mais recentemente, observou-se esta correlação positiva para distintos grupos como besouros (Papadopoulou *et al.* 2011), gastrópodes (Evanno *et al.* 2009), peixes (Blum *et al.* 2012), morcegos (Struebig *et al.* 2011), e plantas (He & Lamont 2010; Whitlock *et al.* 2011; Adams & Vellend 2011). Contrariamente, outras duas meta-análises indicam que, tanto no entorno do Mar Mediterrâneo (Fady & Conord 2010) como na Europa como um todo (Taberlet *et al.* 2012), a riqueza de espécies vegetais e a diversidade genética não estão correlacionadas de modo positivo (Fady & Conord 2010; Taberlet *et al.* 2012). Estes trabalhos ilustram que a relação entre esses níveis hierárquicos é uma questão controversa e, que, como esperado (Vellend & Geber 2005), há uma enorme complexidade nos mecanismos que os relacionam. Isso demonstra também a relevância da avaliação de outros sistemas biológicos para se compreender como a informação é transferida entre esses níveis da hierarquia biológica.

O sistema de nosso interesse, as florestas de mangue, é uma comunidade tropical e subtropical que apresentam uma característica bastante peculiar: a baixa diversidade de espécies (Tomlinson 1986), sendo a riqueza de espécies algumas ordens de grandeza inferior à encontrada em outras florestas como a Mata Atlântica e o cerrado (Myers *et al.* 2000; Zachos & Habel 2011; Joppa *et al.* 2011). A distribuição geográfica das árvores verdadeiras de mangue é bem relatada ao redor do globo, o que também é verdade para o litoral atlântico da América do Sul (Spalding *et al.* 2010). Ao longo desta região, como explicitado anteriormente nos Capítulos II e III, utilizamos marcadores neutros em relação à seleção natural para compreender como processos contemporâneos e históricos moldam e moldaram a diversidade genética de duas espécies de *Avicennia*. Nesta etapa, nosso objetivo foi compreender como a diversidade genética neutra destas árvores se relaciona com a riqueza de espécies de árvores de mangue. Para isso, devido à natureza dos marcadores utilizados (com base nos fundamentos teóricos propostos por Vellend e Geber (2005)) testamos a hipótese de que diversidade genética está correlacionada positivamente com a riqueza de espécies. Avaliamos também o papel da distribuição geográfica de cada amostra e como ela se relaciona com a diversidade genética medida por esses marcadores. Discutimos os resultados preliminares à luz das conclusões dos capítulos anteriores e de avanços recentes realizados por outros grupos de pesquisa.

Material e Métodos

Este estudo em larga-escala geográfica utilizou as amostras de *A. germinans* e de *A. schaueriana* provenientes de distintas localidades do litoral brasileiro (Tabela 1). Cada amostra é identificada por cinco letras: Ag ou As indica as espécies *A. germinans* e *A. schaueriana,* respectivamente e as três letras seguintes indicam o local de amostragem (Tabela 1). As espécies foram diferenciadas em campo com base nos caracteres vegetativos e reprodutivos segundo (Tomlinson 1986). Elas nem sempre

ocorrem no mesmo local e florescem simultaneamente (Duke 1991; Menezes *et al.* 2008; Spalding *et al.* 2010), de modo que não foi possível avaliar indivíduos de ambas as espécies em todas as localidades amostradas.

A variabilidade genética foi avaliada por meio de microssatélites que apresentaram produtos de amplificação nas duas espécies e marcadores de genoma nuclear (nDNA - *ribosomal internal transcribed spacer*, ITS) e cloroplastidial (cpDNA - espaçadores intergênicos *trn*D-*trn*T e *trn*H-*trn*K, os quais foram concatenados), conforme os Capítulos II e III. Apenas amostras com mais de dez indivíduos por localidade foram consideradas para estas análises (Tabela 1). Distintas medidas de diversidade foram calculadas. Utilizando os marcadores microssatélites, avaliamos a heterozigosidade esperada (H_E) e a riqueza alélica (A) por meio do programa GenAlEx 6.5 (Peakall & Smouse 2012). Já considerando os marcadores baseados em sequencia, utilizamos dois estimadores de θ, uma medida da diversidade nucleotídica que combina a quantificação do tamanho efetivo de população e a taxa de mutação. Um estimador de θ, θ_S tem como premissa o modelo de sítios infinitos entre o número de sítios segregantes, tamanho amostral e θ (Watterson 1975). O outro estimador, θ_π, presume o mesmo modelo para o número médio de diferenças pareadas ($\hat{\pi}$) e θ (Tajima 1983). Ambos foram calculados por meio do programa Arlequim 3.5 (Excoffier & Lischer 2010).

Para permitir a análise nesta escala geográfica, a riqueza de espécies de árvores verdadeiras de mangue (*sensu* Tomlinson, 1986) foi adquirida por meio do número de espécies com base no *World Atlas of Mangroves* (Spalding *et al.* 2010). Embora haja alguns problemas no que se refere às distribuições de espécies do hemisfério oriental (Dahdouh-Guebas 2010), na porção ocidental este atlas é consistente com trabalhos que estudaram os manguezais do litoral brasileiro (por exemplo, Schaeffer-Novelli *et al.* 1990; Silva *et al.* 2005; Menezes *et al.* 2008; Cunha-Lignon *et al.* 2011). Consideramos a distribuição contínua das espécies ao longo da região conforme o *World Atlas of*

Mangroves (Spalding *et al.* 2010), para que, em cada local de amostragem, tenhamos a riqueza de espécie esperada.

Avaliamos a relação entre a diversidade genética e a riqueza de espécies de mangue ocorrentes nestas áreas por meio de correlação de Pearson. Como a riqueza de espécies de mangue, em geral, é maior em regiões próximas ao Equador (Tomlinson 1986; Duke *et al.* 1998), construímos também modelos lineares utilizando como variável resposta as diferentes medidas de diversidade (H_E, A, θ_S e θ_π) e como fatores fixos as seguintes variáveis: espécie (*A. germinans* ou *A. schaueriana*), latitude, longitude (medidas em graus decimais) e riqueza de espécies. Estas análises estatísticas foram realizadas considerando ambas as espécies e também cada uma de modo isolado por meio do programa estatístico R 3.0 (R Core Team 2013), adotando um intervalo de confiança de 95%.

Resultados e Discussão

A grande variação em termos de diversidade genética e a distribuição geográfica das amostras encontram-se sumarizadas na Tabela 1. Alguns fatores contemporâneos (como a hibridação interespecífica, o sistema misto de reprodução e as correntes marinhas superficiais) e históricos (por exemplo, as expansões demográficas após a última glaciação) que influenciaram e moldaram esta diversidade já foram discutidos nos Capítulos II e III desta tese e também por diversos trabalhos independentes que estudaram árvores de mangue do hemisfério ocidental (Dodd *et al.* 2002; Nettel & Dodd 2007; Nettel *et al.* 2008; Cerón-Souza *et al.* 2010, 2012; Pil *et al.* 2011; Takayama *et al.* 2013).

Considerando as duas espécies simultaneamente, nós encontramos evidências que apoiam a hipótese de correlação positiva entre diversidade genética e riqueza de espécies apenas quando se consideram as medidas de diversidade genética quantificadas

utilizando microssatélites. Com relação aos marcadores baseados em sequências, apenas as estimativas baseadas em DTHK apresentaram correlações positivas com a riqueza de espécies, embora elas sejam apenas marginalmente significativas (Figura 1).

Tabela 1. Sumário das medidas de diversidade genética e de espécies das localidades amostradas no litoral brasileiro.

Abr.	Local	n	latitude	longitude	N_{sps}	DTHKθ_S	DTHKθ_π	ITSθ_S	ITSθ_π	H_E	A
AgMRJ	Soure,PA	24/31	-0.706	-48.486	6	0.22533	0.2234	0.45066	0.08333	0.587	6.087
AgPAa*	Bragança,PA	25/28	-0.893	-46.687	6	0.89302	0.60082	0.22325	0.1151	0.452	4.913
AgPAb	Bragança,PA	16/27	-1.939	-46.721	6	2.23478	1.42742	1.24154	0.70968	0.569	5.8261
AgALC	Alcântara,MA	21/29	-2.41	-44.406	6	1.8592	2.49942	0.2324	0.41812	0.54	5.2609
AgPNB	Parnaíba,PI	24/29	-2.781	-41.824	6	0	0	0.22533	0.28369	0.35	3.4348
AgTMD	Tamandaré,PE	24/32	-8.59	-35.064	4	0.22533	0.38298	0.67599	0.31915	0.11	1.4783
AsPAR	Bragança,PA	26/115	-0.824	-46.617	6	2.43427	1.98793	0.88519	0.5098	0.284	3.9524
AsALC	Alcântara,MA	22/30	-2.41	-44.406	6	0	0	0	0	0.336	3.1765
AsPRC	Paracuru,CE	16/31	-3.413	-39.057	6	0	0	0	0	0.178	1.6471
AsVER	Vera Cruz,BA	16/31	-12.934	-38.674	4	0.49662	0.24194	1.98647	1.19556	0.213	2.6471
AsGPM	Guapirmirim,RJ	24/35	-22.699	-43.002	3	0.22533	0.08156	1.12664	2.09663	0.216	2.8235
AsUBA	Ubutuba,SP	21/32	-23.49	-45.163	3	0	0	0	0	0.104	1.4706
AsCNN	Cananéia,SP	23/32	-24.897	-47.847	3	0	0	0	0	0.215	2.5882
AsPPR	Pontal do Paraná,PR	23/28	-25.623	-48.355	3	0.22753	0.08502	0.91014	1.3372	0.213	2.8235
AsFLN	Florianópolis,SC	22/66	-27.568	-48.519	3	0.45977	0.17759	0	0	0.188	3

Abr. sigla correspondente à amostra considerada;* indica amostras de localidades bastante próximas geograficamente mas sob regimes distintos de maré; Local município, estado brasileiro de origem das amostras; n número de indivíduos amostrados para análises baseadas em sequências/microssatélites; latitude e longitude em graus decimais; N_{sps} número de espécies de árvores de mangue; DTHKθ_S e DTHKθ_π estimadores de θ considerando os marcadores cloroplastidiais; ITSθ_S e ITSθ_π estimadores de θ levando em conta o marcador nuclear; H_E heterozigosidade esperada referente aos microssatélites, A riqueza alélica com base em marcadores microssatélites.

Há uma série de explicações para observarmos evidências de covariação positiva entre medidas de diversidade genética e de espécies apenas considerando

microssatélites, com suporte estatístico, e o marcador de genoma cloroplastidial DTHK, neste caso com evidências menos claras ($p<0.1$). Os microssatélites apresentam maior taxa de mutação (em algumas ordens de grandeza) quando comparados com marcadores como DTHK e ITS sendo, portanto, mais sensíveis a eventos e processos recentes em termos evolutivos (Sunnucks 2000; Schlötterer 2004; Wang 2010). Já o marcador de cpDNA, apesar da menor taxa de mutação, por ser haploide, apresenta um menor tamanho efetivo de população (Birky *et al.* 1983, 1989) sendo desta maneira, mais impactado pelos efeitos amostrais de populações finitas (deriva genética) e/ou de tamanhos variáveis ao longo do tempo (Hartl & Clark 2007; Hamilton 2011). Estes argumentos estão de acordo com as expectativas teóricas, uma vez que os modelos propostos enfocam escalas temporais mais recentes, ignorando o efeito do processo de formação de espécies (Vellend & Geber 2005). Com relação ao marcador ITS, cuja análise não evidenciou relações robustas entre estes níveis de organização, ele apresenta sinais de maior incidência de recombinação (dados não mostrados). Esta é compatível com marcadores de genoma nuclear, e a recombinação alteraria a variação genética no nível de populações de maneira não previsível (Vellend & Geber 2005).

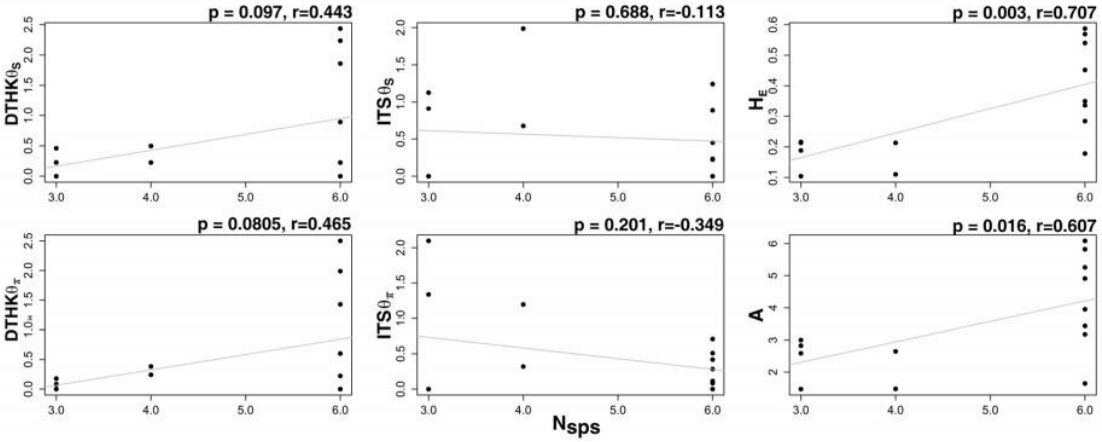

Figura 1. Testes de correção entre diversidade genética e de espécies. Demonstram-se as relações entre medidas de diversidade genética baseadas em sequências ($DTHK\theta_S$, $DTHK\theta_\pi$, $ITS\theta_S$ e $ITS\theta_\pi$) e em microssatélites (H_E e A) e a riqueza de espécies (N_{sps}).

Observamos que no litoral brasileiro há uma maior riqueza de espécies em regiões mais próximas do Equador ($r=0.969$, $p<0.0001$), o que também é observado

considerando os manguezais ao redor do globo (Tomlinson 1986; Duke *et al.* 1998). Novamente, pode-se observar que as variações genéticas, tanto avaliadas por DTHK como por ITS não são explicadas com suporte estatístico por nenhum dos fatores fixos considerados (Tabela 2). Por outro lado, levando em conta as quantificações baseadas em microssatélites, pode-se observar que o fator "espécie" (*A. germinans* ou *A. schaueriana*) e a posição geográfica do local de amostragem, latitude e/ou longitude, explicam a variação observada enquanto que a riqueza de espécies não o faz (Tabela 2). Entretanto, quando cada espécie é avaliada isoladamente, nenhum fator variável explica a diversidade genética observada em nenhum dos casos.

Tabela 2. Sumário das análises dos modelos considerando medidas de diversidade genética (H_E, A, θ_S e θ_π) quantificadas por distintos marcadores moleculares como fatores resposta e como fatores fixo as espécies (*A. germinans* e *A. schaueriana*), latitude, longitude, interação entre latitude e longitude (medidas em graus decimais), e riqueza de espécies (N_{sps}).

	DTHKθ_S	DTHKθ_π	ITSθ_S	ITSθ_π	H_E	A
espécie	-	-	-	-	**	**
Latitude	-	-	-	-	*	-
Longitude	-	-	-	-	**	**
Latitude.Longitude	-	-	-	-	*	-
N_{sps}	-	-	-	-	-	-
A. germinans						
Latitude	-	-	-	-	-	-
Longitude	-	-	-	-	-	-
Latitude.Longitude	-	-	-	-	-	-
N_{sps}	-	-	-	-	-	-
A. schaueriana						
Latitude	-	-	-	-	-	-
Longitude	-	-	-	-	-	-
Latitude. Longitude	-	-	-	-	-	-
N_{sps}	-	-	-	-	-	-

* P<0.05, ** P<0.001, - não significativo

Em conjunto, esses resultados indicam que a diversidade genética de árvores de mangue, embora correlacionadas positivamente com a riqueza de espécies, tem sua variação explicada pela localização geográfica da qual a amostra foi tomada. Isso pode indicar que, embora os modelos teóricos considerem a diversidade em uma ou poucas

espécies (Vellend & Geber 2005), considerando um maior número de espécies seria possível balancear as peculiaridade dos padrões de diversidade genética e história evolutiva de cada táxon individual, levando a uma correlação entre diversidade genética e de espécies consistente (Papadopoulou *et al.* 2011). Como observado principalmente no Capítulo III desta tese, *A. germinans* e *A. schaueriana* apresentam uma história evolutiva recente discrepante, o que, inclusive, pode explicar o padrão de distribuição destas espécies no sul da América do Sul. Este fato pode ser um exemplo de como particularidades de cada táxon, mesmo que bastante próximos filogeneticamente, podem afetar as expectativas dos modelos teóricos que sugerem a correlação positiva entre a variação nestes níveis da hierarquia biológica.

Neste sentido, atualmente há esforços paralelos a este trabalho, que estão sendo desenvolvidos pela bióloga Patrícia Mara Francisco em seu projeto de doutorado, cujo objetivo é estudar a distribuição geográfica da variação genética de *Rhizophora mangle* nos mesmos locais de amostragem do litoral brasileiro considerados neste trabalho. Ao analisar a variação genética em *R. mangle*, poderemos avaliar a sugestão de que a inclusão de mais táxons pode esclarecer as relações entre distintos níveis hierárquicos no sistema de florestas de mangue.

Há também a possibilidade que os modelos teóricos propostos por Vellend e Geber (2005) e Vellend (2005b) não se apliquem à comunidade de mangue, do mesmo modo que uma correlação positiva não foi observada considerando a flora de alta montanha dos Alpes (Taberlet *et al.* 2012) ou as plantas vasculares presentes no entorno do Mar Mediterrâneo (Fady & Conord 2010). Nos exemplos citados, sugere-se que as flutuações demográficas após a última glaciação superariam a potencial covariação entre diversidade genética e de espécies (Fady & Conord 2010; Taberlet *et al.* 2012), o que também pode ser o caso dos manguezais do litoral brasileiro. Como visto no Capítulo III, encontramos evidências de que as populações de *A. germinans* e de *A. schaueriana* expandiram modo desigual entre si após o último período glacial, o que

levaria a uma ausência de correlação positiva entre diversidade genética e riqueza de espécies. Esta hipótese poderá ser analisada de maneira mais consistente com a inclusão de *R. mangle,* conforme sugerido pelos resultados e discussões de Papadopoulou e colaboradores (2011).

Com base nesses resultados preliminares e, considerando a diversidade genética neutra e a riqueza de espécies de árvores de mangue do litoral brasileiro, não foi possível compreender como a informação é transmitida entre o nível genético e o de comunidades, questão chave para se compreender a biota (Levin 1992). Esperamos que no futuro, somando informações referentes a outro gênero de árvore de mangue (*R. mangle*), possamos esclarecer esta questão e entender se os modelos teóricos que explicam os mecanismos paralelos que relacionam estes dois níveis hierárquicos (Vellend & Geber 2005; Vellend 2005) são também válidos para as comunidades de mangue.

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Conclusões e Perspectivas

A passagem é estreita. Nenhuma solução é definitiva. Toda vitória é parcial, cada avanço traz novos desafios e qualquer conquista é passível de retrocesso. A prevenção do mal ajuda, mas não sacia o desejo humano de encontrar o bem. Navegar é preciso. Ouvidos abertos, olho na bússola, mastro na mão.

Eduardo Giannetti, 2005, Auto-Engano

Conclusões

Após o desenvolvimento deste trabalho, trouxemos contribuições no entendimento de como está distribuída a variação genética neutra de espécies de árvore de mangue do gênero *Avicennia* do hemisfério ocidental, com particular atenção para o litoral brasileiro. Foi possível identificar e entender como alguns fatores, históricos e contemporâneos, moldaram e continuam influenciando esta diversidade. Entretanto, não encontramos claras relações entre a diversidade genética neutra e riqueza de espécies de mangue. Para chegar a esta conclusão geral, uma série de etapas tiveram que ser realizadas e os avanços alcançados em cada uma serão sucintamente apresentados a seguir.

Desenvolvemos e disponibilizamos à comunidade científica novos microssatélites específicos para as espécies *A. germinans* e *A. schaueriana*. Tais ferramentas moleculares se mostraram muito úteis para uma série de finalidades, como o estudo da hibridação interespecífica entre as espécies em questão bem como do sistema de cruzamento de *A. schaueriana*.

Embora *A. schaueriana* se reproduza majoritariamente pelo cruzamento entre diferentes indivíduos (alogamia), há considerável taxa de autofecundação, indicando que *A. schaueriana* apresenta um sistema misto de cruzamento, sendo provavelmente também é o caso de *A. germinans* (com base nos resultados da distribuição da diversidade genética desta espécie).

Para as duas espécies, a diversidade genética avaliada por microssatélites aponta a existência de uma organização em distintas escalas geográficas no litoral brasileiro. Esta estrutura é influenciada por fatores contemporâneos intrínsecos a esses organismos (sistema de cruzamento, limitação de dispersão de pólen e de propágulo, mas não a ocorrência de hibridação interespecífica) e extrínsecos (sentido e intensidade de correntes marinhas e regime de marés).

Analisando marcadores de genomas nuclear e cloroplastidial e amostras provenientes de praticamente todo o hemisfério ocidental, observamos novas evidências de dispersão transatlântica para *A. germinans*. Além disso, confirmamos a hibridação entre *A. germinans* e *A. schaueriana* por meio do compartilhamento de haplótipos no litoral norte do Brasil.

Ao associar os resultados obtidos com os marcadores de genomas nuclear e cloroplastidial aos obtidos com microssatélites, explicamos a distribuição atual destas espécies de *Avicennia* no litoral atlântico da América do Sul com base nos diferentes padrões de expansões demográficas após a última glaciação, e no maior fluxo gênico histórico por meio de propágulos observado para *A. schaueriana* em comparação com *A. germinans.*

Não observamos uma correlação positiva entre diversidade genética e riqueza de espécies estritas de mangue ao longo do litoral do Brasil. Um dos possíveis motivos pelo qual não obtivemos o resultado esperado são as flutuações demográficas sofridas por estas espécies após a última glaciação. Esperamos que estas questões possam ser melhor respondidas futuramente, com a inclusão de informações sobre a diversidade genética provenientes de outra espécie de mangue, *Rhizophora mangle*.

Perspectivas

O término do trabalho proposto para esta tese não indica o fim do interesse por este sistema biológico. Ainda há diversas questões em aberto as quais surgiram com os resultados obtidos durante o desenvolvimento do presente trabalho. Algumas delas poderão (e deverão) ser respondidas utilizando os dados gerados neste projeto.

Uma delas é estender as análises filogeográficas realizadas e discutidas no Capítulo III. Por meio de uma abordagem de Filogeografia Estatística (Knowles 2009; Beaumont *et al.* 2010), poderemos testar explicitamente distintos cenários evolutivos e compará-los com base nos dados genéticos. Esses modelos poderão ser construídos com base em revisão bibliográfica em busca de informações paleontológicas (principalmente evidências de pólens fossilizados e dados estratigráficos) sobre a dinâmica temporal das expansões das florestas de mangue discutidas no Capítulo III. Tais cenários evolutivos poderão também se basear em análises de modelagem de distribuição de espécies, cujos resultados preliminares indicam que desde a última glaciação, de fato houve um aumento na área em que as florestas de mangue do hemisfério ocidental se distribuiriam (Figuras 1A e 1B). Utilizando tal estratégia baseada em modelos associado a novas e robustas metodologias de análise de dados genéticos (Kuhner 2009; Csilléry *et al.* 2010; Bloomquist *et al.* 2010), poderemos melhor compreender como as árvores de mangue responderam às alterações globais históricas após a última glaciação.

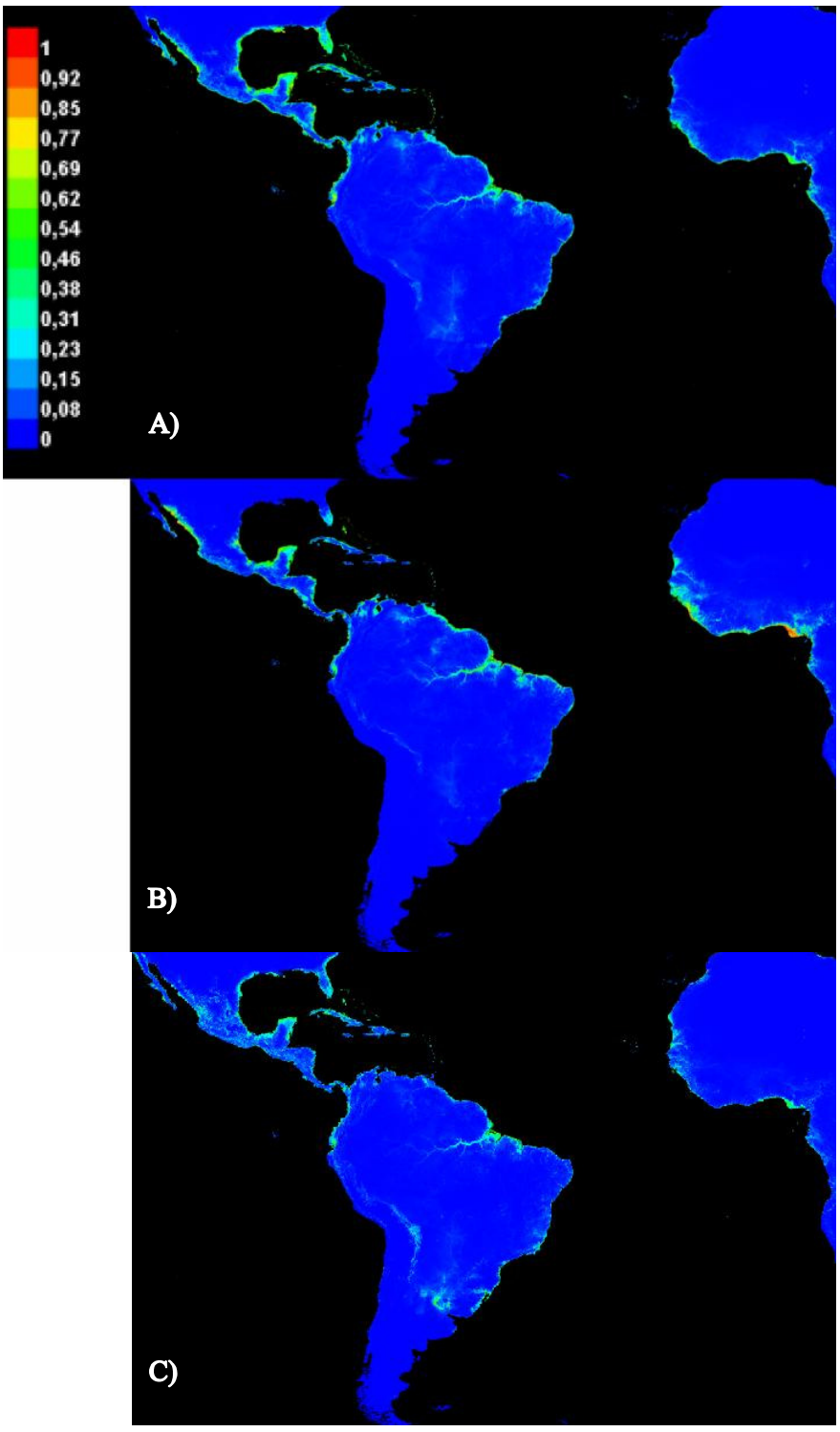

Figura 1. Simulações de distribuição de espécies para *A. germinans* realizadas pelo método de Máxima Entropia (Phillips & Dudík 2008) considerando informações edáficas, de altitude e 19 variáveis bioclimáticas A) atuais, B) inferidas para a última glaciação e C) em cenário A2 estimado para 2050. As cores indicam a probabilidade de ocorrência da espécie em cada célula (*pixel*) do mapa.

Além desta visão em direção ao passado, poderemos também avaliar como as espécies de *Avicennia* estudadas podem vir a responder às mudanças climáticas globais ocorrendo atualmente. Em nossas análises preliminares, consideramos o cenário A2 do Painel Intergovernamental sobre Mudanças Climáticas (IPCC da sigla em inglês) que se caracteriza por ser uma estimativa "pessimista". Neste cenário, há um intenso crescimento populacional humano, baixo desenvolvimento econômico e poucas mudanças tecnológicas (Intergovernmental Panel on Climate Change 2007). Podemos observar que espécies de mangue, neste caso representadas apenas por *A. germinans,* apresentariam futuramente não apenas uma expansão em direção aos pólos, como já vem sendo observado (Perry & Mendelssohn 2009; Osland *et al.* 2013; Saintilan *et al.* 2013), mas também extinções locais (Figura 1C). Poderemos então ter ideia de quais populações poderão se extinguir, levando a perdas de diversidade genética em termos de alelos e de haplótipos e quais delas podem vir a ser favorecidas.

Além da perspectiva acadêmico-científica, do ponto de vista aplicado da Genética da Conservação (Frankham *et al.* 2002; DeSalle & Amato 2004; Frankham 2005), compreender essas potenciais extinções locais e expansões demográficas em direção aos pólos e suas consequências na variabilidade genética é de grande importância. Associando essas projeções futuras às informações obtidas em relação à estrutura genética observada por diferentes marcadores moleculares e aos fatores ecológicos atuais e históricos que moldam esta diversidade, esperamos poder contribuir para a conservação e para o manejo destas florestas. Isso poderá ser realizado por meio de uma aproximação com gestores de unidades de conservação, o que já vem ocorrendo, e também com organizações não-governamentais que atuam diretamente em ações de plantios e reflorestamentos de manguezais.

Além do gênero *Avicennia*, em trabalho realizado pela bióloga Patrícia Mara Francisco em nosso grupo, estuda a diversidade genética de espécies do gênero *Rhizophora* que ocorrem no Brasil, *R. mangle, R. racemosa* e *R. harisonii*. Poderemos

então responder questões similares às descritas ao longo desta tese tendo como sistema de interesse estas espécies de *Rhizophora*. Além disso, por uma abordagem comparativa, poderemos compreender como plantas de gêneros tão distantes filogeneticamente e também tão diferentes ecologicamente, mas que estão co-distribuídas praticamente ao longo de todo hemisfério ocidental, têm suas diversidades genéticas estruturadas. Será então possível compreender, por exemplo, se os fatores históricos e contemporâneos que moldam a diversidade genética das espécies de *Avicennia* são similares aos que influenciam a de *Rhizophora.*

Esperamos que as questões avaliadas durante este trabalho e sumarizadas nesta tese sirvam como um abrir de portas para que novas perguntas sobre este sistema biológico sejam feitas e respondidas. Nosso desejo é que este ciclo de questões e soluções se mantenha por um longo tempo e seja realizado por um número cada vez maior de pesquisadores interessados. Ansiamos que as perspectivas apresentadas indiquem que este seja justamente o caso, e que, passo a passo, possamos descobrir mais sobre as interessantes plantas de mangue.

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"The intellectual challenges
are fascinating, the opportunities
plentiful, and the results can be
personally gratifying."
Michael E Soulé, 1985, BioScience
sobre Biologia da Conservação

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