PATRÍCIA MARA FRANCISCO

CARACTERIZAÇÃO DE POPULAÇÕES NATURAIS DE *RHIZOPHORA* SPP. (RHIZOPHORACEAE) DE MANGUEZAIS DO LITORAL BRASILEIRO E ANÁLISE DE ZONA DE HIBRIDAÇÃO UTILIZANDO MARCADORES MICROSSATÉLITES

CHARACTERIZATION OF NATURAL POPULATIONS OF *RHIZOPHORA* SPP. (RHIZOPHORACEAE) FROM MANGROVE FORESTS ALONG THE BRAZILIAN COAST AND ANALYSIS OF A HYBRIDIZATION ZONE USING MICROSATELLITE MARKERS

CAMPINAS
2015

UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

PATRÍCIA MARA FRANCISCO

Caracterização de populações naturais de *Rhizophora* spp. (Rhizophoraceae) de manguezais do litoral brasileiro e análise de zona de hibridação utilizando marcadores microssatélites

Characterization of natural populations of *Rhizophora* spp. (Rhizophoraceae) from mangrove forests along the Brazilian coast and analysis of a hybridization zone using microsatellite markers

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção de título de Doutora 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/Supervisora: Profa. Dra. Anete Pereira de Souza

Este exemplar corresponde à versão final da tese defendida pela aluna Patrícia Mara Francisco, orientada pela profa. Dra. Anete Pereira de Souza

Profa. Dra. Anete Pereira de Souza

CAMPINAS 2015

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

F847c

Francisco, Patrícia Mara, 1984-
Caracterização de populações naturais de *Rhizophora* spp. (Rhizophoraceae) de manguezais do litoral brasileiro e análise de zona de hibridação utilizando marcadores microssatélites / Patrícia Mara Francisco. – Campinas, SP : [s.n.], 2015.

Orientador: Anete Pereira de Souza.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Genética de populações. 2. Manguezais. 3. Hibridação. 4. *Rhizophora*. I. Souza, Anete Pereira de,1962-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Characterization of natural populations of *Rhizophora* spp. (Rhizophoraceae) from mangroves forests along the Brazilian coast and analysis of a hybridization zone using microsatellite markers
Palavras-chave em inglês:
Population genetics
Mangroves
Hybridization
Rhizophora
Área de concentração: Genética Vegetal e Melhoramento
Titulação: Doutora em Genética e Biologia Molecular
Banca examinadora:
Anete Pereira de Souza [Orientador]
Maria Imaculada Zucchi
Elizabeth Ann Veasey
Evandro Vagner Tambarussi
Marília Cunha Lignon
Data de defesa: 23-02-2015
Programa de Pós-Graduação: Genética e Biologia Molecular

Campinas, **23** de **fevereiro** de **2015**

BANCA EXAMINADORA

Profa. Dra. Anete Pereira de Souza (orientadora) _____
Assinatura

Profa. Dra. Maria Imaculada Zuchi _____
Assinatura

Profa. Dra. Elizabeth Ann Veasey _____
Assinatura

Prof. Dr. Evandro Vagner Tambarussi _____
Assinatura

Profa. Dra. Marília Cunha Lignon _____
Assinatura

Profa. Dra. Eliana Regina Forni-Martins _____
Assinatura

Dra. Prianda Rios Laborda _____
Assinatura

Dr. Miklos Maximiliano Bajay _____
Assinatura

RESUMO

Manguezais são ecossistemas com uma variedade incomum de animais e plantas adaptados às condições de alta salinidade, inundações frequentes e solo lodoso e anaeróbico. Ocorrem em locais onde há o encontro de águas de rios com a do mar. Diversos fatores bióticos e abióticos influenciam os padrões de diversidade de espécies de manguezais, como oceanografia, clima, topografia e condições do solo. A diversidade de plantas de mangue é muito reduzida, quando comparada com outros ecossistemas tropicais. O Brasil possui uma das maiores areas de manguezal do mundo e apresenta três gêneros de angiospermas de mangue. Um deles é *Rhizophora*, composto pelas espécies *Rhizophora mangle, Rhizophora racemosa* e, um possível híbrido, *Rhizophora harrisonii.* O objetivo da presente tese foi isolar e caracterizar locos microssatélites para essas espécies e estimar parâmetros populacionais como fluxo gênico, estruturação populacional, diversidade gênica e tamanho efetivo de população, além de estudar outros aspectos da biologia de *Rhizophora*, como uma possível zona de hibridação na região norte do país, taxa de cruzamento e o sistema reprodutivo. Com este propósito, foram coletados 318 indivíduos de *R. mangle* de 11 localidades ao longo da costa brasileira, e 33 indivíduos de *R. racemosa* e 37 indivíduos de *R. harrisonii* ambas coletadas de duas localidades no litoral brasileiro. Para identificar e caracterizar locos de microssatélites foram desenvolvidas bibliotecas enriquecidas em microssatélites para as três espécies. Utilizando os marcadores desenvolvidos na presente tese, bem como outros que já publicados, observou-se uma diferença significativa entre as populações no padrão de variação genética. A riqueza de alelos, heterozigosidades esperada e observada foram maiores na região norte. Os resultados sugerem que as espécies de *Rhizophora* não compõe apenas uma população

panmítica ao longo do litoral brasileiro, devido à diferenciação existente entre as regiões norte e sul da costa. A análise do sistema reprodutivo de *R. mangle* de uma população do estado do Pará, encontramos valores que indicariam um sistema de reprodução misto. Em relação à hibridação contínua, não foram encontradas evidências de hibridação introgressiva entre as espécies de *Rhizophora.* Concluímos que com os resultados obtidos na presente tese foi possível contribuir para o maior conhecimento genético das espécies de *Rhizophora* spp. do litoral brasileiro.

ABSTRACT

Mangrove are ecosystems with an unusual variety of animals and plants adapted to conditions of high salinity and frequent floods and muddy anaerobic soil. Several abiotic factors influence the patterns of mangrove species diversity, such as oceanography, climate, topographic and soil conditions. The number of mangrove plant species is much reduced compared with other tropical ecosystems. Brazil has the second largest mangrove area in the world and has three genera of mangrove angiosperms. One genera is Rhizophora, composed of *Rhizophora mangle, Rhizophora racemosa* and a possible hybrid*, Rhizophora harrisonii*. The aim of this thesis was to isolate and characterize microsatellite loci for these species and estimate population parameters such as gene flow, population structure, genetic diversity and effective population size, and study other aspects of Rhizophora biology, as a possible hybrid zone in the north region of the Brazilian coast, crossing rate and the reproductive system. For this purpose, 318 individuals of *R. mangle* of 11 locations along the Brazilian coast, 33 individuals of *R. ra*cemosa and 37 individuals of *R. harrisonii* from two locations were collected. To identify and characterize the microsatellite loci, enriched microsatellite libraries for the three species were developed. Using the developed markers, and some others already published, we observed a significant difference between the populations in the pattern of genetic variation. Alleles richness, expected and observed heterozygosity were higher in the north. The results suggest that the species of *R. mangle* is not only composed of a single panmitic population due to differentiation found among the population from locales north and south of the Brazilian Coast. The reproductive system was evaluated studing a population of *R. mangle* from the state of Pará and we find values that would indicate a mixed mating system. Regarding the

ongoing hybridization, we found no evidence of introgressive hybridization among the species leading to a hybrid species. We concluded that with this results it was possible to contribute to further genetic knowledge of *Rhizophora* spp. from the Brazilian coast.

SUMÁRIO

DEDICATÓRIA

Aos meus pais César e Katia,

que dignamente me apresentaram à

importância da família e ao caminho

da honestidade e persistência, dedico.

AGRADECIMENTOS

À Profa. Dra. Anete Pereira de Souza, por me receber de braços abertos como sua aluna em 2007, me dando grandes oportunidades de pesquisa, me orientando e por todo o esforço e apoio oferecido para que eu concluísse meu trabalho. Obrigada pelas conversas que sempre me acalmavam quando eu achava que os problemas não teriam solução.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pelo apoio financeiro no projeto (2008/52045-0 e 2010/50178-2) e pela bolsa de doutorado direto concedida (2008/56404-4).

Aos meus pais, Katia e César, pelo amor, incentivo, apoio incondicional e por sempre apoiarem os caminhos que eu decido trilhar, me acolhendo sempre que eu preciso.

À minha irmã Priscila, pela paciência e amor. Também pela disposição em entrar na lama e me ajudar no trabalho de campo.

Aos meus avôs, avós, tios, tias, primos e primas pelos churrascos, pastéis, cafés da tarde e todos os momentos passados juntos. Pela expressão de surpresa quando eu aparecia em meio a períodos de ausência: "A Patrícia ta aí". Acho que todos sabem o quanto são importantes e quanto prezo pelo convívio com vocês.

À Profa. Dra. Michele Piercey-Normore por ter me aceitado para um estágio na University of Manitoba, Winnipeg, Canadá. Agradeço a todos os alunos do grupo de pesquisa do laboratório na UofM, Kyle, Sarangi, Brinda, Mostafa, Mona, Aida, Chris pela ótima convivência, por me ajudarem com os experimentos e por me fazerem me sentir em casa. Foi muito mais fácil graças a vocês.

Aos membros da qualificação, da pré-banca e da banca de defesa, pela contribuição na melhoria deste trabalho.

Ao Doutor Gustavo Mori por me inserir no mundo dos manguezais, pela orientação, companhia em congressos e por todo auxílio durante essa caminhada.

Aos Doutores Maísa Ciampi, Fábio Matos e Gustavo Mori pelo auxílio nas discussões estatísticas associadas à genética de populações.

Aos funcionários e técnicos do LAGM, Patrícia Zambom, Aline Moraes, Carlos Corona e Dr. Juverlande Lugli, pelo apoio e pela boa convivência, o que tornou o clima de trabalho mais agradável.

Às secretárias do Centro de Biologia Molecular e Engenharia Genética (CBMEG) da UNICAMP, Tânia e Sandra pela prontidão.

Ao Gustavo Mori, Julia V. San Román, Liliane G. D. de Oliveira, João de Oliveira Júnior, Armando Reis Neto por todo o apoio para que o trabalho de campo fosse realizado.

À psicóloga e amiga Iná, que me ajudou a enfrentar as crises existenciais me fazendo compreender que existem muitos meios de se conduzir a própria vida, e nenhum meio é apenas bom ou ruim.

Aos amigos que tive a oportunidade de fazer nesses anos de LAGM. Obrigada a todos vocês que cruzaram meu caminho. Posso afirmar que todos contribuíram de alguma forma para eu me tornar a pessoa que sou hoje.

Em especial, agradeço aos amigos Melina, Estela, Eliza, Carlos, Prianda, Camila, Tatiane, Angela, Fernanda, Juliana, Ketti, Bruno, Iris, Maya, Josh, Luiz Eduardo e muitos outros

que não vou conseguir listar aqui. Amigos de Lab, de Limeira e de lugares por onde passei. Obrigada pelos dias felizes e também pelo ombro amigo, muitas vezes em momentos em que nem eu mesma me aguentava. Espero que continuemos participando da vida um do outro, mesmo que a distância.

Muito obrigada a todos!

ORGANIZAÇÃO DA TESE

Esta tese busca gerar informações que venham corroborar numa melhor compreensão de como a variação genética das espécies de *Rhizophora* (*Rhizophora mangle, R. racemosa* e *R. harrisonii*), da costa brasileira está distribuída, entender fatores que influenciam essa distribuição e estimar parâmetros genéticos, como fluxo gênico, sistema de reprodução e estruturação populacional. Tais parâmetros são essenciais para programas de conservação e manejo, além de permitirem um maior conhecimento sobre os efeitos da fragmentação de manguezais e desflorestamento. O trabalho foi organizado da forma a seguir:

Revisão de literatura atualizada, onde se encontram informações relevantes para o entendimento do trabalho desenvolvido, juntamente com os objetivos propostos. O Capítulo I apresenta os resultados obtidos durante o período de doutoramento, que estão apresentados na forma de um artigo intitulado "Population structure of *Rhizophora* species (Rhizophoraceae) from mangrove forests along the Brazilian coast", refere-se ao desenvolvimento de marcadores moleculares do tipo microssatélite para as espécies de mangue *R. mangle, R. racemosa* e o possível híbrido *R. harrisonii.* Os marcadores desenvolvidos foram utilizados para avaliar a distribuição da diversidade genética em indivíduos de populações dessas espécies, coletados em cline ao longo do litoral brasileiro. Também foi realizada a investigação sobre uma possível zona de hibridação no litoral norte brasileiro entre as espécies *R. mangle* e *R. racemosa*.

No Capítulo II são apresentados, como resultados complementares aos expostos no artigo, estimativas do sistema reprodutivo da espécie *R. mangle.* Assim, foram coletados folhas e propágulos de indivíduos do litoral norte, no município de Salinopólis, PA.

Na última seção são apresentadas as considerações finais de forma sucinta, as conclusões obtidas após o desenvolvimento dessa tese e perspectivas para a continuidade do trabalho.

Por fim, apresento no Anexo I um artigo desenvolvido durante estágio realizado no Departamento de Ciências Biológicas da *University of Manitoba* (Winnipeg, Canadá) sob a orientação da Professora Michele Piercey-Normore.

INTRODUÇÃO

Manguezais: distribuição, importância e diversidade

O termo manguezal (em inglês *mangal*) pode ser definido como um ecossistema de mangue onde populações de animais, micro-organismos e plantas interagem entre si e com o ambiente físico (Schaeffer-Novelli & Cintrón-Molero, 1999); ou ainda como uma comunidade com plantas de mangue, definida tanto pelas espécies que contém como pelo seu ambiente (Tomlinson, 1986). Já a palavra mangue é usada para designar um grupo de árvores e arbustos tropicais, floristicamente diverso com famílias botânicas não relacionadas, mas que compartilham características fisiológicas similares (Schaeffer-Novelli & Cintrón-Molero, 1999). Kathiresan e Bingham (2001), no entanto, definiram mangue como plantas lenhosas que crescem na interface entre terra e mar em latitudes tropicais e subtropicais. Os manguezais são ambientes com alta salinidade, solo lodoso e anaeróbico, que sofre frequentes inundações, e são compostos por uma variedade incomum de animais e plantas adaptados a essas condições (Macintosh & Ashton, 2002). Plantas que são confinadas ao manguezal são chamadas de mangue verdadeiro; plantas que podem ocorrer também em outros lugares são chamadas de vegetaçao associada a florestas de manguezal. O mangue verdadeiro se difere das vegetação associada de florestas de manguezal em características foliares e propriedades osmóticas; são plantas halófitas, enquanto as associações possuem menor tolerância a altas concentrações de sal (Tomlinson, 1986; Wang *et al*., 2010).

A flora dos manguezais possui características específicas que tornam esses ecossistemas funcional e estruturalmente únicos. Características morfológicas e adaptações das árvores incluem raízes aéreas, dispersão de propágulos pelas correntes controladas

pelas marés, rápido crescimento de copa, ausência de anéis de crescimento, eficiente mecanismo de retenção de nutrientes, resistência à ambientes salinos, retenção de água e importante contribuição no balanço de carbono (Alongi, 2002).

Ecossitemas costeiros desempenham papéis importantes na acumulação e estabilização de sedimentos, que proveem diversos serviços ecológicos (Schaeffer-Novelli, 2005). A riqueza biológica dos ecossistemas costeiros faz com que essas áreas sejam os grandes "berçários" naturais para diversas espécies de animais (Barbier, 2014). Além disso, os manguezais proveem proteção e são santuários para pássaros (Macintosh & Ashton, 2002). Também promovem sombra, abundância de substrato para animais sésseis que vivem na região entre marés, matéria orgânica para uma rica cadeia de decompositores e detritívoros e uma variedade de microambientes protegidos da ação das ondas (Por, 1984). As raízes e troncos das plantas de mangue promovem a fixação do solo e são importantes para reduzir a erosão da linha costeira e para proteger essa área contra alagamentos, tsunamis, reduzindo a força das ondas e dos ventos em regiões costeiras (Diegues, 1991; Othman, 1994; Alongi,2008, Mclvor *et al.,* 2012).

Apesar de todos os benefícios trazidos pelos manguezais, as atividades humanas já destruíram 35% dessas florestas no mundo nas últimas duas décadas (Valiela *et al*., 2001). Os principais vetores potenciais geradores de impactos sobre os manguezais incluem a barragem de rios, a agropecuária, incluindo a aquicultura, e a urbanização, que resultam em pressões sobre o balanço de sedimentos e águas em estuários, fluxo de nutrientes e poluentes, além do desmatamento direto das florestas (Valiela *et al*., 2001; Macintosh & Ashton, 2002; Alongi, 2002; Lacerda, 2003). As florestas de mangue são, também, frequentemente perturbadas por ciclones e outras tempestades, raios, tsunamis e

inundações, e sempre levam décadas para se recuperar (Smith *et al.* 1994). O aquecimento global, inclusive, pode afetar os manguezais por causa da elevação do nível do mar, grandes tempestades, mudanças de temperatura, de concentração de CO_2 atmosférico, padrões de circulação marítimos, entre outros (Gilman *et* al., 2008). Os efeitos sobre o ambiente costeiro se dão por meio da erosão e sedimentação, eutrofização e mudança nas cadeias alimentares e na estrutura de comunidades (Lacerda, 2002; Macintosh & Ashton, 2002; Alongi, 2002).

Estima-se que os manguezais cubram uma área estimada de 137760 km^2 ao redor do globo (Giri *et al.,* 2011) e ocorrem ao longo da costa na zona entremarés (Huxham *et al.,* 2010). Os manguezais mais desenvolvidos crescem ao longo de costas tropicais abrigadas e úmidas, por exemplo, em deltas de principais rios como o Ganges (Índia), Mekong (sudeste asiático) e Amazonas (Brasil), e nas linhas costeiras protegidas por grandes massas de areia, como por exemplo, em Madagascar, no arquipélago da Indonésia e em Papua Nova Guiné (Macintosh & Ashton, 2002). Estendem-se em regiões temperadas, e estão mais amplamente distribuídos nas regiões entre 30º N e 30º S do equador (Spalding *et al*., 2010, Giri *et al.*, 2011).

Uma característica distinta de manguezal é a baixa diversidade de plantas superiores (Tomlinson, 1986). Segundo Tomlinson (1986) existem 54 espécies verdadeiras de mangue em 20 gêneros pertencentes a 16 famílias, mais 60 espécies de associações de mangue em 46 gêneros. Em contraste, Duke e colaboradores (1998) incluíram aproximadamente 70 espécies de mangue verdadeiro em 28 gêneros, sendo 17 exclusivamente presentes nesse habitat. Esse ecossistema é bem menos diverso, se comparado a outros ecossistemas tropicais, como a Mata Atlântica e o Cerrado (Valiela, 2001; Macintosh & Ashton, 2002;

Duke *et al.*, 2007, Joppa *et al.* 2011; Zachos & Habel 2011). Por outro lado, os manguezais são taxonomicamente diversos e evoluíram pelo menos 16 vezes em 16 famílias (Macintosh & Ashton, 2002). De 34 espécies que representam os principais componentes de manguezais, 25 pertencem a apenas duas famílias, que são *Acanthaceae* e *Rhizophoraceae.* Essas famílias dominam os manguezais em todo o mundo (Macintosh & Ashton, 2002). A Indonésia, a Austrália, o Brasil e a Nigéria possuem, aproximadamente, 43% das florestas de mangue do mundo (Alongi, 2002; Giri *et al*., 2010).

Tomlinson (1986) mostra que existem dois centros de diversidade de mangue no mundo que apresentam muitas diferenças entre os inventários florísticos, tanto no tamanho como na composição. O primeiro é o grupo oriental (Indo West Pacific - IWP), que inclui a África ocidental, Índia, sudeste da Ásia, Austrália e o oeste do Pacífico. O segundo é o grupo ocidental (Atlantic East Pacific - AEP), que inclui o oeste da África, a porção Atlântica da América do Sul, o Caribe, Flórida, América Central, Pacífico norte e América do Sul. O grupo oriental é cinco vezes maior em espécies de plantas que o ocidental (Tomlinson, 1986). Essa diferença é explicada pela hipótese da vicariância na qual todas as plantas de mangue teriam uma origem comum e, pela deriva continental, teriam sido isoladas em diferentes regiões do globo. O padrão de distribuição de manguezais pelo mundo sugere que as plantas de mangue teriam surgido no Cretáceo Superior e Terciário Inferior no mar de Tétis, de 60 a 50 milhões de anos atrás (Ellison, *et al.*, 1999). Outra explicação para essa diferença no número de espécies entre essas regiões seriam diferenças nas taxas de surgimento de linhagens evolutivas no hemisfério oriental, com áreas maiores de manguezais, favorecendo a migração e adaptação na região IWP, o que levou a um

maior número de espécies (Ricklefs *et al.,* 2006). A figura 1 ilustra a distribuição de manguezal no mundo e a áreas AEP e IWP.

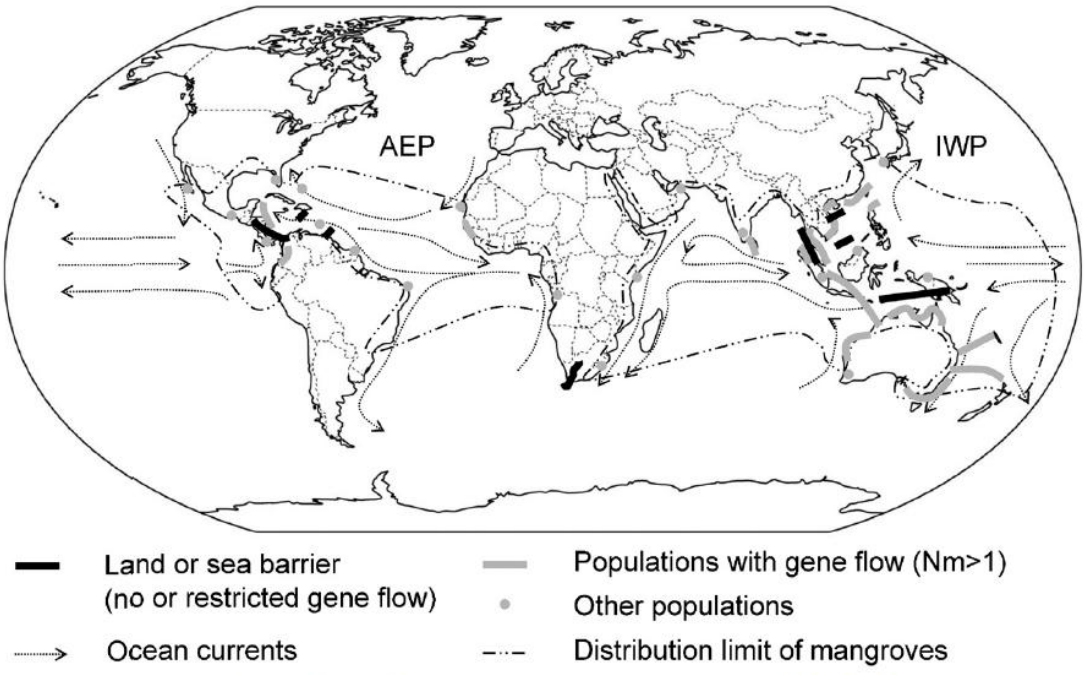

Fig. 5. Conceptual overview of land and oceanic barriers and of coastal zones with high levels of gene flow within the Atlantic East Pacific region and the Indo West Pacific (values and references are given in Tables 2 and 3).

Figura 1. Figura ilustrando a de distribuição de manguezal no mundo (Triest, 2008).

No litoral brasileiro, que tem uma extensão de 7.408 km (CIMA, 1991), com 13.800 km² de área (Kjerfve & Lacerda, 1993), os manguezais estendem-se do extremo norte no Oiapoque, estado do Amapá (4º 30' N), até Santa Catarina (28º28S (Schaeffer-Novelli, 1999; Soares *et al*., 2012). É a segunda maior área de manguezal do mundo, ficando atrás apenas das regiões sul e sudeste da Ásia (Spalding *et al.,* 2010). O litoral dos estados do Amapá, Pará e Maranhão, principalmente entre Belém-PA e São Luís-MA, apresenta 85% da área com manguezais de todo Brasil. Além disso, nesta região os manguezais são maiores e estruturalmente mais complexos (Figura 2). Já o litoral nordeste, do Ceará ao Rio

de Janeiro, embora englobe quase metade do litoral brasileiro apresenta apenas aproximadamente 10% da área total de manguezais do Brasil. Os 5% restantes encontram-se nas regiões sul e sudeste, entre São Paulo e Santa Catarina, onde os manguezais são restritos a interiores de baías. Essas diferenças estão relacionadas a fatores climáticos, pluviométricos, geológicos, antrópicos e relativos ao relevo (Schaeffer-Novelli *et al*., 1990; Lacerda, 2003).

Figura 2. Figura ilustrando a diferença de tamanho de árvores de manguezais na região norte (esquerda) e região sudeste do Brasil (Foto: Gustavo Maruyama Mori).

Devido à importância biológica e econômica das espécies que compõem os manguezais, muitas pesquisas vêm sendo realizadas em todo o mundo, com o uso de marcadores moleculares como as isozimas, RAPD, RFLP e AFLP e marcadores microssatélites (SSRs) para elucidar aspectos relativos à evolução (Duke *et al*., 2002), à origem de híbridos interespecíficos (Parani *et al*., 1997), à relação filogenética com outros

gêneros da família *Rhizophoraceae* (Schwarzbach & Ricklefs, 2000), e à diversidade de filogenia molecular e genética em espécies da tribo *Rhizophoreae* (Lakshmi *et al*.,2002). Pesquisas sobre manguezais avançaram consideravelmente nos últimos anos. O desenvolvimento de técnicas moleculares tem gerado oportunidades de levar a pesquisa sobre manguezais em novas direções para agregar novos conhecimentos acerca do tema (Schwarzbach & Ricklefs, 2001). Muitos estudos foram desenvolvidos na região AEP, e mostram que existe estruturação da diversidade genética entre as populações (Dodd *et al*., 2000, 2002; Pil *et al.*, 2011; Cerón-Souza *et al*., 2012; Takayama *et al.*, 2013). Ou seja, a taxa de fluxo gênico seria baixa, o que pode ser explicado pelo sistema misto de cruzamento dessas espécies, com altos níveis de autofecundação e cruzamento biparental, limitando o fluxo gênico por meio de pólen (Cerón-Souza *et al.,* 2012; Nettel *et al*., 2013; Yahya *et al.,* 2014). No entanto, a maioria das espécies de mangue são dispersas pela água por meio de propágulos flutuantes que são adaptados para dispersão a longas distâncias, com a vantagem de utilizar correntes oceânicas para colonizar novos habitats e reabastecer áreas de manguezais já existentes (Duke *et al.,* 1988; Nathan *et al.,* 2008). Evidências raras de dispersão a longa distância foram levantadas para as espécies *Avicennia germinans*, que pode apresentar dispersão transatlântica (Nettel & Dodd, 2007), e *Rhizophora mangle,* que além de se dispersar entre os oceanos, há também evidência de movimento de indivíduos entre as ilhas do sul do Pacífico e a costa americana do Oceano Pacífico (Takayama *et al.,* 2013). A vicariância, isolamento por distância e as correntes marítimas superficiais são fatores importantes que podem afetar o fluxo de genes e a distribuição dessas espécies, como apresentado por estudos recentes sobre a dispersão e distribuição de manguezais modernos (Yahya *et al.,* 2014; Lo *et al.,* 2014). No Brasil, correntes marinhas também são

um fator importante na distribuição da diversidade genética de plantas de mangue. Pil *et al.* (2011) mostraram que a bifurcação da corrente marinha Sul Equatorial no estado do Rio Grande do Norte em correntes do Norte do Brasil (com sentido norte) e corrente do Brasil (sentido sul), explicaria a diferenciação encontrada para populações da espécie *Rhizophora mangle* presentes nessas regiões.

Como citado anteriormente, a maior área de manguezais do Brasil se encontra na região norte do país, onde também se encontra um maior número de espécies (Schaeffer-Novelli *et al*., 1990, Menezes *et al.*, 2008). Pil *et al.* (2011) evidenciaram que a diversidade genética também é maior nessa região, quando comparada com outras regiões do país no que diz respeito à espécie *R. mangle*. Esse cenário se repete em outras partes do globo, tanto para *A. germinans* quando para *R. mangle.* Uma hipótese que poderia explicar esse cenário de diferenças e estruturação genética são mudanças climáticas que ocorreram no último período glacial, durante o Quaternário. O clima na costa do Atlântico Ocidental era mais frio com frequentes oscilações de temperatura. Durante essas glaciações, as placas de gelo que se formavam limitaram as zonas de vegetação em regiões equatoriais, com clima mais ameno (Hewit, 2000, 2004). Como a distribuição de manguezais é fortemente influenciada por fatores climáticos, como temperatura e umidade (Duke *et al.*, 2002; Krauss *et al.* 2008), durante as glaciações, os manguezais ficaram restritos a essas zonas (Saenger, 1998). Com o aumento da temperatura e da umidade, após o fim do período glacial, os manguezais foram se expandindo gradualmente para latitudes maiores (Pil *et al.*, 2011). Essa hipótese corrobora com dados paleológicos sobre manguezais no Brasil que mostram que os manguezais na região norte são mais antigos que os da região mais ao sul do litoral (Vedel *et al.,* 2006; Amaral *et al.,* 2006). Concluindo, o que se acredita é que as regiões ao

sul foram colonizadas mais recentemente, o que explica a baixa diversidade genética, consequência de consecutivos eventos de fundação seguidos de colonização (Pil *et al.,* 2011).

Possíveis híbridos estão sendo reportados em gêneros como *Rhizophora, Sonneratia, Lumnitzera* e *Bruguiera* (Tomlinson, 1986; Duke & Ge, 2011; Ng & Szmidt, 2014). A maioria desses híbridos são identificados com base em características morfológicas intermediárias de dois ou mais possíveis genitores (Chan, 1996; Duke, 2010; Kathiresan, 1995, 1999). Muitos estudos recorreram ao uso de ferramentas moleculares para estabelecer a identidade de híbridos (Parani *et al.,* 1997; Zhou *et al.,* 2005, 2008; Qiu *et al*., 2008; Céron-Souza *et al.,* 2010; Ng & Szmidt, 2014). Esses estudos demonstram que espécies de mangue, como *Rhizophora mangle* e *Avicennia germinans,* não possuem limites evolutivos absolutos e estão sujeitas ao processo de hibridação natural interespecífica onde suas distribuições se sobrepõe (Nettel *et al*., 2008; Céron-Souza *et al.,* 2010; Takayama *et al.,* 2013). Céron-Souza e colaboradores (2010) apresentaram resultados que indicam que existe uma hibridação introgressiva e que vem ocorrendo há muito tempo entre as espécies de *Rhizophora* do Novo Mundo. Resultados obtidos com o estudos de espécies de *Rhizophora* da região IWP mostram que está ocorrendo hibridação entre as espécies *R. mucronata* e *R. stylosa* com diferentes níveis de introgressão e podem ocorrer em todas as direções (Ng & Szmidt, 2014). No entanto, a maioria dos híbridos reportados são limitados a geração "F_1" e poucos são resultado de introgressão interespecífica (Cerón-Souza *et al.,* 2010; Sun & Lo, 2011; Ng *et al.,* 2013). Apesar de todos os estudos existentes sobre híbridos de mangue, ainda são necessários esforços para

compreender como esses processos de hibridação podem influenciar a evolução dessas espécies (Barton, 2001; Soltis & Soltis, 2009; Abbott *et al.,* 2013).

O desenvolvimento de microssatélites em espécies de mangue pode facilitar estudos sobre ecologia molecular dessas populações com uma precisão muito maior do que alozimas e um poder analítico também maior do que marcadores dominantes (RAPD, AFLP), tanto para o nível do indivíduo como para assembleias agrupadas de (sub) populações (Triest, 2008). Rosero-Galindo e colaboradores (2002) desenvolveram dez marcadores do tipo microssatélites para *Rhizophora mangle*. Estes foram utilizados para avaliar o grau de diversidade genética em cinco populações desta espécie na Colômbia (Arbeláez-Cortes *et al*., 2007). Em 2007, Takayama e colaboradores, caracterizaram outros 14 microssatélites para esta mesma espécie, e testaram sua transferência para outras cinco espécies do mesmo gênero coletadas no México e na Costa Rica, sendo que alguns desses marcadores amplificaram em todas elas.

Microssatélites ou SSRs (*Simple Sequence Repeats*) são motivos de 2 a 6 pares de base repetidos em "*tandem"* e encontrados em quase todos os organismos (Schlötterer, 2000). São considerados os marcadores que mais se aproximam do ideal para estudos genéticos devido às seguintes vantagens: 1) são abundantes e uniformemente distribuídos pelo genoma; 2) são codominantes; 3) são usualmente multialélicos, apresentando grande conteúdo informativo por loco gênico; 4) apresentam, geralmente, alto grau de heterozigozidade e 5) são marcadores de baixo custo e fácil aplicação à análise de um grande número de plantas, quando a técnica está estabelecida para a espécie (Ferreira & Grattapaglia, 1998).

Como as espécies do gênero *Rhizophora* são amplamente distribuídas e dominam muitos manguezais tropicais em todo o mundo, acredita-se que tenham um papel fundamental para o ecossistema manguezal (Duke & Allen, 2006). Nesse contexto, este trabalho pretende gerar informações para melhor compreender como a variação genética das espécies de *Rhizophora* (*R. mangle, R. racemosa* e *R. harrisonii*), da costa brasileira está distribuída e entender fatores que influenciam essa distribuição com o desenvolvimento e uso de marcadores moleculares do tipo microssatélite que provêm uma alta resolução e sensibilidade para estimar parâmetros genéticos, como fluxo gênico, taxa de endocruzamento e estruturação populacional.

Espécies do gênero *Rhizophora* L. do litoral brasileiro

O gênero *Rhizophora* é composto com espécies conhecidas como mangue vermelho, e é um gênero antigo e amplamente distribuído, presente nos neotrópicos desde o Eoceno (50 Ma). Dez milhões de anos depois, essas espécies se espalharam pelo mundo (Figura 3) (Ellison *et al.,* 1999; Duke *et al.,* 2002; Tyagi *et al.*, 2003). Atualmente *Rhizophora* é o gênero mais notável em ecossistemas manguezal, e três espécies são reconhecidas nos neotropicos: *R. mangle, R. samoensis, R. racemosa,* e um possível híbrido, *R. harrisonii.*

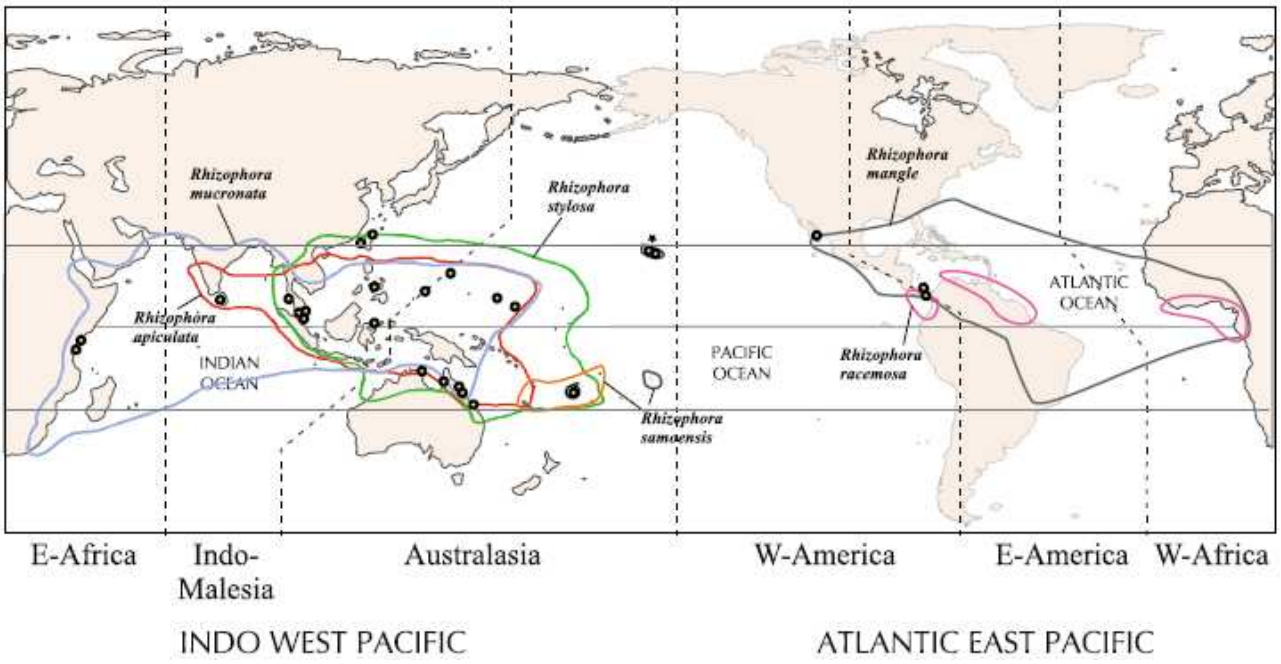

Figura 3. Mapa mostrando a distribuição de espécies de *Rhizophora* no mundo (Lo *et al.,* 2014).

Rhizophora encontra-se geralmente nas franjas de bosques em contato com o mar, ao longo dos canais, na desembocadura de rios ou, nas partes internas dos estuários onde a salinidade não é muita elevada. É caracterizado por árvores ou arbustos com numerosas troncos tipo escora (rizóforos), inflorescências cimosas, flores hermafroditas pedunculares e reprodução por viviparidade, onde o embrião germina dentro do fruto ainda ligado à planta-mãe (Figura 4) (Prance *et al*., 1975). Evidências mostram que as espécies deste gênero são polinizadas principalmente pelo vento, pois possuem um número muito maior de grãos de pólen em relação ao número de ovários por flor (Tomlinson, 1986). As espécies encontradas ao longo do litoral brasileiro são: *Rhizophora mangle* L., da desembocadura do rio Oiapoque, até a latitude da Ilha de Santa Catarina; e *R. racemosa* G. F. W. Meyer e *R.* x *harrisonii* Leech., encontram-se da região norte até o Delta do Rio Parnaíba, no Piauí (Schaeffer-Novelli & Cintrón, 1986).

Figura 3. Propágulos da espécie *Rhizophora mangle* (Foto: Gustavo Maruyama Mori).

A *Rhizophora mangle*, também conhecida popularmente como mangue verdadeiro, tem como principal característica suas raízes aéreas que, partindo do tronco em formato de arcos, atinge o solo, o que permite uma maior sustentação em solos pouco consolidados. Pode alcançar uma altura de até 19 metros e apresenta um diâmetro médio de 30 centímetros. Suas folhas apresentam ápice estreito e curto; o botão floral é comprido com ponta aguda; a inflorescência axilar agrupa geralmente até quatro flores e apresenta-se com nenhuma, ou mais ramificações (Figura S1, Capítulo I); a polinização é realizada principalmente pelo vento, sendo os insetos agentes secundários e podendo também apresentar autopolinização; com fertilidade em torno de 97 a 100% (Breteler, 1969; Prance *et al*. 1975; Menezes & Melo, 1997; Luz, 2006).

R. racemosa são árvores que podem medir de três até 16,5 metros de altura; com casca marrom-amarelada; as raízes no fuste podem chegar a dois metros e meio de altura; suas folhas possuem o ápice mais largo e comprido, quando comparado com *R. mangle* (Luz, 2006); o botão floral é curto, normalmente com ápice obtuso e pouco acuminado; a inflorescência pode ramificar três ou mais vezes apresentando muitas flores (Figura S1, Capítulo I); podem apresetar fertilidade em torno de 97% (Breteler, 1969; Prance *et al*., 1975).

Estudos realizados sobre a ecologia e morfologia demonstraram que *R. harrisonii* apresenta uma posição intermediária entre *R. mangle* e *R. racemosa.* O botão floral de *R. harrisonii* é mais fino, de formato ovado, com ápice e comprimento intermediário quando comparados a *R. mangle* e *R. racemosa* (Figura S1, Capítulo I)*;* suas inflorescências embora apresentem muitas flores, não possuem tantas ramificações quanto *R. racemosa*; a porcentagem de fertilização está entre 20 a 60% e seu nível de tolerância à salinidade é intermediária entre *R. mangle* e *R. racemosa* (Prance *et al*., 1975). Estas características indicam uma possível natureza híbrida da espécie (Breteler, 1969; Prance *et al.*, 1975; Tomlinson, 1986; Luz, 2006; Duke & Allen, 2006, Céron-Souza *et al.,* 2010).

OBJETIVOS

Objetivo Geral

Para caracterizar a diversidade genética e a estrutura populacional das espécies do gênero *Rhizophora (R. mangle, R. racemosa* e *R. harrisonii*) do litoral brasileiro.

Objetivos específicos

- ✓ Desenvolver bibliotecas enriquecidas em microssatélites para as espécies *R. mangle, R. racemosa* e *R. harrisonii*
- ✓ Sequenciar e avaliar os locos dos marcadores do tipo microssatélites isolados, e desenvolver *primers* para amplificar os locos de microssatélites
- ✓ Genotipar indivíduos das espécies *R. mangle, R. racemosa* e *R. harrisonii* com os marcadores microssatélites polimórficos
- ✓ Estimar parâmetros populacionais para as três espécies como estruturação populacional, diversidade gênica e tamanho efetivo de população
- ✓ Estudar o sistema reprodutivo de *R. mangle*
- ✓ Avaliar a existência de uma zona de hibridação entre *R. mangle* e *R. racemosa* em determinadas populações do litoral norte do Brasil por meio de marcadores microssatélites

CAPÍTULO I

Population structure of *Rhizophora* species from mangrove forests along the Brazilian coast

Patrícia M. Francisco[1], Gustavo M. Mori[1, 2], Fábio de M. Alves[1], Maisa B. Ciampi-Guillardi[1,3], Anete P. Souza[1,4*]

[1] Center for Molecular Biology and Genetic Engineering, University of Campinas, CP 6010, Campinas, São Paulo, Brazil, CEP 13083-875. E-mail: patricia_francisco@ymail.com

[2] São Paulo Agency for Agribusiness Technology, Piracicaba, São Paulo, Brazil, CEP 13400-970.

[3] Department of Plant Pathology, ESALQ, University of São Paulo, Piracicaba, São Paulo, Brazil, CEP 13418-900

[4] Department of Plant Biology, Institute of Biology, University of Campinas, Campinas, São Paulo, Brazil, CEP 13083-862.

[*] Corresponding author

E-mail: anete@unicamp.br (APS)

Abstract

Rhizophora L., the red mangrove, is an ancient and widely distributed mangrove tree genera which occur in estuarine regions where freshwater outflow reaches the ocean or in lagoons along the shoreline. In Brazil, this genera is compound by *Rhizophora mangle*, *Rhizophora racemosa*, and a putative hybrid species, *Rhizophora harrisonii*. Several factors influence the patterns of genetic diversity and evolution of these species, such as oceanography, climate and topographic. Thus, the aim of this study was to investigate the genetic variation and population structure of *Rhizophora* species along the Brazilian coast, developing and using microsatellite molecular markers. We genotyped 318 individuals of *Rhizophora mangle* at nine microsatellite loci, and 33 individuals of *R. racemosa* and 37 individuals of *R. harrisonii* at 16 microsatellites. We found genetic diversity among populations of *R. mangle* from northern and southern coast. We also found a mixed mating system with tendency to alogamy. Northern region populations showed higher genetic diversity. These results were confirmed by population structure analyses that indicated the existat ence of two distinct group of genetic diversity in *R. mangle.* The variation found among the populations from sites on the northern and southern Brazilian coast suggests that the species *R. mangle* is not composed of a single panmictic population. In addition, when comparing the three species of *Rhizophora,* we found no evidence of introgressive hybridization among the species that would lead to a hybrid. These results are of great importance for understanding the dynamics of Brazilian mangrove ecosystems.

Introduction

Mangroves are heterogeneous habitats with an uncommon variety of animals and plants that are adapted to conditions of high salinity, frequent flooding and muddy anaerobic soil [1] and cover approximately 137,700 km^2 worldwide [2]. The most developed mangroves grow in sheltered, moist areas, for example, in deltas of major rivers such as the Ganges, the Mekong and along the Amazon tropical coast and along coastlines protected by large masses of sand, such as in Madagascar, the Indonesian archipelago and Papua New Guinea [1].

The species of mangroves are distributed mostly between the latitudes 30° N and 30° S [3], However, their diversity is not uniform There are two centers of diversity, an Eastern - Indo-West Pacific (IWP) group and a Western - Atlantic-East Pacific (AEP) group; the former has five times the number of species compared to the latter [4]. Ellison and colleagues [5] explain this difference according to the vicariance hypothesis, in which all mangrove species have a common origin and have been isolated in different regions of the world via continental drift. Furthermore, in both regions, the number of different species decreases as the latitude increases [6, 7]. Several abiotic factors influence these patterns of species diversity, such as oceanography, climate, and topographic and soil conditions [6]. These patterns are also maintained by biotic factors such as the limited effective dispersion, floating capabilities and salt tolerance of seedlings with varying degrees of viviparity; the continued development of embryos without dormancy also affects the species composition [6, 8, 9].

A distinctive feature of mangrove communities is their low diversity of higher plants [4]. This ecosystem is far less diverse, compared to other tropical ecosystems such as the Atlantic forests [1, 10-13]. In the Brazilian coast, the red mangrove, *Rhizophora* is one of the genus that characterize the mangrove forests [16]; and it is compound by *Rhizophora mangle* L., *Rhizophora racemosa* G.F.W. Meyer, and a putative hybrid species, *Rhizophora harrisonii* Leechman. *Rhizophora* L. distribution expanded worldwide during the Middle Eocene [5, 14, 15]. On the

Brazilian coast, *R mangle* has the largest distribution, occurring along all the mangrove area in Brazil. *R. racemosa* and *R. harrisonii* occur in the northern region [16].

As the species of the *Rhizophora* genus dominate and are widely distributed in many tropical mangroves worldwide, is believed to have a pivotal role in mangrove ecosystems [17]. These mangrove trees have morphological characteristics and adaptations including aerial roots and fast-growing canopy which contributes with an efficient nutrient retention mechanism, water retention and balance sheet carbon [18, 19].

Evidence shows that the species of this genus are mainly pollinated by the wind, as they have a much larger number of pollen grains relative to number of ovaries per flower [4]. Their viviparous propagules are dispersed by water and their dispersion are under ocean current influences [9].

Because of the biological and economic importance of the species that make up the mangroves, studies have been performed worldwide. The development of molecular techniques has generated opportunities to bring research on mangroves in new directions to add knowledge on the subject [20, 21]. Many studies have been developed in AEP region, and show that there is structure of genetic diversity among populations of *Rhizophora* [22-24]. Also, it was found low gene flow rate, evidences of a mixed mating system, high levels of self-pollination and biparental crossing, limiting gene flow through pollen [23]. Recent studies have hypothesized that the major land and sea barriers to the dispersal of propagules are important evolutionary factors influencing mangrove distribution, and ancient and ongoing interspecific hybridization has been recorded [23-25]. Additionally, substantial genetic differences have been observed in different genera [26, 27].

Aiming to better understand the processes that influence the dynamics of Brazilian mangrove ecosystems, in this study we investigated genetic patterns such as genetic diversity and population structure of *Rhizophora* species along the coast, developing and using microsatellite molecular markers Based on previous studies [22, 23], our hypotheses are (1) that distinct genetic structures differentiate *Rhizophora* L. populations in two clusters on the Brazilian seashore, one on the noth and other on the south coast due to vicariance events and oceanographic patterns; (2) and that *R. harrisonii* is an hybrid species originated from the crossing of *R. mangle* and *R. racemosa.*.

Materials and Methods

Plant material and sampling strategy

We sampled 318 genotypes of *R. mangle* from 11 sites, 33 individuals of *R. racemosa* and 37 individuals of *R. harrisonii* from two localities along the Brazilian coast, covering more than 4,900 km of coastline (Fig. 1, Table 1). We recorded latitude and longitude using a Global Positioning System receiver (Garmin 76CSx, WGS-84 standard, Garmin International Inc., Olathe, KS, USA). Licenses (17159 and 17130) to collect leaves of these species were obtained from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA, currently Instituto Chico Mendes de Conservação da Biodiversidade, ICMBio).

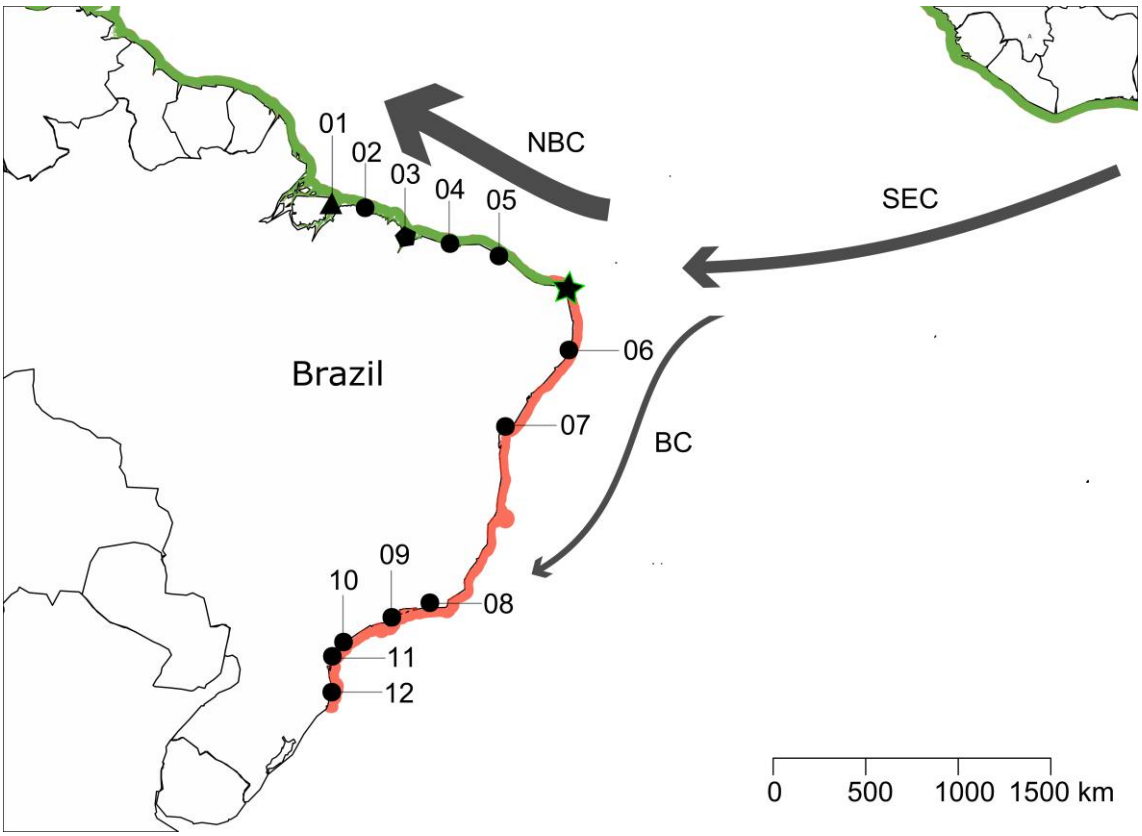

Fig. 1. Geographic distribution of *Rhizophora mangle, R. racemosa* and *R. harrisonii* along the Brazilian coast. Map of South Atlantic displaying the geographic distribution of red *R. mangle* (red line) and the occurrence of the three species (*R. mangle, Racemosa* and the putative hybrid *R. harrisonii* - green line); and the collection points of *R. mangle* (circles), *R. racemosa* and *R. harrisonii* (triangle), and where the three species were collected (pentagon). 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 mangrove species range: the South-Equatorial (SEC), North Brazil (NBC) and Brazil currents (BC). Arrow size and line widths illustrate the mean current speed [73].

Table 1. Description of the locations of the samples of *Rhizophora mangle, R. racemosa* and *R. harrisonii* from the Brazilian coast.

Rm	Rr	Rh	Locality (City, State)	Geographic Coordinates	Position in Fig. 1
	RrSPA (11)	RhSPA (7)	Soure, Pará	0° 43' 26" S, 48° 29' 24" W	1
RmBPA (30)			Bragança, Pará	0° 49' 12" S, 46° 36' 56" W	2
RmAMA (31)			Alcântara, Maranhão	2° 24' 37" S, 44° 24' 22" W	3
RmPPI (13)	RrPPI (22)	RhPPI (30)	Parnaíba, Piauí	2° 46' 42" S, 41° 49' 20" W	4
RmPCE (34)			Paracuru, Ceará	3° 24' 47" S, 39° 3' 23" W	5
RmTPE (20)			Tamandaré, Pernambuco	8° 31' 35" S, 35° 0' 48" W	6
RmVBA (25)			Vera Cruz, Bahia	12° 59' 1" S, 38° 41' 5" W	7
RmGRJ (25)			Guapimirim, Rio de Janeiro	22° 42' 5" S, 43° 0' 26" W	8
RmUBA (32)			Ubatuba, São Paulo	23° 29' 22" S, 45° 9' 52" W	9
RmCNN (35)			Cananéia, São Paulo	25° 1' 12" S, 47° 55' 5" W	10
RmPPR (24)			Pontal do Paraná, Paraná	25° 34' 30" S, 48° 21' 9" W	11
RmFSC (49)			Florianópolis, Santa Catarina	27° 34' 37" S, 48° 31' 8" W	12

Sampled populations of *R. mangle* (Rm), *R. racemosa* (Rr) and *R. harrisonii* (Rh) are indicated by three capital letters. Sample sizes are indicated in parentheses. The city and state in Brazil, geographic coordinates and numbers corresponding to Fig. 1 are denoted for each site.

The geographic information of the localities from which the samples of each species were taken is shown in Table 1, with Rm, Rr and Rh indicating *R. mangle, R. racemosa* and *R. harrisonii*, respectively, and with a three-letter code indicating the locality from which each sample was obtained. We distinguished the species in the field using both vegetative and reproductive traits to minimize misidentification. The three species are similar and they can be identified by the number of flowers per inflorescence. *R. mangle* axillary

inflorescence groups usually contain up to four flowers with zero, one or two branches. *R. harrisonii* has a multi-branched, many-flowered inflorescence like *R. racemosa*, but the bud shape, the branching type, and the longer pedicels resemble those of *R. mangle* (S1 Fig.) [4, 28].

S1 Fig. Representation of differences in number of branches and bud shape of three Western hemisphere *Rhizophora* species (photos by Gustavo Maruyama Mori).

We deposited voucher specimens from every location in the University of Campinas (UEC) and Embrapa Amazônia Oriental (IAN) herbaria, which are both in Brazil. For genetic analyses, we sampled leaves from flowering trees that were at least 20 m from any other sampled trees and kept the leaves in zip-lock bags containing silica gel. This leaf material was lyophilized and stored at -20°C prior to DNA isolation.

Construction of a microsatellite-enriched library

To identify and characterize microsatellites, we developed microsatellite markers for *R. mangle, R. racemosa* and *R. harrisonii*. Using the DNeasy® Plant Mini Kit (Qiagen, Hilden, DE, Germany) according to the manufacturer's instructions, we isolated the genomic DNA from one individual of each species sampled in northern Brazil (0°43 26" S,

48°29'24" W for *R.* mangle and 0°49'12"S, 46°36 56" W for *R. racemosa* and *R. harrisonii*).

We constructed microsatellite-enriched libraries for the three species as previously described by Billotte and colleagues [29]. The enrichment with streptavidin-coated magnetic beads (Streptavidin MagneSphere Paramagnetic Particles, Promega, Madison, WI) and biotinylated $(CT)_8$ and $(GT)_8$ probes was done followed by genomic DNA digestion was with *Afa*I. The fragments were PCR amplified and cloned into the pGEM-T vector (Promega, Madison, WI). We cultivated the XL1-Blue *Escherichia coli* competent cells transformed with the recombinant plasmids on agar medium containing ampicillin (100 mg/ml), X-galactosidase 2% (100 μg/ml) and IPTG (100 mM). We selected and sequenced clones using Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI 377 sequencer (Applied Biosystems, Foster City, CA). We aligned and edited the sequences using SeqMan Software (DNAStar, Madison, WI), and removed the adapters and restriction sites using Microsat Software (A. M. Risterucci, CIRAD, personal communication). For the identification of microsatellite-enriched regions, we used the Simple Sequence Repeat Identification Tool (SSRIT) [30] and defined the following criteria for the number of repeats of motifs: five or more repeats for dinucleotides, four or more repeats for trinucleotides and three or more repeats for pentanucleotides. We designed primers using the PrimerSelect software (DNAStar, Madison, WI).

To amplify the fragments we used 20 μl polymerase chain reactions (PCR) containing 2 ng of template DNA, 2 mM $MgCl_2$, 50 mM KCl, 20 mM Tris–HCl (pH 8.4), 0.2 mM dNTPs, 0.19 mg/ml BSA (bovine serum albumin), 0.15 mM of each primer and 1

U of *Taq* DNA polymerase. PCR was performed according to a touchdown thermo cycling program: 94°C for 2 min, 2 × [10 cycles of 94°C for 1 min, 65°C (−1°C/cycle) for 1 min 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. The amplified samples were genotyped by vertical electrophoresis using 6% denaturing polyacrylamide gels, and DNA bands were visualized using silver nitrate [31]; the sizes of the resulting fragments were estimated by comparison to a 10-bp DNA ladder (Invitrogen, Carlsbad, CA).

Analysis of genetic diversity

Linkage disequilibrium and adherence to the HW equilibrium were tested for all used markers with Genepop v.1.2 [32] software according to the Fisher exact test using 10,000 interactions with 100 batches. The analysis of null alleles was performed using the algorithm of Dempster *et al*. [33] with FreeNA [34] software, accepting a frequency of n>0.20 as the presence of null alleles. The number of alleles (N_a) was calculated in GenALEx 6.4 [35] and the polymorphism information content (PIC) using PIC Calculator [36]. The effective number of alleles (N_e), the expected heterozygosity (H_E), and the observed heterozygosity (H_O) were calculated using GenALEX 6.4 [35]. Estimates of allelic richness (A_R) and the F-statistics of Weir and Cockerham (F_{IT} (F), F_{ST} (θ) and F_{IS} (f)) [37] based on 10,000 resamplings and a 5% nominal adjustment were performed using the FSTAT 2.9.3.2 [37, 38].

To assess the level of population genetic structure, we used a series of complementary approaches, considering different sets of microsatellite markers for each species because we have not tested the markers developed for *R. harrisonii* in the species *R. mangle*, and we could not transfer all the markers of *R. mangle* in *R. racemosa* and vice

versa; for *R. harrisonii* and *R. racemosa* was possible to use the same set of markers (S2 and S3 Tables in S1 File). For this reason, the analyses for *R. harrisonii* and *R. racemosa* were performed together. The apparent rate of inbreeding (t_a) was obtained through the expression $t_{a=}\frac{(1-f)}{(1+f)}$ [39]. We calculated the multilocus Weir & Cockerham estimator of F_{ST} between all pairs of populations using FSTAT and used these statistics to compare the average levels of between-population differentiation in the studied regions [40, 38].

The Bayesian inference of the population genetic structure and grouping of 318 individuals of *Rhizophora mangle*, 37 individuals of *R. racemosa* and 39 individuals *R. harrisonii* was performed with the Structure v.2.3.2 program [40]. We used admixture models that disregarded information about the individuals source and considered co-related allele frequencies between loci in admixed populations. For *R. mangle,* we evaluated the number of groups (*K*) varying from 1 to 15, corresponding to the sampled sites, with 50 repetitions each with the length of the Markov Chain Monte Carlo (MCMC) performed with a 500,000 steps following a burn-in period of100,000 steps. For *R. racemosa* and *R. harrisonii,* we used the same model and evaluated *K* ranging from 1 to 5, to investigate the occurrence of substructure. The most likely *K* was selected using the *ad hoc* statistics ΔK [41] which was estimated using the online tool Structure Harvester [42]. We used CLUMPP software [43] to combine the results from multiple replicates for the K with the highest likelihood, using the Greedy algorithm. The output was plotted using DISTRUCT v. 1.1 [44].We also performed hierarchical analyses of molecular variance (AMOVA) in GenAlEx 6.4 software with 9999 random permutations to test for significance to partition genetic variation among populations and regions, and within populations [35, 45].

Ongoing hybridization between species

We used a different set of markers to evaluate the ongoing hybridization between the *Rhizophora* species because we only considered microsatellites that cross-amplified for *R. mangle, R. racemosa* and *R. harrisonii*. We applied 13 microsatellites in these analyses (S4 Table S4 in S1 File).

For these investigations, the same methods used to study the genetic diversity and the population structure were employed. We also used the Discriminant Analysis of Principal Components (DAPC), a multivariate method [46], which was implemented in the R package ADEGENET 1.3.7 [47], to investigate how the genetic diversity is structured among these species. In addition, we used the same model-based clustering approach described above [41, 45], with *K* values ranging from 1 to 6 because we consider three groups in *Rhizophora mangle* (the results for this species showed *K*=2 and *K*=5, but we could see three groups) and the other two species.

We applied a model-based method implemented in the NewHybrids 1.1 beta software to evaluated the existence of hybrids and categories ("F_1", "F_2" and backcrosses between a pure individual and "F_1") of eventual two-generation hybrids between each “pure species” [48]. The analyses were conducted with the evaluation of the posterior distributions using five independent chains of 10^6 interactions of MCMC after 5×10^5 burn-in steps, without using any prior information on allele frequency and considering Jeffrey-type and uniform distribution priors for Θ and π. Besides, we used hierarchical AMOVA in GenAlEx 6.4 software to evaluate the groups identified with these approaches and assess how the genetic diversity is organized among the three species [35].

Ethics Statement

We confirm that we obtained two licenses (Nos. 17159 and 17130) from the Brazilian Institute of the Environment and Natural Renewable Resources - IBAMA (currently Chico Mendes Institute for Biodiversity Conservation - ICMBio) to collect the leaves of *Rhizophora mangle, R. racemosa* and *R. harrisonii*. We confirm that these are not endangered or protected species.

Results

Microsatellite development

Through automatic genotyping, 96 clones of *R. mangle*, 96 clones of *R. racemosa* and 84 clones of *R. harrisonii* were evaluated. We designed a total of 118 primer pairs: 44 for *R. mangle,* 37 for *R. racemosa* and 37 for *R. harrisonii*. Out of 44 markers for *R. mangle,* 22 amplified properly and seven presented intraspecific polymorphism. For *R. racemosa,* the number of properly amplified loci was 23, and six were polymorphic in the species. For *R. harrisonii,* nine out of 30 markers that amplified properly were polymorphic. Seven markers from *R. mangle* successfully cross-amplified and were polymorphic for the tested samples of the other two species. Four markers developed for *R. racemosa* cross-amplified and were polymorphic for *R. mangle* (S1 Table in S1 File). All primer pairs that were polymorphic for *R. racemosa* amplified *R. harrisonii* samples and were also polymorphic in this species. The same occurred from *R. harrisonii* to *R. racemosa.* We did not test the transferability of *R. harrisonii* primer pairs in the *R. mangle* samples yet. For *R. mangle* primer pairs, the mean number of alleles was 6.14 (form four to eight alleles) and the Polymorphism Information Content (PIC) varied from 0.147 to 0.513. For *R. racemosa* and *R. harrisonii* primer pairs, the mean number of alleles was 3 (from two to four alleles) and the PIC varied from 0.263 to 0.595. We found no interference from null alleles.

Intraspecific genetic diversity and genetic structure

We used a total of nine primer pairs, including some that were previously published [49]. The set of microsatellites used for the genetic diversity analyses of *R. mangle* is shown in S2 Table in S1 File. All loci were polymorphic and generated a total of 48 alleles. The populations with the highest number of alleles were Rm BPA (34), RmAMA (28), RmPPI (26) and RmPCE (22). The remaining populations showed no more than 16 alleles each. Populations RmBPA, RmAMA, RmPCE and RmGRJ had private alleles, with eight, four, two and two alleles, respectively. We found a varying degree of polymorphism among the markers within and among samples in terms of N_e, A_R, H_E and H_O (Table 2). We also observed F_{IS} values ranging from -1.00 to 0.070, with an average of 0.087. A comparison across geographic regions indicated significant differences in the patterns of genetic variation. A_R, H_O and H_E, were higher in the RmBPA, RmAMA, RmPPI, and RmPCE populations comparing to the others (Table 2).. The average t_a estimated was 0.841, ranging from 0.345 to 1 (Table 2).

Table 2. Intraspecific genetic diversity of *Rhizophora mangle, R. racemosa* and *R. harrisonii* samples from the Brazilian coast. Sample codes are denoted as in Table 1.

Sample	N_a		A_R		H_O		H_E		F_{IS}		t_a
***R. mangle* (n=318)**											
RmBPA	1.985	(0.235)	3.778	(0.465)	0.386	(0.082)	0.427	(0.078)	0.070	(0.097)	0.870
RmAMA	1.678	(0.132)	3.111	(0.423)	0.300	(0.067)	0.371	(0.053)	0.233	(0.125)	0.623
RmPPI	1.753	(0.138)	2.889	(0.351)	0.326	(0.072)	0.392	(0.061)	0.177	(0.127)	0.709
RmPCE	1.496	(0.155)	2.444	(0.444)	0.217	(0.095)	0.271	(0.074)	0.189	(0.189)	0.682
RmTPE	1.111	(0.111)	1.111	(0.111)	0.111	(0.111)	0.056	(0.056)	-1.000	(0.057)	-
RmVBA	1.111	(0.111)	1.111	(0.111)	0.111	(0.111)	0.056	(0.056)	-1.000	(0.057)	-
RmGRJ	1.187	(0.113)	1.778	(0.324)	0.139	(0.109)	0.113	(0.059)	-0.047	(0.221)	1.000
RmUBA	1.111	(0.111)	1.111	(0.111)	0.111	(0.111)	0.056	(0.056)	-1.000	(0.056)	-
RmCNN	1.139	(0.108)	1.667	(0.236)	0.114	(0.111)	0.082	(0.053)	0.498	(0.289)	0.345
RmPPR	1.111	(0.111)	1.111	(0.111)	0.111	(0.111)	0.056	(0.056)	-1.000	(0.057)	-
RmFSC	1.083	(0.073)	1.333	(0.167)	0.066	(0.061)	0.053	(0.044)	0.199	(0.239)	0.678
Average	1.342	(0.049)	1.949	(0.259)	0.181	(0.030)	0.176	(0.023)	0.087	(0.059)	0.841
***R. racemosa* (n=33)**											
RrPPI	1.538	(0.117)	2.813	(0.292)	0.212	(0.042)	0.301	(0.044)	0.214	(0.122)	0.647
RrSPA	1.912	(0.208)	2.750	(0.371)	0.241	(0.051)	0.410	(0.047)	0.347	(0.121)	0.485
Average	1.725	(0.122)	2.781	(0.331)	0.227	(0.033)	0.356	(0.033)	0.281	(0.085)	0.562
***R. harrisonii* (n=37)**											
RhPPI	2.234	(0.177)	3.125	(0.455)	0.574	(0.105)	0.522	(0.027)	-0.067	(0.201)	1.000
RhSPA	2.138	(0.187)	2.750	(0.281)	0.427	(0.099)	0.477	(0.046)	0.130	(0.182)	0.770
Average	2.186	(0.127)	2.938	(0.368)	0.501	(0.072)	0.499	(0.027)	0.028	(0.135)	0.945

Average effective number of alleles (N_e), allelic richness (A_R), observed (H_O) and expected (H_E) heterozygosities, inbreeding coefficient (F_{IS}), and outcrossing apparent rate (t_a) are denoted. The standard errors are in parentheses.

The set of markers used for the genetic diversity analyses was the same for *R. racemosa* and *R. harrisonii* (S3 Table in S1 File). We used a total of 16 polymorphic microsatellites, including some that were previously published [49, 50]. The species showed a similar number of alleles and *R. harrisonii* had more private alleles (six alleles) than *R. racemosa* (2 alleles).

We also found a varying degree of polymorphism among the markers within samples in terms of N_e, H_E and H_O (Table 2) and a significant departure from HWE. We observed F_{IS} values ranging from 0.214 to 0.347 for *R. racemosa*; and -0.067 to 0.130 for *R. harrisonii*. The level of inbreeding among individuals was lower for *R. harrisonii* (average of F_{IS}= 0.028±0.135 and t_a= 0.945) compared to the other two species. *R. racemosa* had higher values for F_{IS} (0.281±0.085) and t_a (0.562), which may indicate that these species have a mixed maing system, but *R. harrisonii* has a tendency to alogamy..

R. harrisonii had the lowest value of population differentiation (F_{ST}= 0.067±0.024), whereas *R. mangle* species had higher average genetic differentiation (F_{ST}= 0.346±0.095). The population differentiation of *R. racemosa* was intermediate (F_{ST}= 0.135±0.065) (Table 3).

Table 3. Statistical estimate of Weir and Cockerham F-statistics for *Rhizophora mangle, R. racemosa* and *R. harrisonii* with the respective values of the confidence interval.

	F_{IS} (f)		F_{ST} (θ)		F_{IT} (F)	
R. mangle						
Estimate	-0.087	(0.317)	0.346	(0.095)	0.314	(0.267)
(IC) Lower limit	-0.397		0.202		-0.092	
(IC) Upper limit	0.395		0.507		0.670	
R. racemosa						
Estimate	0.379	(0.120)	0.135	(0.065)	0.462	(0.110)
(IC) Lower limit	0.137		0.027		0.229	
(IC) Upper limit	0.586		0.258		0.648	
R. harrisonii						
Estimate	-0.039	(0.183)	0.067	(0.024)	0.033	(0.184)
(IC) Lower limit	-0.375		0.024		-0.312	
(IC) Upper limit	0.321		0.117		0.383	
Three *Rhizophora* spp.						
Estimate	0.100	(0.156)	0.537	(0.045)	0.585	(0.09)
(IC) Lower limit	-0.171		0.452		0.415	
(IC) Upper limit	0.400		0.621		0.749	

F_{IT} - Total population inbreeding; F_{ST}- inbreeding by subdivision and divergence between populations; F_{IS} - inbreeding due to the reproductive system, with respective standard errors in parentheses. Confidence interval (CI) (95%) obtained after 10,000 resamplings.

Pairwise F_{ST} values, representing the degree of genetic differentiation between *R. mangle* populations, ranged from 0 to 0.437 (Table 4). The highest average genetic differentiation was observed between RmBPA and RmTPE, RmVBA, RmUBA, RmPPR populations (F_{ST}=0.437), whereas the RmTPE showed no significant genetic differentiation from RmVBA, RmUBA and RmPPR populations (Table 4). For *R. racemosa* and *R. harrisonii,* the F_{ST} values ranged from 0.067 to 0.337. The lowest F_{ST} value was between the two populations of *R. harrisonii* (RhPPi and RhSPA). The highest average value was between *R. racemosa* (RrPPI) and *R. harrisonii* from (RhSPA) (F_{ST}=0.337) (Table 5). These results indicates a genetic structure in the three species, primarily when samples from north and south of the brazilian coast are considered (Figure 1).

Table 4. Statistical estimate of pairwise F_{ST} (θ) between populations of *Rhizophora mangle*.

	RmBPA	RmAMA	RmPPI	RmPCE	RmTPE	RmVBA	RmGRJ	RmUBA	RmCNN	RmPPR	RmFSC
RmBPA	0.000	***	***	***	***	***	***	***	***	***	***
RmAMA	0.128	0.000	***	***	***	***	***	***	***	***	***
RmPPI	0.210	0.056	0.000	***	***	**	***	***	***	***	***
RmPCE	0.253	0.049	0.080	0.000	***	***	***	***	***	***	***
RmTPE	0.437	0.170	0.165	0.101	0.000	NA	NS	NA	NS	NA	NS
RmVBA	0.437	0.170	0.165	0.101	0.000	0.000	NS	NA	NS	NA	NS
RmGRJ	0.368	0.151	0.122	0.074	0.024	0.024	0.000	**	NS	NS	***
RmUBA	0.437	0.170	0.165	0.101	0.000	0.000	0.024	0.000	NS	NA	***
RmCNN	0.378	0.158	0.137	0.081	0.012	0.012	0.014	0.012	0.000	NS	***
RmPPR	0.437	0.170	0.165	0.101	0.000	0.000	0.024	0.000	0.012	0.000	NS
RmFSC	0.419	0.160	0.151	0.093	0.025	0.025	0.028	0.025	0.019	0.025	0.000

The lower diagonal presents the structure of populations pairwise (F_{ST}). The upper diagonal presents the statistical significance of each pairwaise comparison after standard Bonferroni corrections. P value obtained after 55,000 permutations.

*** = significance at the 0.1% nominal level; ** = significance at the 1% nominal level; NS = non-significant and NA = not available.

Table 5. Statistical estimate of pairwise F_{ST} (θ) between populations of *Rhizophora racemosa* and *Rhizophora harrisonii*.

	RrPPI	RrSPA	RhPPI	RhSPA
RrPPI	0.000	*	**	**
RrSPA	0.133	0.000	**	NS
RhPPI	0.259	0.163	0.000	**
RhSPA	0.337	0.104	0.067	0.000

The lower diagonal presents the structure of populations pairwise (F_{ST}). The upper diagonal presents the pairwise significance after standard Bonferroni corrections. P values obtained after 55,000 permutations.
*** = significance at the 0.1% nominal level; ** = significance at the 1% nominal level; NS = non-significant.

Based on data obtained through the Bayesian analysis of the population genetic structure using the Structure software, for the *R. mangle,* we observed that there are two distinct groups (*K*=2) for *R. mangle*, suggesting the occurrence of two populations (Fig. 2). In this scenario, we observed a structure that separates the species into two groups: one along the northern, including the populations RmBPA, RmPPI, RmPCE; and the other along the southern Brazilian coast, including the populations: RmTPE, RmVBA, RmGRJ, RmUBA, RmCNN, RmPPR, RmFSC (Fig. 3A). For *R. racemosa* and *R. harrisonii,* the most likely number of populations was *K*=2, which indicates two different species (Figs. 2B and 4). There was no structure in the individuals of the same species.

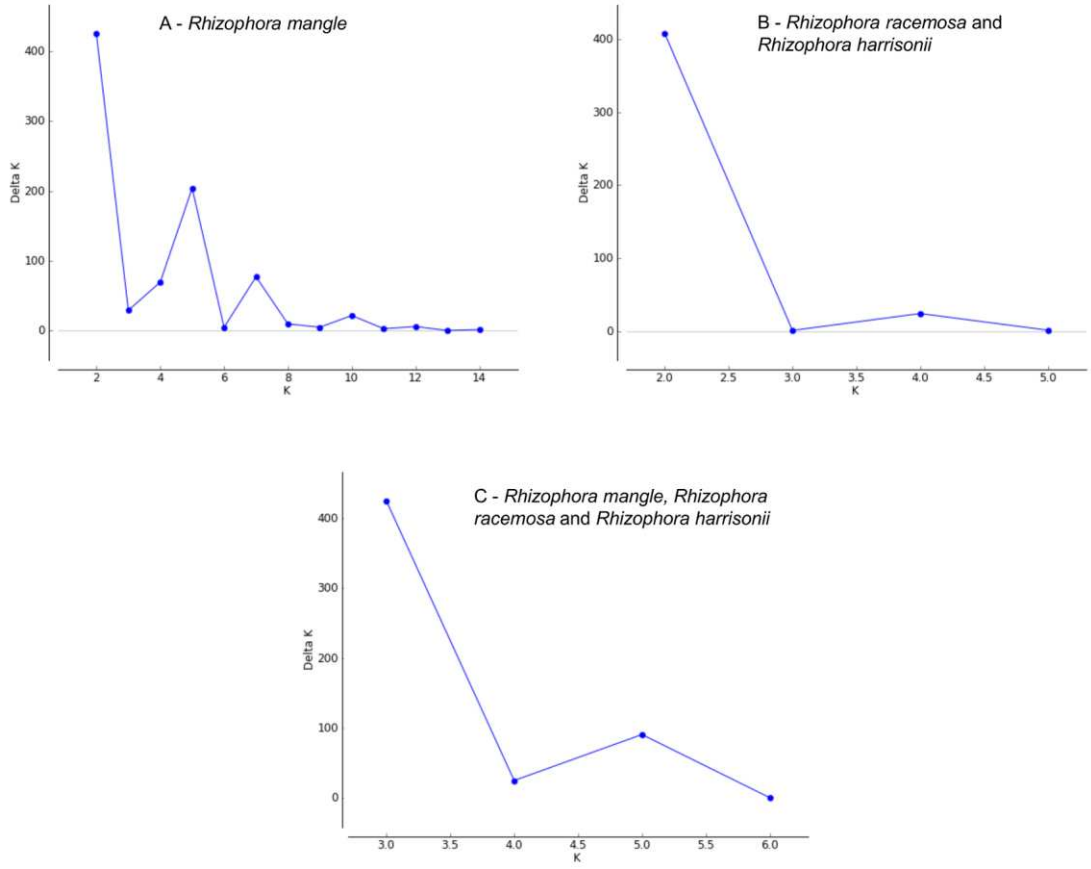

Fig. 2. **Bayesian inference of the number of clusters (*K*).** The mean values of Δ*K* for A) samples of *Rhizophora mangle;* B) samples of *R. racemosa* and *R. harrisonii*; C) and samples of *R. mangle, R. racemosa* and *R. harrisonii* analyzed with different microsatellite primer sets (S4 Table in S1File).

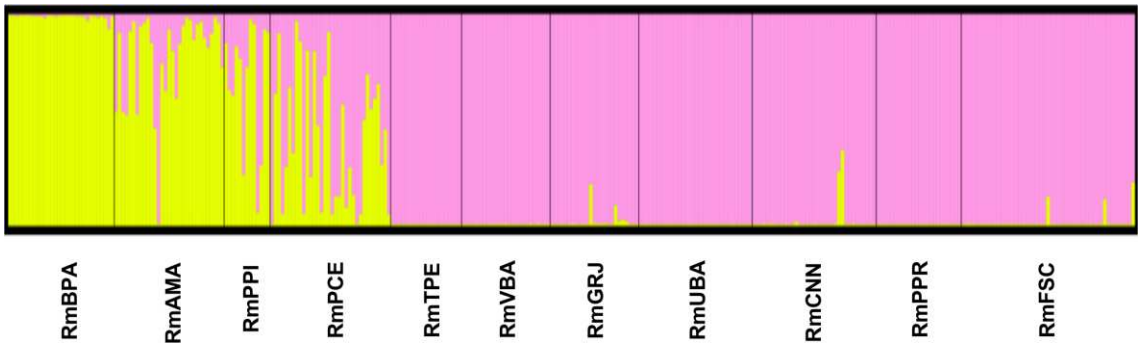

Fig. 3. ***Rhizophora mangle*** **population structure.** Bayesian clustering analyses [41] considering *K*=2, where each individual is represented by a vertical line with populations separated by black lines, and each color refers to one inferred cluster: samples from North (yellow) and South (pink) of the Brazilian Coast.

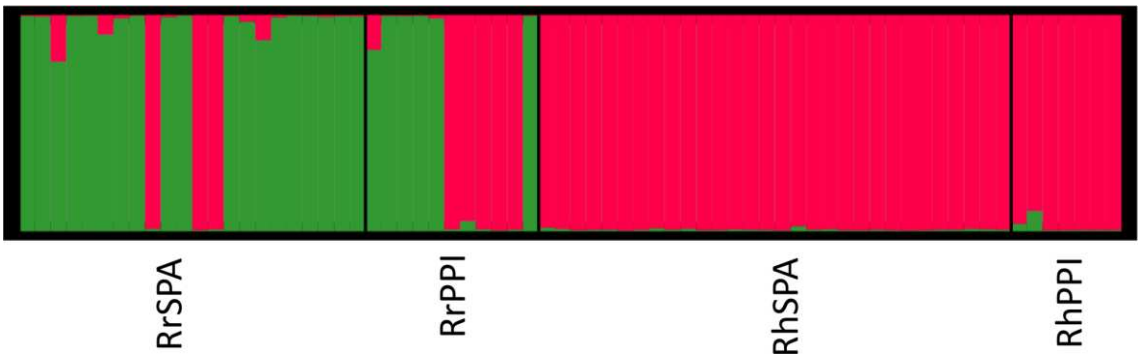

Fig. 4. *Rhizophora racemosa* and *R. harrisonii* population structure along the Brazilian coast. A) Model-based clustering analyses [41] considering K=2, where each individual is represented by a vertical line, each color refers to one inferred cluster.

Analyses of molecular variance were also held for population differentiation to additional evaluation of the genetic structure (Table 6). Genetic variance was observed among the two groups of *R. mangle* populations (P<0.0001), determined according to the results of the structure analysis, and explained 51% of the total variance. Only 3.86% of the total genetic variance was found among populations, indicating higher genetic differentiation within individuals than among the populations and the emergence of genetic differentiation within populations (45.14%). In *R. racemosa,* the variation within populations represented 84.5% of the total variance, and 15.5% the variance was between the two populations, which correlates with the F_{ST} value (0.027) that showed there was greater variance within each population than among populations. For *R. harrisonii* the pattern was the same, with 12.6% of variance among the populations and 87.4% within each population.

Table 6 Analysis of molecular variance (AMOVA) for *Rhizophora mangle, R. racemosa* and *R. harrisonii*

A) *R. mangle*

Source of variation	df	Sum of squares	Mean squares	Variance component	Total variance (%)	P-value
Among groups	1	301.092	301.092	1.951	51.00	0.0001
Among populations	9	191.679	21.298	0.668	3.86	0.0001
Within populations	307	712.839	2.322	2.322	45.14	0.0001

B) *R. racemosa*

Source of variation	df	Sum of squares	Mean squares	Variance component	Total variance (%)	P-value
Among populations	1	32.742	32.742	1.628	15.53	0.0001
Within populations	31	274.591	8.858	8.858	84.47	0.0001

C) *R. harrisonii*

Source of variation	df	Sum of squares	Mean squares	Variance component	Total variance (%)	P-value
Among populations	1	23.385	23.385	1.279	12.60	0.0004
Within populations	35	310.452	8.870	8.870	87.40	0.0004

D) Three *Rhizophora* spp.

Source of variation	df	Sum of squares	Mean squares	Variance component	Total variance (%)	P-value
Among species	2	1409.923	704.961	10.741	61.250	0.0001
Within species	391	2656.737	6.795	6.795	38.750	0.0001

Results of the hierarchical analysis of molecular variance (AMOVA) considering A) R. mangle populations and northern and Southern groups to the Northeast extreme of South America with K=2 according to Fig. 3; B) R. racemosa populations; C) R. harrisonii populations; and D) R. mangle, R. racemosa and R. harrisonii species comparison.

Ongoing hybridization between *Rhizophora* species

Characterizing all the samples collected together with thirteen microsatellite loci (S4 Table in S1 File), we detected that the differentiation observed among the species was greater than the differentiation observed among the samples within each species, with an F_{ST} value of 0.537 (Table 3), which was higher than the values observed for individual species. This result is consistent with the hierarchical AMOVA approach among populations and species, which revealed that the majority of the variation (61.25%) occurred among the species (Table 4).

Regarding the DAPC, we observed an optimum of 3 clusters, revealing the difference between two groups of *R. mangle* and the other two species (Fig. 5A). The pattern of genetic structure within each species was consistent with the results presented above when each taxon was evaluated separately, including the clear divergence between northern and southern samples for *R. mangle*. DAPC revealed no clear difference between *R. racemosa* and *R. harrisonii* (Fig.5A). Considering the model implemented in the Structure software [41, 42], the most likely *K* was only 3 according to ΔK (Figs. 2C and 5B). There was no differentiation between *R. racemosa* and *R. harrisonii*. 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 (Fig. 5C). There was no differentiation between the Jeffrey-like and uniform priors results. These results indicate that there is no evidence that the species are hybridizing (Fig. 5C).

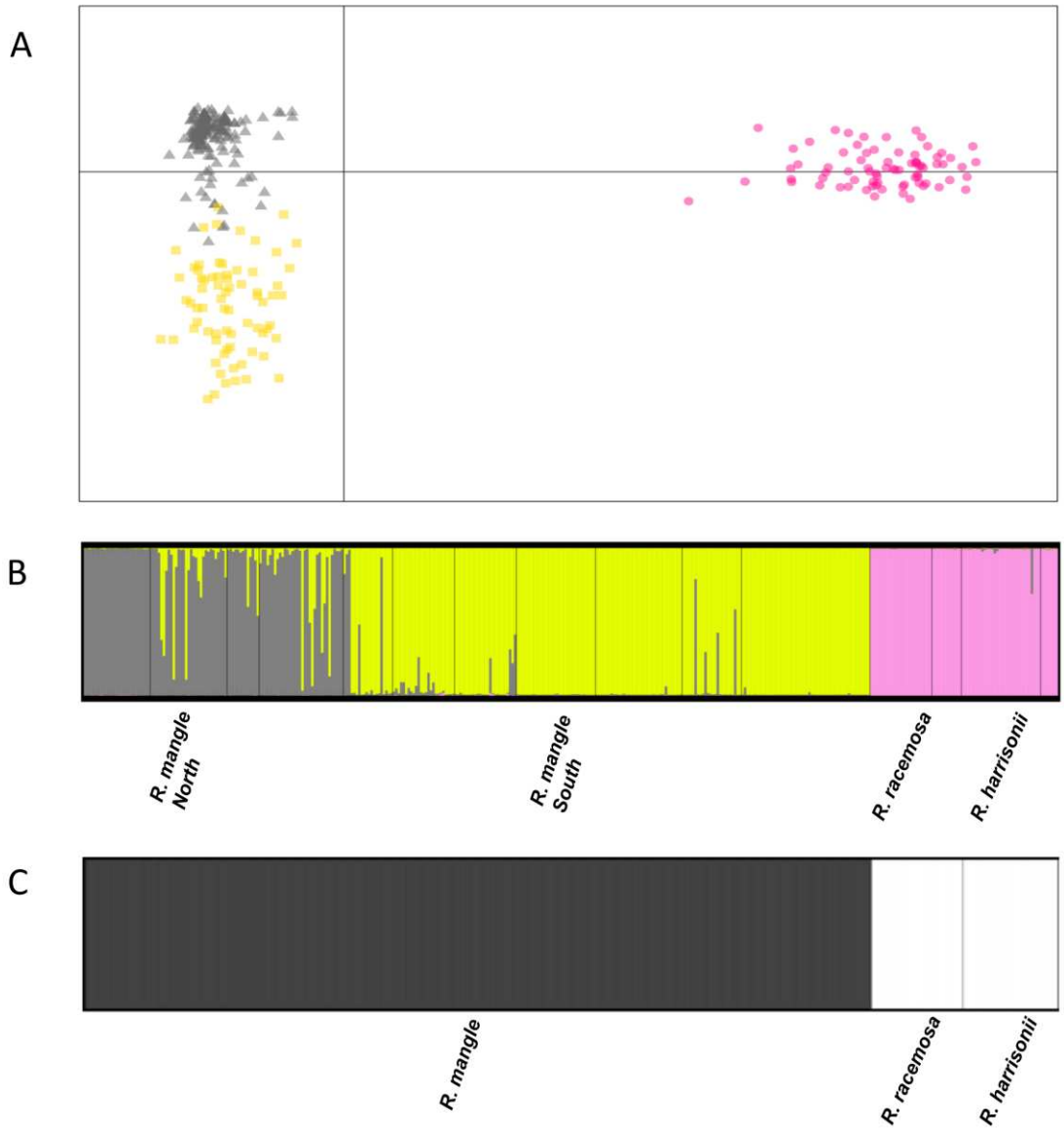

Fig. 5. **Investigation of ongoing hybridization between *Rhizophora mangle* and *R. racemosa.*** A) Multivariate analysis of DAPC [46]. Each color indicates the group to which each individual was assigned. The gray represents one group of *R. mangle* with Northern populations, yellow represents the second group of *R. mangle* with Southern populations, pink are *R. racemosa* and *R. harrisonii* individuals, which were grouped together. B) Bayesian clustering analyses [41] considering *K*=3, where each individual is represented by a vertical line, each color refers to one cluster. The groups and colors are the same as A. C) Evaluation of the existence of hybrids and categories (F1, F2 and backcrosses between a pure individual and F1) in NewHybrids [48]. Each vertical line refers to an

individual, and different colors indicate distinct classes of individuals: dark gray: “pure” *R. mangle*; white: "pure" *R. racemosa* and *R. harrisonii.*

Discussion

Microsatellite markers

Studies have previously reported the isolation of microsatellite markers for *Rhizophora mangle*, but the reported allelic diversity is rather low [49, 51], as we observed herein. However, a more recent study of the same species showed a high number of private alleles [52]. In the present study, we found a variable degree of polymorphism among loci and a high transferability rate between *R, mangle, R. racemosa* and *R. harrisonii* species (S1 Table in S1 File). These results indicate that these markers are valuable molecular tools for genetic studies such as the investigation of these species genetic structure, genetic diversity, mating system and for the evaluation of hybridization areas between them.

Intraspecific genetic diversity, mating system and structure

With the set of primer pairs used in this study for *R. mangle*, we found 48 alleles and the allelic richness ranged from 3.8 to 1.1 (Table 2). This number of alleles was higher than that obtained by Pill *et al*. [22] for the same species from the Brazilian coast using a different set of eight microsatellite primer pairs and 145 individuals across 10 populations. Likewise, the range of allelic richness for the northern region of Brazil was slightly higher than that described by Pill *et al.* [22]. In Mexico, Sandoval-Castro *et al*. [53] found reduced genetic diversity and increased inbreeding. Evidence of low genetic diversity was detected in other previous studies of mangrove species [6, 22, 54, 55]. However, populations of *R. mangle* from the Pacific Coast presented a higher genetic diversity and genetic differentiation among populations, especially between populations from the northern coast

and the other populations [56, 57], which was consistent with the findings reported in the present study.

We observed lower values for inbreeding for *R. mangle* and *R. harrisonii* and an intermediate value for *R. racemosa* (Table 2). The results for these species indicate that these populations have a mixed mating system, although most of the sampled areas presents tendency to cross-pollination [58]. *Rhizophora* species have a wide variety of pollination mechanisms such as flower visitors (bees, butterflies, and birds in some species of the genus) but are considered primarily wind pollinated [59, 60]. However, the mixed mating system strategy seems to be particularly important for mangrove trees as colonizers because of the small number of individuals present in the area at first [4], and self-pollination [58]. This breeding strategy is also used by other species of mangrove trees such as *Avicennia* spp. [61].

A comparison across geographic regions indicated significant differences in the pattern of genetic variation for *R. mangle*. Some populations contained more heterozygotes than expected (Table 2). Allelic richness, H_O and H_E were higher in the RmBPA, RmAMA, RmPPI, and RmPCE populations, and a reduction of these parameters was observed in the populations in estuaries farther south (Table 2). The number of private alleles in northern populations was also higher when compared to the number in southern populations. The same pattern was observed in another study in the same region [22]. The F-statistics estimated for *R. mangle* showed that the variation in population structure among the populations studied is higher than the differentiation within the populations (Table 3). Little genetic structure was present for the species *R. racemosa* and *R. harrisonii.* This finding was expected because the two locations where these species were collected were relatively

close and the distribution of these species in the Brazilian coast is restricted to the northern region. The value of genetic structure among populations (F_{ST}) was high when compared to that of the three species of *Rhizophora* (Table 3).

The observed pattern of genetic differentiation can be associated with the climatic changes that occurred during the last millennia and the biological characteristics of *Rhizophora* spp. [22]. During Quaternary glaciations, the climate of the western Atlantic coast was colder but exhibited frequent temperature oscillations. During this glacial period, the ice extended into the Northern Hemisphere, limiting the vegetation zones to the equatorial regions, whereas in the Southern Hemisphere, the ice sheets were not as extensive [62, 63]. Because mangrove distribution is strongly determined by temperature and humidity [6], these species distribution was likely restricted to equatorial regions during the cooling of the Quaternary [64]. When the temperature increased, the mangroves could have extended their distribution to latitudes farther south [22]. This scenario may explain the differences in genetic variability and number of species between the northern and southern mangrove forests [22, 65], which is supported by this study. The low species richness and number of alleles of *R. mangle* in southern regions would be the result of consecutive founder effects associated with a stepping-stone dispersion pattern as this species colonized estuaries farther from the equatorial region [65, 66]. Pil *et al.* found elevated homozygosity in these populations [22]. However, in the present study, the homozygosity was not high, which may indicate that there is a non-negligible genetic diversity even though this diversity is low compared to northern populations. This difference could be explained by the number of microsatellites loci used in each study, which was higher in ours.

In *R. mangle,* pairwise comparisons indicate that the most significant differences occurred between RmBPA, RmAMA, RmPPI and RmPCE and the other populations (Fig. 1, S5 Table in File S1), which correlates with the findings of a previous study about the same species [22]. These differences are reported for other sea-dispersed species. Mori and colleagues [61] found strong differentiation between *A. germinans* and A. *schaueriana* populations sampled in North and South of the northeastern extremity of South America.

The differences between the northern and southern populations were also evident in the other analyses. Considering genetic structure using the Bayesian population assignment method, we observed a scenario for the *R. mangle* analysis suggesting the occurrence of two populations: one on the northern and another on the southern Brazilian coast (Fig. 3). This result provides additional support for the findings regarding the evaluation of pairwise F_{ST}. For *R. racemosa* and *R. harrisonii,* it was not found structure among individuals of the same species was found and the difference was only present between the two species. AMOVA results also supported this genetic structure pattern because both species had significant fixation index values when the variation among populations within groups was considered (Table 4). The variation among groups (north and south) was higher than that among populations.

A study investigating the phylogeographic pattern of *Rhizophora* worldwide reveal the importance of a combination of vicariant events and oceanic long-distance dispersals to account for the historical diversification and present-day biogeographic patterns of mangroves [67]. New findings for *Avicennia germinans,* another widespread mangrove species, reinforce the role of long-distance dispersal for which was observed signs of transatlantic dispersal, a process that has likely contributed to the large extent this species

distribution [68, 69]. Although long distance dispersal is found in mangrove species, there are important evolutionary factors that differentiate mangrove populations such as large land and ocean barriers to propagule dispersal [24]. *Rhizophora* spp. propagules are water dispersed and take advantage of estuarine, coastal and ocean currents to restock or colonize habitats [54]. The northern and southern regions studied here are under distinct hydrographic regimes [70], and this pattern possibly plays an important role as barriers to dispersal, as expected from the water-based dispersal of mangrove propagules. The distinctions in allelic and species richness of the northern and southern groups of the Brazilian coast are related to the bifurcation of superficial currents and this same pattern was found in *Hibiscus pernambucensis* and *Avicennia germinans* and *A. schaueriana* species [68, 71]. The southern branch of the South Equatorial Current into the Brazil Current (BC) and the cross-equatorial North Brazil Current (NBC) (Fig. 1) [72] likely constrains the movement of propagules between the northern and southern groups [22, 24 71]. The higher frequency admixture events in the northern regions can be explained by the fact that the NBC is faster than the BC [72], which could favor the migration of individuals from Southeast at the beginning of the BC to the North region, as observed in this study (Fig. 3). Therefore, the current dispersal of these species influences their genetic structure. This constraint would amplify the founder effect on the putative stepping-stone-colonizing dispersion that results in the low variability of the newly colonized regions in the south [22]. Similar to the findings reported in other studies on mangrove trees [22, 23, 71], substantial divergence within the *Rhizophora* spp. exists between samples from locales on the northern and southern of Brazilian Coast.

Ongoing hybridization between *Rhizophora* species

Imply *Rhizophora* species relationships in the New World is complicated because they overlap in the continental margins of the eastern Atlantic Ocean and in the eastern Pacific Ocean and present extreme phenotypic plasticity of main diagnostic characters [3, 6, 14].

Studies in the Indo-West Pacific indicate considerable hybridization between *Rhizophora* species [73, 74-76]. Hybridization is common in plants and has an important role in the evolution of species and promoting speciation [77]. In Brazil, it was reported the first evidence of ongoing hybridization between *Avicennia* species. It was found that hybrids are fertile, but this interspecific crossing has not contributed to an increase in the genetic diversity of the populations [61]. An investigation about the phylogeographic patterns of neotropical *Avicennia* species using nuclear and chloroplast genome markers indicate that introgressive hybridization between *A. bicolor* and *A. germinans* and between *A. schaueriana* and *A. germinans* is a relevant evolutionary process [68, 78].

In this study, when comparing only the species *R. racemosa* and *R. harrisonii,* we observed that they are very similar, more so than when compared to *R. mangle.* With the Bayesian analyses using microsatellites only for *R. racemosa* and *R. harrisonii* (S3 Table in F1 File), it was possible to detect two distinct groups with little admixture between them, representing the two species. To evaluate whether hybridization is occurring between *R. racemosa* and *R. mangle,* we used a different set of microsatellites (S4 Table in F1 File). The principal difference in the data set is that we did not used markers developed for *R. harrisonii* for this hybridization study. The results of this study show differentiation among the three species that was greater than among the samples within each species. This result is

consistent with the AMOVA approach, which revealed that the majority of the variation was present among the species.

Although we found this differentiation among species, we observed an optimum of 3 clusters from the DAPC results, revealing the difference between two groups of *R. mangle,* the same found when only this species was evaluated, and the other two species grouped together. The same happened in the STRUCTURE, considering the three species (Fig. 5A) where there were no significant differences between *R. racemosa* and *R. harrisonii,* the probable hybrid. The pattern of genetic structure within each species was consistent with the multi-scale results presented above when each taxon was evaluated separately, including the clear divergence between northern and southern samples of *R. mangle*. The Bayesian analyses [40] indicated that the genetic structure was the same (Fig. 5B). There was also no differentiation between *R. racemosa* and *R. harrisonii*. However, some individuals of *R. harrisonii* showed an admixture with *R. mangle* from the southern region, where the three species occur concomitantly on the Brazilian coast. We used another model-based approach to verify the presence of different classes of hybrids in up to two generations. Again, there was no differentiation among species *R. racemosa* and *R. mangle*. Results presented in the above section showed differentiation between the species *R. racemosa* and *R. harrisoni* (Fig. 4). One of the factors that may have modified this result is that the microsatellite loci set used were different.

The results of this study show no evidence that mangrove species are hybridizing because it was expected that hybrid species present heterogeneous gene pools and hybrid individuals exhibit disproportional mix of parental gene pools, and this pattern was not observed [75]. There is reported evidence that the species *Rhizophora harrisonii* is a result

of ancient and persistent introgressive hybridization among New World *Rhizophora mangle* and *Rhizophora racemosa* [23]. However, in the present study we found no evidence that *R. harrisonii* is a hybrid of *R. mangle* and *R. racemosa*; rather, *R. harrisonii* and *R. racemosa* are "pure species" only and may be the same species. The differences between this first study and the present one can be result of a methodological bias, because different markers were used.

Conclusions

These results suggest that *R. mangle* is not composed of a single population, as indicated by genetic differentiation found among the population from locales on the northern and southern of the Brazilian Coast. The higher genetic diversity found in populations in the North compared to populations in the South may be related to dispersion of propagules by ocean currents which also favors admixture in the North, and recent foundation events and colonization could explain the lower genetic diversity of populations in the South.

Regarding ongoing hybridization, we found no evidence of introgressive hybridization between species leading to a hybrid species, which was indicated in a previous study [23]. The biological processes that determine how the genetic diversity of the *Rhizophora* species is distributed is still an open question, but our results can contribute to the understanding of these issues.

Acknowledgments

The authors thank I. C. Menezes, I. Sampaio, L. G. Dantas de Oliveira, and M. A. Louzada for sampling support and assistance in the field and P. Zambon, A. Moares and D. A. Sforça for support in the laboratory.

Financial Disclosure

The authors gratefully acknowledge the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, 2008/52045-0 and 2010/50178-2) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES – Computational Biology Program, project number 51) for financial support and scholarships to PMF (DD 2008/56404-4) and GMM (MS 2007/57021-9, DD 2010/50033-4 and PD 2013/08086-1) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for research fellowships to APS. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data Availability

The authors confirm that all data underlying the findings are fully available without restriction. All sequence files are available from the GenBank database (accession numbers: KJ740653 - KJ40692; KM870531- KM870562; KM280821 - KM280856).

Supporting Information

S1 Table. Polymorphic microsatellites developed for *Rhizophora mangle* (Rma3), *R. racemosa* (Rra1) and *R. harrisonii* (Rha1).

Marker	Primer sequence (5'-3')	Size (bp)	Repeat motif	AC(ºC)	GenBank	Transferability
Rma3-05	F: CAAGGTCAATGGGTGTAG R: AATGAAGCAAATAAGAGATAAG	170	$(tc)_{18}$	TD 65-55	KJ740656	Rr, Rh
Rma3-14	F: AAATGCATAAAAGTTGAAGATA R: AAAAGGATGTGATGAGACTGTT	160	$(tc)_{18}$	TD 65-55	KJ740663	Rr, Rh
Rma3-17	F: TTCATCACCAGCACCAAAGT R: TGACCTCGCAATCTACACAAA	210	$(tg)_{15}$	TD 65-55	KJ740669	-
Rma3-20	F: AATTTGCACACCACTTATCTTT R: TCTGTTAGTTGACCTTTTCCTT	280	$(ag)_{23}$	TD 65-55	KJ740667	Rr, Rh
Rma3-23	F: AAGTGGGTCATGTTTAGAA R: CTTATGGTATGTGTATTAGGTC	230	$(ct)_{19}$	TD 65-55	KJ740675	Rr, Rh
Rma3-37	F: AGGCCATTTATACTCTCACACC R: TTACGGCGAACCACACTT	240	$(ag)_5$	TD 65-55	KJ740686	Rr, Rh
Rma3-38	F: TGGCAGATGTGTCTTCCTGA R: CCTCAGACTTGAATCAGCAGTG	260	$(ag)_5...(aga)_{10}$	TD 65-55	KJ740686	-
Rra1-09	F: AACACCAATCATTTCCTTCACTAT R: TCCTTAATCTATCTCTGGCTTCTT	129	$(ttcc)_3$	TD 65-55	KM870534	Rm, Rh
Rra1-14	F: GATTCGCCTCATGCACTGTCTG R: CTTTCCCTGCTTTCCCTTTGATT	251	$(tta)_5$	TD 65-55	KM870543	Rm, Rh
Rra1-18	F: TGTGGGTGCATGGATTAGATTTAT R: CACGCGCCTTGGATTCATTT	239	$(tg)_5(gt)_{12}$	TD 65-55	KM870545	Rm, Rh
Rra1-20	F: GATTCGCCTCATGCACTGTCTG R: CTTTCCCTGCTTTCCCTTTGATT	297	$(tc)_{18}$	TD 65-55	KM870547	Rm, Rh

Rra1-33	F: GACCAGTGAGTAAAAAGGGAGTAG R: CTGGGCCATGCAATAGTGA	214	$(ag)_{11}$	TD 65-55	KM870558	Rm, Rh
Rra1-35	F: TTCTGAGCTCAAATGTCT R: TTCAGCCTCTTCCAATA	355	$(ca)_{19}$	TD 65-55	KM870556	Rh
Rha1-07	F: CCCTCGTGAGTTATTGTCAT R: CTTGGTGGACCTTTTCTTTT	180	$(ag)_{10}$	TD 65-55	KM280827	Rr
Rha1-09	F: GTAACTGGTGCAGCTTTGA R: GTGTGTATCTTGGGTGCAG	223	$(tc)5(ta)(tc)5(c)(ct)_5(ca)_9$	TD 65-55	KM280829	Rr
Rha1-11	F: ACCCAAACTATCCCTAGCTC R: GGCATTTCCTGAGATAAAAG	208	$(tc)_{17}$	TD 65-55	KM280831	Rr
Rha1-17	F: GGAAAGTTGTATGCAGGAGA R: CTCTCTTCAGCCCAAAATAC	206	$(ga)_{17}$	TD 65-55	KM280837	Rr
Rha1-18	F: GCATCGTGAGATTGGACTAT R: CAGAGGAACACGGGATATT	237	$(gt)_9$	TD 65-55	KM280838	Rr
Rha1-21	F: GCGTGGACTAACTTTTTCTC R: AACTTTGGGTCTCTTGTGC	208	$(tg)_8$	TD 65-55	KM280841	Rr
Rha1-24	F: CAACCATTTCATGTGCAAG R: GATCAGGCATAGGTGGACT	208	$(tg)_9(ga)_9(a)(ag)_5$	TD 65-55	KM280844	Rr
Rha1-32	F: CAAGAAACGGATGAGAAAAC R: CCTTGGAAAATAAGGTTGG	232	$(ct)_{16}$	TD 65-55	KM280852	Rr
Rha1-34	F: AGAGTTACGAAATGGGGAAT R: GTGATGGAGGATAAGTTGGA	209	$(ga)_{11}(g)(ga)_6$	TD 65-55	KM280854	Rr

Characteristics of seven polymorphic microsatellite markers developed for *Rhizophora mangle,* six polymorphic microsatellite markers developed for *Rhizophora racemosa*, and nine polymorphic microsatellite markers developed for *Rhizophora harrisonii.* 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. The last column indicates the primers that were transferred to the other species (Rm=*R. mangle;* Rr=*R. racemosa;* Rh=*R. harrisonii).*

S2 Table. Microsatellite markers used in this study for *R. mangle.*

Marker	Repeat motif	Reference	N_a	PIC
Rma3-5	$(tc)_{18}$	This study	7	0.209
Rma3-14	$(tc)_{18}$	This study	5	0.264
Rma3-17	$(tg)_{15}$	This study	5	0.050
Rma3-20	$(ag)_{23}$	This study	8	0.513
Rma3-23	$(ct)_{19}$	This study	8	0.296
Rma3-37	$(ag)_5$	This study	4	0.147
Rma3-38	$(ag)_5...(aga)_{10}$	This study	6	0.308
Rra1-18	$(tg)_5(gt)_{12}$	This study	7	0.397
M36	$(ga)_{21}$	[49]	3	0.508
M41	$(ga)_{25}$	[49]	4	0.289

Characteristics of polymorphic microsatellite markers used for *Rhizophora mangle* in this study and previously developed markers for *Rhizophora* spp. [49]. Repeat motifs, number of alleles (N_a) and polymorphism information content (PIC) are shown for each marker.

S3 Table. Microsatellite markers used in this study for *R. racemosa* and *R. harrisonii.*

Marker	Repeat motif	Reference	N_a	PIC
Rma3-5	$(tc)_{18}$	This study	5	0.564
Rma3-14	$(tc)_{18}$	This study	5	0.516
Rma3-23	$(ct)_{19}$	This study	9	0.781
Rma3-37	$(ag)_5$	This study	3	0.433
Rra1-9	$(ttcc)_3$	This study	3	0.346
Rra1-14	$(tta)_5$	This study	2	0.263
Rra1-18	$(tg)_5(gt)_{12}$	This study	4	0.376
Rra1-20	$(tc)_{18}$	This study	4	0.374
Rra1-35	$(ca)_{19}$	This study	2	0.469
Rha1-7	$(ag)_{10}$	This study	2	0.383
Rha1-11	$(tc)_{17}$	This study	4	0.409
Rha1-18	$(gt)_9$	This study	2	0.370
Rha1-21	$(tg)_8$	This study	2	0.370
Rha1-24	$(tg)_9(ga)_9(a)(ag)_5$	This study	4	0.595
Rha1-34	$(ga)_{11}(g)(ga)_6$	This study	2	0.351
M38	$(ca)_8$	[49]	3	0.543
Rhstcp01	$(a)_{11}$	[50]	4	0.435

Characteristics of polymorphic microsatellite markers used for *Rhizophora racemosa* and *R. harrisonii* in this study and previously developed markers for *Rhizophora* spp. [49, 50]. Repeat motifs, number of alleles (N_a) and polymorphism information content (PIC) are shown for each marker.

S4 Table. Microsatellite markers used in this study for hybrid analyses.

Marker	Repeat motif	Reference	N_a	PIC
Rma3-5	$(tc)_{18}$	This study	5	0.564
Rma3-14	$(tc)_{18}$	This study	5	0.516
Rma3-20	$(ag)_{23}$	This study	7	0.793
Rma3-23	$(ct)_{19}$	This study	9	0.781
Rma3-37	$(ag)_5$	This study	3	0.433
Rra1-9	$(ttcc)_3$	This study	3	0.346
Rra1-14	$(tta)_5$	This study	2	0.263
Rra1-18	$(tg)_5(gt)_{12}$	This study	4	0.376
Rra1-20	$(tc)_{18}$	This study	4	0.374
Rra1-33	$(ag)_{11}$	This study	2	0.333
M38	$(ca)_8$	[49]	3	0.386
M41	$(ga)_{25}$	[49]	2	0.383
Rhstcp01	$(a)_{11}$	[50]	4	0.435

Characteristics of polymorphic microsatellite markers used for *R. mangle, R. racemosa* and *R. harrisonii* in this study and previously developed markers for *Rhizophora* spp. [49, 50]. Repeat motifs, number of alleles (N_a) and polymorphism information content (PIC) are shown for each marker.

References

1. Macintosh DJ, Ashton, EC. A Review of Mangrove Biodiversity Conservation and Management. Centre for Tropical Ecosystems Research, University of Aarhus, Denmark (pdf file); 2002.
2. Giri C, Ochieng E, Tieszen LL, Zhu Z, Singh A, Loveland, T, et al. Status and distribution of mangrove forests of the world using earth observation satellite data. Glob Ecol Biogeogr. 2011; 20: 154–159.
3. Spalding M, Kainuma M, Collins L. World Atlas of Mangroves. London Department of Zoology, University of Cambridge, Ed; 2010.
4. Tomlinson PB. The botany of mangroves. 1st ed. Ashton PS, Hubbel SP, Janzen DH, Raven PH, Tomlinson PB, editors. Cambridge, MA. Cambridge University Press; 1986.
5. Ellison AM, Farnworth EJ, Merkt RE. Origins of mangrove ecosystems and the mangrove biodiversity anomaly. Global Ecology and Biogeography. 1999; 8:95–115.
6. Duke NC, Ball MC, Ellison JC. Factors influencing biodiversity and distributional gradients in mangroves. Glob Ecol Biogeogr Lett. 1998; 7: 27–47.
7. Ellison AM. Macroecology of mangroves: large-scale patterns and processes in tropical coastal forests. Trees. 2002; 16: 181–194.
8. Clarke PJ, Kerrigan RA, Westphal CJ. Dispersal potential and early growth in 14 tropical mangroves: do early life history traits correlate with patterns of adult distribution? J Ecol. 2001; 89: 648–659.
9. Rabinowitz D. Dispersal properties of mangrove propagules. Biotropica. 1978; 10: 47–57.
10. Valiela I, Bowen JL, York JK. Mangrove forests: One of the World's threatened major tropical environments. BioScience. 2001; 51 (10): 807-815.
11. Duke NC, Meynecke O, Dittmann S, Ellison AM, Anger K, Berger U, et al. A World without mangroves? Science. 2007; 317:41-42.
12. Joppa LN, Roberts DL, Myers N, Pimm SL. Biodiversity hotspots house most undiscovered plant species. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108, 13171–6.
13. Zachos FE, Habel JC. Biodiversity Hotspots - Distribution and Protection of Conservation Priority Areas. New York. Springer; 2011.
14. Duke NC, Lo Y, Sun M. Global distribution and genetic discontinuities of mangroves – emerging patterns in the evolution of *Rhizophora*. Trees. 2002; 16: 65–79.
15. Tyag AP. Location and Interseasonal Variation in Flowering, Propagule Setting and Propagule Size inMangroves Species of the Family Rhizophoraceae. Wetland ecology and management. 2003; 11:167-174.

16. Schaeffer-Novelli Y, Cintrón G. Guia para estudo de áreas de manguezal. Estrutura, função e flora. Caribbean Ecological Research, São Paulo, 1986.

17. Duke NC, Allen JA. *Rhizophora mangle*, *R. samoensis*, *R. racemosa*, *R. x harrisonii* (Atlantic-East Pacific red mangrove), ver. 2.1. *In*: ELEVITCH, C.R. (ed.). Hölualoa, Hawaii. Species Profiles for Pacific Island Agroforestry. Permanent Agriculture Resources (PAR); 2006. Available: http://www.traditionaltree.org.

18. Alongi DM. Present state and future of the world's mangrove forests. Environmental Conservation. 2002; 29(3):331-349.

19. Othman MA. Value of magroves in coastal protection. Hydrobiology. 1994; 285:343-351.

20. Schwarzbach A, Ricklefs R. The use of molecular data in mangrove plant research. Wetlands Ecol. Manage. 2001; 9:195-201.

21. Triest L. Molecular ecology and biogeography of mangrove trees towards conceptual insights on gene flow and barriers: A review. Aquat Bot. 2008; 89: 138–154.

22. Pil MW, Boeger MRT, Muschner VC, Pie MR, Ostrensky A, Boeger WA. Postglacial north-south expansion of populations of *Rhizophora mangle* (Rhizophoraceae) along the Brazilian coast revealed by microsatellite analysis. Am J Bot. 2011; 98: 1031–1039.

23. Cerón-Souza I, Rivera-Ocasio E, Medina E, Jiménez JA, McMillan WO, Bermingham E. Hybridization and introgression in New World red mangroves, *Rhizophora* (Rhizophoraceae). Am J Bot. 2010; 97: 945–957.

24. Takayama K, Tamura M, Tateishi Y, Webb EL, Kajita T. Strong genetic structure over the American continents and transoceanic dispersal in the mangrove genus *Rhizophora* (Rhizophoraceae) revealed by broad-scale nuclear and chloroplast DNA analysis. Am J Bot. 2013; 100: 1–11.

25. Sun M, Lo EYY. Genomic markers reveal introgressive hybridization in the Indo-West Pacific mangroves: a case study. PLoS One. 2011; 6: e19671.

26 Cerón-Souza I, Bermingham E, McMillan WO, Jones FA. Comparative genetic structure of two mangrove species in Caribbean and Pacific estuaries of Panama. BMC Evol Biol. 2012; 12: 205.

27. Geng Q, Lian C, Goto S, Tao J, Kimura M, Islam MS, et al. Mating system, pollen and propagule dispersal, and spatial genetic structure in a high-density population of the mangrove tree *Kandelia candel*. Mol Ecol. 2008; 17: 4724–4739.

28. Breteler FJ. The Atlantic species of Rhizophora. Acta Botanica Neerlandica. 1969; 18: 434–441.

29. Billotte N, Lagoda PJL, Risterucci AM, Baurens FC. Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits. 1999; 54(4)**:**277–288.

30. Temnykh S, DeClerck G, Lukashova A, Lipovich L, Cartinhour S, McCouch S. Computational and experimental analysis of microsatellites in rice (Oryza sativa L.):

Frequency, length variation, transposon associations, and genetic marker potential. Genome Res. 2001; 11:1441–1452.

31. Creste S, Tulmann-Neto A, Figueira A. Detection of single repeat polymorphisms in denaturing polyacrilamide sequencing gels by silver staining. Plant Mol Biol Report. 2001; 19**:**229–306.

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

33. Dempster AP, Laird NM, Rubin DB. Maximum likelihood from incomplete data via the EM algorithm. Journal of the Royal Statistical Society. 1977; 39(1):1-38.

34. Chapouis MP, Estoup A. Microsatellite null alleles and estimation of population differentiation. Molecular Biology and Evolution. 2007; 24(3):621-631.

35. Peakall RS, Mouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Molecular Ecology Notes. 2006; 6, 288-295.

36. Kemp, S. PIC Calculator Extra; 2002. Available: http://www.genomics.liv.ac.uk/animal /pic.html.

37. Goudet J. FSTAT (version 1.2) a computer program to calculate F-statistics. Journal of Heredity. 2001; 86:485-486.

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

39. Fyfe J, Bailey NTJ. Plant breeding studies in leguminous forage crops I. Natural cross-breeding in winter beans. Journal of Agricultural Science. 1951; 41: 371–378.

40. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000; 155:945-959.

41. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Molecular Ecology. 2005; 14:2611-2620.

42. Earl DA, vonHoldt BM. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conservation Genetics Resources. 2012; 4(2):359-361

43. Jakobsson M, Rosenberg NA. CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics. 2007; 23: 1801–1806.

44. Rosenberg N. DISTRUCT: a program for the graphical display of population structure. Molecular Ecology Notes. 2014; 4: 137–138.

45. Excoffier L, Smouse PE, Quattro JM. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics. 1992; 131: 479-491.

46. Jombart T, Devillard S, Balloux F. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. BMC Genet. 2010; 11: 94.

47. Jombart T, Ahmed I. Adegenet 1.3-1: new tools for the analysis of genome-wide SNP data. Bioinformatics. 2011; 27: 3070–3071.

48. Anderson EC, Thompson EA. A model-based method for identifying species hybrids using multilocus genetic data. Genetics. 2002; 160: 1217–1229.

49. Rosero-Galindo C, Gaitan-Solis E, Cárdenas-Henao H, Tohme J, Toro-Perea N. Polymorphic microsatellites in a mangrove species, *Rhizophora mangle* L. (Rhizophoraceae). Molecular Ecology Notes. 2002; 2 : 281-283.

50. Islam NS, Lian C, Kameyama N, Wu B, Hogetsu T. Development of microsatellite markers in *Rhizophora stylosa* using a dual-suppression-polymerase chain reaction technique. Molecular Ecology. 2004; 4:110-112.

51. Takayama K, Tamura M, Tateishi Y, Kajita T. Isolation and characterization of microsatellite loci in the red mangrove *Rhizophora mangle* (Rhizophoraceae) and its related species.Conservation Genetics. 2008; 9: 1323–1325.

52. Ribeiro DO, Vinson CC, Nascimento DSS, Mehlig U, Menezes MPM, Sampaio I, Silva MB. Isolation of microsatellite markers for the red mangrove, *Rhizophora mangle* (Rhizophoraceae). Appl Plant Sci. 2013; 1(9): apps.1300003.

53. Sandoval-Castro E, Muñiz-Salazar R, Enríquez-Paredes LM, Riosmena-Rodríguez R, Dodd RS, Tovilla-Hernández C, Arredondo-García M. Genetic population structure of red mangrove (*Rhizophora mangle* L.) along the northwestern coast of Mexico. Aquatic Botany. 2012; 99: 20–26.

54. Lakshmi M, Rajalakshmi S, Parani M, Anuratha CS, Parida AK. Molecular phylogeny of mangroves I. Use of molecular markers in assessing the intraspecific genetic variability in the mangrove species *Acanthus ilicifolius* Linn. (Acanthaceae). Theoretical and Applied Genetics. 1997; 94 : 1121 – 1127 .

55. Sun M, KC Wong, JSY Lee. Reproductive biology and population genetic structure of *Kandelia candel* (Rhizophoraceae), a viviparous mangrove species. American Journal of Botany. 1998; 85: 1631 – 1637.

56. Arbeláez-Cortes E, Castillo-Cárdenas MF, Toro-Perea N, Cárdenas-Henao H. Genetic structure of the red mangrove (*Rhizophora mangle* L.) on the Colombian Pacific detected by microsatellite molecular markers. Hydrobiology. 2007; 583: 321–330.

57. Núñez-Farfán J, Domínguez CA, Eguiarte LE, Cornejo A, Quijano M, Vargas J, and Dirzo R. Genetic divergence among Mexican populations of red mangrove (*Rhizophora mangle*): Geographic and historic effects. Evolutionary Ecology Research 2002; 4: 1049 – 1064.

58. Goodwillie C, Kalisz S, Eckert CG. The evolutionary enigma of mixed mating systems in plants: Occurrence, theoretical explanations, and empirical evidence. Annu Rev Ecol Evol Syst. 2005; 36: 47–79.

59. Tomlinson PB, Primack RB, Bunt JS. Preliminary observations on floral biology in mangrove Rhizophoraceae. Biotropica. 1979; 11: 256-277.

60. Menezes MPM, Oliveira D, Mello CF. Pollination of red mangrove, *Rhizophora mangle*, in northern Brazil. Acta Hortic. 1997; 347: 431-434.

61. Mori GM, Zuchi MI, Souza AP. Multiple-Geographic-Scale Genetic Structure of Two Mangrove Tree Species: The Roles of Mating System, Hybridization, Limited Dispersal and Extrinsic Factors. PLoS One - Submitted.

62. Hewitt G. The genetic legacy of the Quaternary ice ages. Nature. 2000; 405 : 907–913 .

63. Hewitt G. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society, B .Biological Sciences. 2004; 359 : 183–195 .

64. Saenger P. Mangrove vegetation: An evolutionary perspective. Marine & Freshwater Research. 1998; 49 : 277 – 286.

65. Palumbi SR. Population genetics, demographic connectivity, and the design of marine reserves. Ecological Applications. 2003; 13 : 146 – 158.

66. Petit RJ, Aguinagalde I, Beaulieu J de, Bittkau C, Brewer S, Cheddadi R, Ennos R, *et al*. Glacial refugia: Hotspots but not melting pots of genetic diversity. Science. 2003; 300 : 1563–1565.

67. Lo EYY, Duke NC, Sun M. Phylogeographic pattern of Rhizophora(Rhizophoraceae) reveals the importance of both vicariance and long-distance oceanic dispersal to modern mangrove distribution. BMC Evolutionary Biology. 2014; 14:83.

68. Mori GM, , Zuchi MI, Sampaio I, Souza A. Western *Avicennia* species distribution, introgressive hybridization and trans-Atlantic dispersal unveiled by phylogeographic patterns. BMC Evol Biol. 2015; 15:61..

69. Nettel A, Dodd RS. Drifting propagules and receding swamps: genetic footprints of mangrove recolonization and dispersal along tropical coasts. Evolution. 2007; 61: 958–971.

70. Menezes MPM De, Berger U, Mehlig U. Mangrove vegetation in Amazonia: a review of studies from the coast of Pará and Maranhão States, north Brazil. Acta Amazonica. 2008; 38: 403 – 420.

71. Takayama K, Tateishi Y, Murata J, Kajita T. Gene flow and population subdivision in a pantropical plant with sea-drifted seeds *Hibiscus tiliaceus* and its allied species: evidence from microsatellite analyses. Mol Ecol. 2008; 17: 2730–2742.

72. Lumpkin R, Johnson GC. Global ocean surface velocities from drifters: Mean, variance, El Niño-Southern Oscillation response, and seasonal cycle. J Geophys Res Ocean. 2013; 118: 2992–3006.

73. Parani M, Rao CS, Mathan N, Anuratha CS, Narayanan KK, Parida A. Molecular Phylogeny of mangroves III Parentage analysis of a Rhizophora hybrid using random amplified polymorphic DNA and restriction fragment length poly-morphism markers. Aquat. Bot. 1997; 58, 165–172.

74. Tyagi AP. Chromosomal pairing and pollen viability in Rhizophora mangle and Rhizophora stylosa hybrids. S. Pac. J. Nat. Sci. 2002; 20, 1–3.

75. Lo EYY. Testing hybridization hypotheses and evaluating the evolutionary potential of hybrids in mangrove plant species. J. Evol. Biol. 2010; 23, 2249–2261.

76. Ng WL, Szmidt AE. Introgressive hybridization in two Indo-West Pacific *Rhizophora* mangrove species, *R. mucronata* and *R. stylosa*. Aquatic Botany. 2014; 120, 222-228.

77. Soltis PS, Soltis DE. The role of hybridization in plant speciation. Ann. Rev.Plant Biol. 2009; 60, 561–588.

78. Nettel A, Dodd RS, Afzal-Rafii Z, Tovilla-Hernández C. Genetic diversity enhanced by ancient introgression and secondary contact in East Pacific black mangroves. Mol Ecol. 2008; 17: 2680–2690.

CAPÍTULO II

RESULTADOS COMPLEMENTARES

Sistema Reprodutivo de *Rhizophora mangle*

Com intuito de melhor entender a biologia de *Rhizophora mangle,* a espécie mais abundante no litoral brasileiro entre as estudadas nesse trabalho, um dos objetivos dessa tese foi realizar a análise de sistema reprodutivo dessa espécie. Para tanto, foi realizada uma coleta na cidade de Salinópolis, no estado do Pará (00°36.294'S, 047°22.417'O) (Figura 1), onde foi encontrada uma diversidade genética maior que em outras áreas, através de análises preliminares. Foram coletadas folhas de 30 árvores e, aproximadamente, 10 propágulos de cada uma, com uma distância mínima de dez metros entre as árvores. Foram coletadas informações sobre coordenada geográfica e altura de cada árvore de onde foram retirados os propágulos.

As extrações de DNA foram realizadas utilizando o kit de extração DNeasy® Plant Mini Kit (Qiagen, Hilden, DE). As amplificações foram realizadas com os mesmos nove locos utilizados no artigo apresentado no Capítulo I para *R. mangle* (Tabela S2), mas apenas quatro locos apresentaram boa amplificação (Rma3-14, Rma3-23, Rma3-37 e Rma3-38).

A análise de sistema de reprodução foi efetuada por meio do programa de análise multilocos MLTR v.3.4 (Ritland, 2002). Esta análise leva em consideração o sistema de reprodução baseado em modelo misto (Ritland and Jain, 1981) que assume taxas de endocruzamento como *s* (*selfing*) e o cruzamento de pólen proveniente de diferentes plantas, bem como o modelo de cruzamento correlacionado (Ritland, 1989), que pode

estimar grau de progênies irmãs provenientes da planta paterna. Para a análise das famílias foi adotado o método numérico EM (*Expectation Maximization*), com 95% de intervalo de confiança e 10.000 reamostragens *bootstrap*. As estimativas obtidas pelo software foram taxa de cruzamento multilocos (t_m), taxa de cruzamento uni-locos (t_s), cruzamento entre aparentados ($t_m - t_s$), correlação de endocruzamento multilocos (r_s), correlação multilocos de pólen ($r_{p(m)}$), respectivamente.

A frequência alélica de óvulos e pólen nos quatro locos examinados apontou a ocorrência de 12 alelos. Foi observada pouca diferença na frequência de pólen e óvulos em todos os locos avaliados, mostrando assim uma frequência pouco heterogênea para a espécie *R. mangle* (Tabela 1).

Tabela 1 Frequência alélica de pólen e óvulos de *R. mangle* de Salinópolis - PA, com base em 4 marcadores de microssatélites.

Loco	Alelo	Pólen	Óvulo
Rma3-14	158	0.966 (0.031)	0.971 (0.008)
Rma3-14	164	0.034 (0.031)	0.029 (0.008)
Rma3-23	210	0.583 (0.091)	0.549 (0.024)
Rma3-23	228	0.392 (0.094)	0.443 (0.025)
Rma3-23	220	0.013 (0.017)	0.007 (0.004)
Rma3-23	200	0.012 (0.007)	0.001 (0.002)
Rma3-37	250	0.615 (0.083)	0.704 (0.014)
Rma3-37	270	0.348 (0.086)	0.274 (0.013)
Rma3-37	260	0.037 (0.036)	0.021 (0.008)
Rma3-38	242	0.757 (0.082)	0.701 (0.020)
Rma3-38	274	0.159 (0.043)	0.271 (0.014)
Rma3-38	264	0.084 (0.071)	0.027 (0.015)

Intervalo de confiança entre parênteses, com 95% de probabilidade.

A taxa de cruzamento uniloco (ts = 0,378±0,064) foi menor que a taxa de cruzamento multiloco (tm= 0.421±0.070), e esses valores indicam um sistema reprodutivo

misto (Tabela 2). A diferença entre *tm* e *ts* foi igual a 0,043±0,026, o que representa aproximadamente 4% de endogamia biparental. Esses resultados indicam que ocorreram cruzamentos entre indivíduos parentes dentro das populações, e existe possível estrutura genética espacial. A proporção de autofecundação estimada (*s*) por meio da diferença de 1 menos a taxa de cruzamento multilocos sugere aproximadamente 57,9% de autofecundação. A estimativa multilocos de pólen ($r_{p(m)}$), que determina a correlação do grau de parentesco entre as progênies, indicam que grande proporção das progênies foram geradas por cruzamentos biparentais e são parentes no grau de irmãs completas ($r_{p(m)}$ = 0,822 ±0,216) (Tabela 2). O valor encontrado indica a ocorrência de cruzamentos entre parentes e, segundo Ritland (2002), diferenças negativas entre estes dois parâmetros sugerem que os genitores paternos eram parentes entre si. O número efetivo de árvores doadoras de pólen (N_{ep}) foi baixo e representou aproximadamente 1,21 indivíduo contribuindo por árvore materna.

Tabela 2. Estimativas de taxa de cruzamento total em *R. mangle* de Salinópolis - PA.

Parâmetros estatísticos	Resultado (Erro Padrão)
Número de sementes - árvore (propágulos)	30 (10)
Taxa uniloco (t_s)	0,378 (0,064)
Taxa multiloco (t_m)	0,421 (0,070)
Taxa de cruzamento entre parentes (t_p)	0,043 (0,013)
Correlação de autofecundação (r_s)	0,726 (0,103)
Proporção autofecundação (s)	0,579
Correlação multiloco de paternidade ($r_{p(m)}$)	0,822 (0,216)
Número médio de doadores pólen (N_{ep})	1,21
Proporção de irmãs de autofecundação (P_{SS})	34%
Proporção irmãs completas e meio irmãs (P_{SHS})	49%
Proporção irmãs-completas (P_{FS})	15%
Proporção de meio-irmãs (P_{HS})	3%
Coancestria (entre propágulos)	0.370
Variação tamanho efetivo (N_e)	1.35
Número de sementes-árvores (m)	111

Erro padrão entre parênteses, estimado através de 10.000 reamostragens em bootstrap através do programa MLTR.

Ainda na Tabela 2, os resultados obtidos pelo grau de parentesco entre as progênies, que abordam as categorias: (S_{FS}) *Self full-sibs*, (irmãs formadas por autofecundação); (P_{FS}) *Full-sibs*, (irmãs originadas de uma única árvore paterna); (P_{SHS}) *Self half-sibs*, (irmã originada via autofecundação e outra proveniente de pólen de outra árvore paterna); e (P_{HS}) *Half-sibs*, (originadas de duas árvores paternas distintas, sem autofunção) descrito por Squillace (1974). Por meio desta análise obteu-se a proporção de 15% para P_{FS}; uma proporção de 49% para P_{SHS} ; uma quantidade de 34% em P_{SS}; e uma baixa amostragem de meias irmãs (P_{HS}), com apenas 3%. Estas análises de Squillace (1974) sustentam que existe um número reduzido de plantas doadoras de pólen conforme demonstrado pelas proporções elevadas de irmãs-completas (P_{FS}) e irmãs completas e meio-irmãs (P_{SHS}). Este resultado pode estar relacionado ao fato de que a região em que essas amostras foram coletadas a

espécie *R. mangle* era visivelmente predominante, e se encontrava próxima a um estacionamento (Figura 1), área possivelmente perturbada por fatores antrópicos, aparentando ser resultado uma colonização mais recente, onde ocorre uma perda de número de alelos e consequentemente uma elevada homozigose (Nettel & Dodd, 2007).

Figura 1. Figura mostrando a região onde as amostras foram coletadas, no município de Salinópolis - PA (Imagem do Google Maps, acessado em 21 de abril de 2015).

CONSIDERAÇÕES GERAIS

No presente estudo, foram desenvolvidos 22 locos microssatélites que apresentaram boa amplificação e polimorfismo intraespecífico. Entre eles, sete foram desenvolvidos para *R. mangle,* seis para *R. racemosa* e nove para *R. harrisonii*. Verificou-se um elevado polimorfismo entre os locos e uma alta taxa de transferência de locos de uma espécie para a outra (Tabela S1 no Capítulo I). Os resultados indicam que estes marcadores são ferramentas valiosas para estudos genético moleculares, tais como para as investigações da estrutura e diversidade genética, sistema de reprodução e para a avaliação das áreas de hibridação entre espécies. Além disso, até onde temos conhecimento, este é o primeiro trabalho que apresenta o desenvolvimento de marcadores microssatélites moleculares para as espécies *R. racemosa* e *R. harrisonii.*

Utilizando esses marcadores e dois outros encontrados na literatura (Rosero-Galindo *et al.,* 2002; Islam *et al.,* 2004), encontramos que indica o sistema reprodutivo de *R. mangle* seria misto com tendencia a alogamia. No estudo realizado sobre a taxa de cruzamento da espécie *R. mangle,* novamente encontramos valores que indicariam sistema reprodutivo misto. A diferença entre os resultados pode estar relacionada com o número de marcadores utilizados em cada um dos experimentos (nove no primeiro e quatro no segundo, pois alguns locos não amplificaram apropriadamente) e também, com o fato de que na primeira análise foram avaliados indivíduos de toda a costa brasileira, enquanto que no segundo avaliamos apenas uma região no norte, Salinópolis-PA. Essa região era dominada por *R. mangle,* o que pode significar que seja uma área recentemente colonizada por essa espécie.

Encontramos diferenciação entre as populações estudadas e uma diversidade genética maior, com um número maior de alelos privados, em populações de regiões ao norte da costa brasileira. Resultados como os encontrados eram esperados, uma vez que as áreas de mangue em regiões equatoriais são maiores e possuem uma diversidade de espécies maior.

As análises realizadas nesta tese também apresentam uma diferenciação entre as populações do norte e do sul para a espécie *R. mangle,* formando dois grupos distintos de diversidade. Esses resultados revelam que o padrão de distribuição de *Rhizophora* pode ser influenciado por uma combinação de eventos vicariantes e de dispersão oceânica. Mesmo existindo evidências de que os propágulos dessas espécies podem se dispersar a longas distâncias, no presente estudo não encontramos esse padrão. A distribuição da diversidade genética no Brasil para essa espécie estaria sendo influenciada por correntes oceânicas superficiais, conforme discutido no Capítulo I, o que forma o padrão de estruturação encontrado.

Há também evidências na literatura de que a espécie *Rhizophora harrisonii* é resultado da hibridação introgressiva, antiga e persistente entre *R. mangle* e *R. racemosa*. A hibridação natural é comum em plantas e desempenha um papel importante na evolução da espécie ao promover especiação. No entanto, os resultados deste trabalho não mostram evidências de que *R. harrisonii* seja um híbrido interespecífico e, junto com *R. racemosa*, seriam "espécies puras".

Os manguezais são compostos por espécies únicas de árvores e arbustos (Macintosh & Ashton, 2002). São considerados “berçários” naturais para muitas espécies de animais, que se tornam fonte de alimentação para populações humanas, entre outros benefícios.

Contribuem também com a redução da erosão da linha costeira. (Diegues, 1991; Othman, 1994; Schaeffer-Novelli, 1999) e diminuem o impacto de catástrofes naturais (Kathiresan & Rajendran, 2005; Dahdouh-Guebas *et al*., 2005). O tsunami de 26 de dezembro de 2004 causou desastres em vários países asiáticos e africanos, mas seus danos foram menores em locais onde os manguezais estavam bem preservados (Kathiresan & Rajendran, 2005). Mas a escala de impactos humanos nos manguezais tem crescido dramaticamente nas últimas três décadas, com muitos países mostrando perdas de 60 a 80% ou mais de cobertura de florestas de mangue, em relação ao que existia nos anos 60 (Macintosh & Ashton, 2002).

Sendo assim, os resultados obtidos nessa tese de doutorado são de grande relevância para o entendimento da dinâmica de populações brasileiras de ecossistemas de mangue. Tais resultados são essenciais para serem utilizados em programas de conservação e manejo, além de permitirem um maior conhecimento sobre os efeitos da fragmentação de manguezais e desflorestamento (Frankham *et al.*, 2004; Freeland, 2005).

RESUMO DOS RESULTADOS

Capítulo I

- Foram desenvolvidos novos locos microssatélites para as espécies estudadas. Para *R. mangle* sete apresentaram polimorfismo intraespecífico e os valores de PIC variaram de 0,147 a 0,513; para *R. racemosa* foram seis, e para *R. harrisonii* foram nove, com valores de PIC variando de 0,263 a 0,595

- Uma comparação entre as regiões geográficas indicaram diferenças significativas nos padrões de variação genética. A riqueza alélica, a heterozigosidade observada (H_O) e diversidade genética (H_E) foram significativamente maiores nas populações do Pará, Maranhão, Piauí e Ceará

- A taxa de cruzamento aparente média estimada foi de 0,841. *R. racemosa* apresentou valores mais elevados de F_{IS} (0,281 ± 0,085) e t_a (0,562), o que pode indicar que possui sistema reprodutivo misto. Os valores encontrados para *R. harrisonii* foram parecidos com o da espécie *R. mangle*

- Em *R. mangle,* os valores de F_{ST} par a par variaram de 0 a 0.437. A menor diferenciação genética média entre as populações foi observada entre Pernambuco, Bahia, Rio de Janeiro, Ubatuba, Cananéia, Paraná, Santa Catarina, enquanto a população do Pará teve o maior valor médio de diferenciação. Para *R. racemosa* e *R. harrisonii* os valores de F_{ST} foram mais baixos entre as duas populações de *R. harrisonii*. O valor médio mais elevado foi entre *R. racemosa* e *R. harrisonii*

- Por meio da análise Bayesiana observamos que há dois grupos distintos ($K = 2$) para *R. mangle,* sugerindo a ocorrência de dois grupos de diversidade genética: um ao norte e outro ao sul da costa brasileira. Para *R. racemosa* e *R. harrisonii*, o número mais provável de populações foi de $K = 2$, o que indica as duas diferentes espécies.

- Em relação à ocorrência de hibridação, todas as análises realizadas não mostraram diferenças entre as espécies *R. racemosa* e *R. harrisonii*. Estes resultados indicam que não há nenhuma evidência de que as espécies estejam hibridando

Capítulo II

- Na análise de taxa de cruzamento de *R. mangle,* o número efetivo de árvores doadoras de pólen (N_{ep}) encontrado foi baixo e representou aproximadamente 1,21 indivíduos contribuindo por árvore materna
- A taxa de cruzamento foi de 0,421, sugerindo sistema reprodutivo misto na população avaliada
- Existe um número reduzido de plantas doadoras de pólen conforme demonstrado pelas proporções elevadas de irmãs-completas (P_{FS}) e irmãs completas e meio-irmãs (P_{SHS})

CONCLUSÕES

O desenvolvimento e a utilização de novos locos de marcadores do tipo microssatélite para as espécies *Rhizophora mangle, R. racemosa* e *R. harrisonii* nesta tese permitem concluir que existe uma diferença significativa entre as populações de *R. mangle* no padrão de variação genética em diferentes regiões do litoral brasileiro. Ou seja, essa espécie não é composta de uma única população, o que é indicado pela diferenciação encontrada entre a população de localidades no litoral norte e sul do Brasil. *R. racemosa* e *R. harrisonii* apresentaram menor diferenciação de uma região para a outra, mas as diferenças genéticas ainda estavam presentes.

O resultados encontrados indicam que o sistema reprodutivo para estas espécies é misto com tendência a alogamia. Além disso, não foram encontradas evidências de hibridação introgressiva entre as espécies de *Rhizophora*. Concluindo assim que a presente tese alcançou seus objetivos iniciais gerando importantes contribuições para o conhecimento genético das espécies de *Rhizophora* spp. do litoral brasileiro.

PERSPECTIVAS

Os marcadores microssatélites da espécie *Rhizophora harrisonii,* descritos no Capítulo I, serão transferidos para a espécie *R. mangle*, bem como será avaliado como os mesmo se comportam nessa espécie. Também deverá ser investigada a taxa de cruzamento de *R. mangle*, onde outros marcadores desenvolvidos poderão ser utilizados e novas análises serão realizadas, para melhor entender o mecanismo de reprodução dessa espécie. Os resultados aqui relatados são valiosos e abrem caminho para futuras pesquisas sobre o ecossistema de manguezal. Esses permitem um maior conhecimento sobre os efeitos da fragmentação de manguezais e desflorestamento (Frankham *et al.*, 2004; Freeland, 2005). Sendo assim, esperamos que os mesmos possam ser associados a programas de manejo e preservação da espécie e de manguezais como um todo.

Além de estudos de espécies do gênero *Rhizophora,* existem outros trabalhos em nosso laboratório relacionados a outras espécies e ao ecossistema de mangue. Uma abordagem que está sendo realizada é avaliar como as espécies de *Rhizophora* e *Avicennia* podem vir a responder às mudanças climáticas globais que vêm ocorrendo atualmente.

LITERATURA CITADA

Abbott R, Albach D, Ansell S *et al.* Hybridization and speciation. Journal of Evolutionary Biology. 2013; 26, 229–46.

Alongi DM. Present state and future of the world's mangrove forests. Environmental Conservation. 2002; 29(3):331-349.

Alongi DM. Mangrove forests: resilience, protection from tsunamis, and responses to global climate change. Estuarine, Coastal and Shelf Science. 2008; 76(1), 1-13.

Amaral PGC do, Ledru M, Branco FR, Giannini PCF. Late Holocene development of a mangrove ecosystem in southeastern Brazil (Itanhaém, state of São Paulo). Palaeogeography, Palaeoclimatology, Palaeoecology. 2006; 241 : 608–620.

Arbeláez E, Castilho ME, Totro N, Cárdenas H. Genetic structure of red mangrove (*Rhizophora mangle* L.) on the Colombian Pacific detected by microsatellite molecular markers. Hydrobiologia. 2007; 583:321-330.

Barbier EB. A global strategy for protecting vulnerable coastal populations. Science 2014; 345.6202, 1250-1251.

Barton N. The role of hybridization in evolution. Molecular Ecology. 2001; 10, 551-568.

Breteler, FJ. The Atlantic species of *Rhizophora*. Acta Bot. Neerl. 1969; 18 3, pp. 434–441.

Cerón-Souza I, Rivera-Ocasio E, Medina E *et al.* Hybridization and introgression in New World red mangroves, Rhizophora (Rhizophoraceae). American Journal of Botany. 2010; 97, 945–57.

Cerón-Souza I, Bermingham E, McMillan WO, Jones FA. Comparative genetic structure of two mangrove species in Caribbean and Pacific estuaries of Panama. BMC Evolutionary Biology. 2012; 12, 205.

CIMA. Subsídios técnicos para elaboração do relatório nacional do Brasil para a Conferência das Nações Unidas sobre Meio Ambiente e Desenvolvimento. Brasília, Comissão Interministerial para a preparação da preparação a Conferência das Nações Unidas sobre Meio Ambiente e Desenvolvimento; 1991.

Chan HT. A note on the discovery of *Rhizophora x lamarckii* in PeninsularMalaysia. J. Trop. For. Sci. 1996; 9, 128–130.

Dahdouh-Guebas F, Jayatissa LP, Di Nitto D *et al.* How effective were mangroves as a defence against the recent tsunami? Current Bio*l*ogy. 2005; 15 (12):443-447.

Diegues AC. Comunidades humanas e os manguezais do Brasil. *In:* CPRH, 1991. Alternativas de uso e proteção dos manguezais do Nordeste.Recife, Companhia Pernambucana de Controle da Poluição Ambiental e de Administração de Recursos Hídricos. Série Publicações Técnicas. 1991; 03:38-45.

Dodd RS, Afzal-Rafii Z, Bousquet-Mélou A. Evolutionary divergence in the pan-Atlantic mangrove Avicennia germinans. New Phytologist. 2000; 145, 115–125.

Dodd RS, Afzal-Rafii Z, Kashani N, Budrick J. Land barriers and open oceans: effects on gene diversity and population structure in *Avicennia germinans* L.(Avicenniaceae). Molecular Ecology. 2002; 11, 1327 – 1338.

Duke NC, Ball M, Ellison J. Factors influencing biodiversity and distributional gradients in mangroves. Global Ecol. Biogeogr. Lett. 1998; 7:27-47.

Duke NC, Lo YY, Sun M. Global distribution and genetic discontinuities of mangrove - emerging patterns in the evolution of Rhizophora. Trees. 2002; 16:65-79.

Duke NC, Allen JA. *Rhizophora mangle*, *R. samoensis*, *R. racemosa*, *R. x harrisonii* (Atlantic-East Pacific red mangrove), ver. 2.1. *In*: ELEVITCH, C.R. (ed.). Species Profiles for Pacific Island Agroforestry. Permanent Agriculture Resources (PAR), Hölualoa, Hawaii; 2006. Available: http://traditionaltree.org.

Duke NC, Meynecke O, Dittmann S *et al.* A World without mangroves? Science. 2007; 317:41-42.

Duke NC. Overlap of eastern and western mangroves in the South-western Pacific: hybridization of all three *Rhizophora* (Rhizophoraceae) combinations in New Caledonia. Blumea - Biodiversity, Evolution and Biogeography of Plants. 2010; 55, 171–188.

Duke, NC, Ge XJ. Bruguiera (Rhizophoraceae) in the Indo-West Pacific:a morphometric assessment of hybridization within single-flowered taxa.Blumea-Biodiversity. Evol. Biogeogr. Plants. 2011; 56, 36–48.

Ellison AM, Farnworth EJ, Merkt RE. Origins of mangrove ecosystems and the mangrove biodiversity anomaly. Global Ecology and Biogeography. 1999; 8:95–115.

Ferreira ME, Grattapaglia D. Introdução ao uso de marcadores moleculares em análise genética 3 ed. Embrapa Recursos Genéticos e Biotecnologia, Brasília; 1998.

Frankham R, Ballou JD, Briscoe DA. A primer of conservation genetics. Cambrigde University Press; 2004.

Freeland JR. Molecular Ecology. John Wiley & Son Ltd, Chichester; 2005.

Gilman EL, Ellison J, Duke NC, Field C. Threats to mangrove from climate change and adaptation options: A review. Aquatic Botany. 2008; 89:237-350.

Giri C, Ochieng E, Tieszen LL *et al.* Status and distribution of mangrove forests of the world using earth observation satellite data. Global Ecology and Biogeography. 2011; 20, 154–159.

Hewitt G. The genetic legacy of the Quaternary ice ages. Nature 2000; 405 : 907 – 913.

Hewitt G. Genetic consequences of climatic oscillations in the Quaternary. Philosophical Transactions of the Royal Society, B . Biological Sciences. 2004; 359 : 183 – 195.

Huxham M, Kumara MP, Jayatissa LP *et al*. Intra-and interspecific facilitation in mangroves may increase resilience to climate change threats. Philosophical Transactions of the Society B: Biological Sciences. 2010; 365(1549), 2127-2135.

Joppa LN, Roberts DL, Myers N, Pimm SL. Biodiversity hotspots house most undiscovered plant species. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108, 13171–6.

Kathiresan K. Rhizophora annamalayana: A new species of mangroves. Environment and Ecology. 1995; 13 : 240 – 241.

Kathiresan K. Rhizophora x annamalayana Kathir (Rhizophoraceae), a new nothospecies from Pichavaram Mangrove Forest in southeastern peninsular India. Environ. Ecol. 1999; 17, 500–501.

Kathiresan K, Bingham BL. Biology of Mangroves and Mangrove Ecosystems. Advances in Marine Biology. 2001; 40: 81-251.

Kathiresan K, Rajendran N. Coastal magrove forests mitigated tsunami. Estuarine, Coastal and Shelf Science. 2005; 65:601-606.

Kjerfve B, Lacerda LD. Mangroves of Brazil. *In:* LACERDA, L.D. (Ed.) Conservation and sustainable utilization of mangrove forest in Latin America and Africa regions. Part I - Latin America. Magrove Ecosystem Techinical Report No. 2. ITTO/ISME, Okinawa; 1993.

Krauss KW, Lovelock CE, McKee KL *et al.* Environmental drivers in mangrove establishment and early development: A review. Aquatic Botany. 2008; 89, 105–127.

Lacerda LD. Mangrove Ecosystems: Function and Management. Springer Verlag, Berlin; 2002.

Lacerda LD. Os Manguezais do Brasil. *In*: VANNUCCI, M. Os manguezais e nós: uma síntese de percepções. 2ª ed; Editora da USP, São Paulo; 2003.

Lakshmi M, Parani M, Parida A. Molecular phylogeny of mangroves IX. Molecular marker assisted intra-specific relationships in the Indian mangrove tribe Rhizophoreae. Aquatic Botany. 2002; 74:110-115.

Lo, E.Y.Y.; Duke, N.; Sun, M. Phylogeographic pattern of *Rhizophora* (Rhizophoraceae) reveals the importance of both vicariance and long-distance oceanic dispersal to modern mangrove distribution. BMC Evolutionary Biology. 2014; 14:83.

Luz V. Diferenciação e caracterização morfológica das três espécies do gênero Rhizophora da costa do Pará. Trabalho de Conclusão de Curso. (Graduação em Licenciatura em Biologia) - Universidade Federal do Pará. Orientador: Moirah Paula Machado de Menezes. 2006.

Macintosh DJ, Ashton EC. A Review of Mangrove Biodiversity Conservation and Management. Centre for Tropical Ecosystems Research, University of Aarthus, Denmark (artigo pdf); 2002.

Menezes MPM, Oliveira D, Mello CF. Pollination of red Mangrove *Rhizophora mangle* in the Northern of Brazil . Acta Horticulturae. 1997; 437: 431-434.

Menezes MPM De, Berger U, Mehlig U. Mangrove vegetation in Amazonia: a review of studies from the coast of Pará and Maranhão States, north Brazil. Acta Amazonica. 2008; 38, 403–420.

McIvor AL, Möller I, Spencer T, Spalding M. Reduction of wind and swell waves by mangroves. The Nature Conservancy and Wetlands International. 2012.

Nathan R, Schurr FM, Spiegel O *et al.* Mechanisms of long-distance seed dispersal. Trends in Ecology & Evolution. 2008; 23, 638–47.

Nettel A, Dodd RS. Drifting propagules and receding swamps: genetic footprints of mangrove recolonization and dispersal along tropical coasts. Evolution. 2007; 61, 958–71.

Nettel A, Dodd RS, Afzal-Rafii Z, Tovilla-Hernández C. Genetic diversity enhanced by ancient introgression and secondary contact in East Pacific black mangroves. Molecular Ecology. 2008; 17, 2680–90.

Nettel A, Dodd RS, Ochoa-Zavala M, Tovilla-Hernández C, Días-Gallegos JR. Mating System Analyses of tropical populations of the black mangrove *Avicennia germinans* (L.) L. (Avicenniaceae). Botanical Sciences. 2013; 91, 115–117.

Ng WL, Chan HT, Szmidt AE. Molecular identification of natural mangrove hybrids of Rhizophora in Peninsular Malaysia. Tree Genet. Genom. 2013; 9, 1151–1160

Ng WL, Szmidt AE. Introgressive hybridization in two Indo-West Pacific *Rhizophora* mangrove species*, R. mucronata* and *R. stylosa*. Aquatic Botany. 2014, 120, 222–228.

Othman MA. Value of magroves in coastal protection. Hydrobiology. 1994; 285:343-351.

Parani M, Rao CS, Mathan N *et al.* Molecular Phylogeny of mangroves III Parentage analysis of a Rhizophora hybrid using random amplified polymorphic DNA and restriction fragment length polymorphism markers. Aquatic botany. 1997; 58:165-172.

Pil MW, Boeger MRT, Muschner VC *et al.* Postglacial north-south expansion of populations of *Rhizophora mangle* (Rhizophoraceae) along the Brazilian coast revealed by microsatellite analysis. American Journal of Botany. 2011; 98, 1031–9.

Por FD. The ecosystem of the Mangal: general considerations. *In*: D. POR E I. DOR (Eds) *Hydrobiology of the Mangal*. Dr. W. Junk Publishers, The Hague. 1984; pp.1-26.

Prance GT, Silva MF, Albuquerque BW *et al.* Revisão taxonômica das espécies amazônicas de Rhizophoraceae. Acta Amazonica. 1975; 5(1): 5-22.

Qiu S, Zhou R, Li Y, Havanond S. Molecular evidence for natural hybridization between *Sonneratia alba* and *S. griffithii*. Journal of Systematics and Evolution. 2008; 46, 391–395.

Ricklefs RE, Schwarzbach AE, Renner SS. Rate of lineage origin explains the diversity anomaly in the Wold's mangrove vegetation. The American Naturalist. 2006; 168, 805–810.

Ritland K. Extensions of models for the estimation of mating systems using n independent loci. Heredity. 2002; 88:221-228.

Ritland K, Jain S. A model for the estimation of outcrossing rate and gene frequencies using n independent loci. Heredity. 1981; 47:35-52.

Ritland K. Correlated matings in the partial selfer, *Mimulus guttatus*. Evolution. 1989; 43:848-859

Rosero-Galindo C, Gaitan E, Cárdenas, H *et al.* Polymorphic microssatellites in a mangrove species, *Rhizophora mangle* L. (Rhizophoraceae). Mol. Ecol. Notes. 2002; 2:281-283.

Saenger P. Mangrove vegetation: an evolutionary perspective. Marine Freshwater Research. 1998; 49, 277–286.

Schaeffer-Novelli Y, Cintrón G. Guia para estudo de áreas de manguezal. Estrutura, função e flora. Caribbean Ecological Research, São Paulo; 1986.

Schaeffer-Novelli Y, Cintrón-Molero G. Brazilian mangroves: A hitorical ecology. Journal of Brazilian Association for the Advancement of Science. 1999; 51:3/4, 274-283.

Schaeffer-Novelli Y, Cintrón-Molero G, Adaime RR, Camargo TMD. Variability of mangrove ecosystems along the Brazilian coast. Estuaries. 1990; 13:204-218.

Schaeffer-Novelli Y. Relatório do grupo manguezal, marisma e apicum. *In*: Workshop para avaliação e ações prioritárias para a conservação da biodiversidade das zonas costeira e marinha: Relatório técnico, 1, Porto Seguro: MMA; 1999.

Schaeffer-Novelli Y, Cintrón-Molero G, Cunha-Lignon M, Coelho-Jr C. A Conceptual Hierarchical Framework for Marine Coastal Management and Conservation: a" Janus-Like" Approach. Journal of Coastal Research. 2005; 191-197.

Schlötterer C. Evolutionary dynamics of microsatellite DNA. Chromossoma. 2000; 109: 365-371.

Schwarzbach A, Ricklefs R. Systemic affinities of Rhizophoraceae and Anisophylleaceae, and intergeneric relationships within Rhizophoraceae, based on chloroplast DNA, nuclear ribossomal DNA, and morphology. American Journal Botany. 2000; 87:547-564.

Schwarzbach A, Ricklefs R. The use of molecular data in mangrove plant research. Wetlands Ecol. Manage. 2001; 9:195-201.

Squillace AE. Average correlations among offspring from open-pollinated forest trees. Silvae Genetica. 1974; 23(5):149-156

Smith III TJ, Robblee MB, Wanless HR, Doyle TW. Mangroves, hurricanes, and lightning strikes: assessment of Hurricane Andrew sugests and interactions across two differing scales of disturbance. BioScience. 1994; 44:256-262.

Spalding M, Kainuma M, Collins L. World Atlas of Mangroves (U Spalding, Mark (Department of Zoology, University of Cambridge, Ed,). Earthscan, London; 2010.

Soares MLG, Estrada GCD, Fernandez V, Tognella MMP. Southern limit of the Western South Atlantic mangroves: assessment of the potential effects of global warming from

a biogeographical perspective. Estuarine, Coastal and Shelf Science. 2012; 101, 44-53.

Soltis PS, Soltis DE. The role of hybridization in plant speciation. Annual review of plant biology. 2009; 60, 561–88.

Sun M, Lo EYY. Genomic markers reveal introgressive hybridization in the Indo-West Pacific mangroves: a case study. PloS One. 2011; 6, e19671.

Takayama K, Tamura M, Tateishi Y, Kaijta T. Isolation and characterization of microsatellite loci in the red mangrove *Rhizophora mangle* (Rhizophoraceae) and its related species. Conservation Genetics. 2007; 9(5):1323-1325.

Takayama K, Tamura M, Tateishi Y, Webb EL, Kajita T. Strong genetic structure over the American continents and transoceanic dispersal in the mangrove genus *Rhizophora* (Rhizophoraceae) revealed by broad-scale nuclear and chloroplast DNA analysis. American Journal of Botany. 2013; 100, 1–11.

Tomlinson PB. The botany of mangroves. Cambridge University Press, Cambridge, Inglaterra; 1986.

Triest L. Molecular ecology and biogeography of mangrove trees towards conceptual insights on gene flow and barriers: A review. Aquatic Botany. 2008; 89:138-154.

Tyagi AP (2002) Chromosomal pairing and pollen viability in *Rhizophora mangle* and *Rhizophora stylosa* hybrids. *South Pacific Journal of Natural Science* 20: 1 – 3.

Valiela I, Bowen JL, York JK. Mangrove forests: One of the World's threatened major tropical environments. BioScience. 2001; 51 (10): 807-815.

Vedel V, Behling H, Cohen M, Lara R. Holocene mangrove dynamics and sea-level changes in northern Brazil, inferences from the Taperebal core in northeastern Pará State. Vegetation History and Archaeobotany. 2006; 15 : 115 – 123.

Yahya AF, Hyun JO, Lee JH, Kim YY *et al.* Genetic variation and population genetic structure of *Rhizophora apiculata* (Rhizophoraceae) in the greater Sunda Islands, Indonesia using microsatellite markers. J. Plant Res. 2014; 127:287–297.

Zachos FE, Habel JC. Biodiversity Hotspots - Distribution and Protection of Conservation Priority Areas (FE Zachos, JC Habel, Eds,). Springer, New York; 2011.

Zhou R, Shi S, Wu CI. Molecular criteria for determining new hybridspecies—an application to the Sonneratia hybrids. Mol. Phylogenet. Evol. 2005; 35,595–601.

Zhou R, Gong X, Boufford D, Wu C-I, Shi S. Testing a hypothesis of unidirectional hybridization in plants: observations on Sonneratia, Bruguiera and Ligularia. BMC Evolutionary Biology. 2008; 8, 149.

Wang L, Mu M, Li X, Lin P, Wang W. Differentiation between true mangroves and mangrove associates based on leaf traits and salt contents. Journal of Plant Ecology. 2010; rtq008.

ANEXO I

The Lichenologist **44**(5): 649–660 (2012)
doi:10.1017/S0024282912000217

Diversity of *Ramalina sinensis* and its photobiont in local populations

P. M. FRANCISCO DE OLIVEIRA, B. TIMSINA and M. D. PIERCEY-NORMORE

Abstract: *Ramalina sinensis* is a widespread lichen in the Northern Hemisphere with sparse local populations, and its potential to adapt to changing environmental conditions is unknown. The objectives of this study were to determine whether geographical distance reflects fungal phylogenetic patterns, and to infer algal identity and its pattern of geographical distribution. Twenty-three samples of *R. sinensis* were collected from three geographical regions in Manitoba. The internal transcribed spacer of ribosomal DNA (ITS rDNA) was sequenced from each of the algal and fungal partners, and phylogenetic analyses were performed. Algal haplotypes were estimated and placed on a map of the geographical regions. Although the fungal partner showed no geographical segregation within Manitoba, the divergence of three samples added to the phylogeny from GenBank suggested that a pattern may be evident if broader geographical distances were examined. The photobiont sequence was determined to be most similar to that of *Trebouxia impressa* and *T. potteri*, two widely distributed algal species. The algal partner showed no geographical structure with sequence polymorphism or haplotype analyses. The abundance of sexual reproduction might explain widespread occurrence and the absence of geographical segregation of the fungus. This study suggests that the diversity in each of the symbionts of *R. sinensis* should not be a limiting factor for adaptation.

Key words: adaptation, lichen, sexual reproduction

Accepted for publication 6 *March* 2012

Introduction

Phylogenetic studies have been used to examine algal selection by lichen-forming fungi within and among populations (Kroken & Taylor 2000; Helms *et al.* 2001; Piercey-Normore 2006), investigating photosymbiodemes (Goffinet & Bayer 1997), and algal adaptation to environmental conditions in wide geographical ranges of lichen distribution (Blaha *et al.* 2006). Co-dispersal or the coupling of algal and fungal haplotypes has been reported (Wagner *et al.* 2005) and examined further by comparing algal and fungal partners using phylogenetic analysis (Piercey-Normore & DePriest 2001; Ohmura *et al.* 2006; Yahr *et al.* 2006; Piercey-Normore 2006). The co-dispersal or coupling of symbiont partners suggests dispersal through vegetative propagules that contain both partners. On the other hand, after sexual reproduction and spore dispersal of the fungal partner, the fungal ascospore must germinate within the vicinity of a compatible algal partner. The alga may be from a free-living cell (Mukhtar *et al.* 1994; Friedl & Büdel 2008) or cells that are already established in a lichen thallus or vegetative propagule (Ohmura *et al.* 2006; Yahr *et al.* 2006). Airborne cells of chlorophytes, including species of *Trebouxia*, have also been reported (Handa *et al.* 2007). The union of an ascospore with an alga after a dispersal event will uncouple the previous fungal-algal association of the parent lichen, resulting in a new combination of algal-fungal genotypes. In the absence of sexual reproduction, the parent genotypes remain coupled within the vegetative propagules resulting in the same combinations of

P. M. Francisco de Oliveira: Center for Molecular Biology and Genetic Engineering (CBMEG), State University of Campinas, Campinas, SP, Brazil.
B. Timsina and M. D. Piercey-Normore (corresponding author): Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada.
Email: pierceyn@cc.umanitoba.ca

genotypes that were present in the parent genotype (Wagner *et al.* 2005), except in *Lepraria* (Nelsen & Gargas 2008).

Most lichen associations studied show a clustering of algal partners with geographical or ecological conditions (Kroken & Taylor 2000; Piercey-Normore 2004; Ohmura *et al.* 2006; Werth & Sork 2010). The selection of algae by the fungal partner may reflect selection from a pool of algae available within a habitat. The taxonomic preference of algae by the fungal partner reflects specificity. Further explanations of selectivity and specificity are available (Honegger 1996). However, some cases of a one-to-one reciprocal specificity have been reported (Summerfield *et al.* 2002; Otálora *et al.* 2010). A single, exclusively lichenized lineage of *Scytonema* photobionts is reported to associate with members of the genus *Stereocaulon* (Lucking *et al.* 2009), suggesting algal selection by the fungal partner. Similarly, the cyanobacterial lineages associated with *Pseudocyphellaria crocata* and *P. neglecta* were specific to the fungal species (Summerfield *et al.* 2002). The photobiont variation in *R. menziesii*, a sexually-reproducing lichen, did not correspond with that of the fungal partner, but it did correspond with the host tree, suggesting ecological specialization of the photobiont (Werth & Sork 2010). Similarly, the algal and fungal combinations for *Cladonia subtenuis* did not correspond with one another, and ecological specialization was also reported for the photobiont (Yahr *et al.* 2006). Habitat variation was used as an explanation for uncoupled genotypic combinations of fungal and algal partners in *Evernia mesomorpha* (Piercey-Normore 2006). On a broad geographical scale, the algae that associate with *Tephromela atra* are segregated according to Mediterranean and temperate climates (Muggia *et al.* 2010). The uncoupling of the symbiont combinations to produce the inferred ecological specializations of these lichens was partially explained by sexual reproduction of the lichen fungus, where each fungal spore may combine with different algal partners at each dispersal event. Diverse symbiont combinations, reflected in diverse allele combinations between alga and fungus, may increase the likelihood of survival of the lichen under changing habitat conditions (Werth & Sork 2010).

Ramalina sinensis Jatta is a widespread species in North America, Europe and Asia (Hermansson & Kudryatseva 1995; Brodo *et al.* 2001; Hedenås *et al.* 2006; Li *et al.* 2007) but uncommon in the Canadian prairies, being restricted to dry sandy habitats. It has been reported to be present but uncommon in the Kuril Islands (Joneson 2003) and to be locally common in Russia (Hermansson & Kudryatseva 1995). It is a broad, fan-shaped *Ramalina* that reproduces sexually by apothecia. Since no vegetative propagules are produced, the fungal partner may associate with different algal partners at each dispersal event. Narrow thalli can be confused with *R. americana*, which overlaps in its distribution extending into southern Manitoba. Since the only known method of reproduction in *R. sinensis* is sexual, we would expect to see mixed combinations of fungal and algal genotypes within and between geographical locations. However, if the algal partner reflects an ecological specialization, we should see clustering of the algal haplotypes within geographical locations.

The general goal of this study was to investigate the extent of algal selection by the lichen-forming fungus, *R. sinensis*, and infer its potential for adaptation to changing habitat conditions. The two objectives were: 1) to examine evolution within *Ramalina sinensis* to determine whether individuals are segregated by geographical location, and 2) to infer the identity and determine the pattern of distribution of the algal partner associated with each fungal partner to investigate ecological specialization.

Materials and Methods

Lichen material

Twenty-three samples of *Ramalina sinensis* and three samples of *R. americana* were collected from three geographical regions in southern Manitoba, Canada (Table 1). The vegetation in Spruce Woods Provincial Park (SWPP) is comprised of poplar, oak, and spruce forests, riparian habitats, mixed grass prairie, forested and open sand dunes. The specimens were collected in open spruce and poplar forests. The Sandilands Provincial

TABLE 1. *List of specimens used in this study, location and date of collection, and GenBank accession numbers for algal and fungal ITS rDNA sequences The same collection number refers to the same host tree and different letters after each collection number refer to different individual thalli*

Specimen	Location of collection, source, and TLC results	Algal Haplotype	Algal ITS rDNA accession number	Fungal ITS rDNA accession number
Evernia mesomorpha	Canada, Manitoba, Sandilands Provincial Park (Piercey-Normore 2006)	–	ns	DQ087986
Ramalina americana	Strain "9" (LaGreca 1999)	–	ns	AF109235
R. americana	Strain "1b" (LaGreca 1999)	–	ns	AF109237
R. americana 4358-A	Canada, Manitoba, Sandilands Provincial Forest. Between St. Labre and Whitemouth Lake. N49°17′21″; W95°45′22″; 2005. TLC: Usnic acid	I	JQ085336	JQ085312
4358-B	Canada, Manitoba, Sandilands Provincial Forest. Between St. Labre and Whitemouth Lake. N49°17′21″; W95°45′22″; 2005. TLC: Usnic acid	III	JQ085335	JQ085305
7328	Canada, Manitoba, Sandilands Provincial Forest. On Hwy 525. N49ff16′20.7″; W95°12′41.6″; 2007. TLC: Usnic acid and unknown (Tr.) (unknown is purple-yellow after H+; Rf = 4.	II	JQ085327	????
R. sinensis	Unknown location. LaGreca & Lumbsch, unpub. (Submitted to GenBank 28 March 2000)	–	ns	AF249905
R. sinensis	China. Hur, unpub. (Submitted to GenBank 31 January 2006)	–	ns	DQ383646
R. sinensis	China. Lim, Hur, & Wang, unpub. (Submitted to GenBank 7 April 2005)	–	ns	DQ001299
R. sinensis 7334-A	Canada, Manitoba, Sandilands Provincial Forest. On Hwy 525. N49°16′20.7″; W95°12′41.6″; 2007.TLC: Usnic acid	–	–	JQ085303
7334-B	Canada, Manitoba, Sandilands Provincial Forest. On Hwy 525. N49°16′20.7″; W95°12′41.6″; 2007. TLC: Usnic acid	XI	JQ085338	JQ085302
7319-A	Canada, Manitoba, Sandilands Provincial Forest. On Hwy 308, cedar-aspen forest. N49°32′45.9″; W95°34′30.5″; 2007. TLC: Usnic acid	–	–	JQ085307
7319-B	Canada, Manitoba, Sandilands Provincial Forest. On Hwy 308, cedar-aspen forest. N49°32′45.9″; W95°34′30.5″; 2007. TLC: Usnic acid	I	JQ085324	JQ085301

TABLE 1. *Continued*

Specimen	Location of collection, source, and TLC results	Algal Haplotype	Algal ITS rDNA accession number	Fungal ITS rDNA accession number
9477-A	Canada, Manitoba, Spruce Woods Provincial Park. N49°39′39.1″; W99°16′6.5″; 2009. TLC: Usnic acid	XI	JQ085325	JQ085320
9477-B	Canada, Manitoba, Spruce Woods Provincial Park. N49°39′39.1″; W99°16′6.5″; 2009. TLC: Usnic acid	V	JQ085326	JQ085310
9477-C	Canada, Manitoba, Spruce Woods Provincial Park. N49°39′39.1″; W99°16′6.5″; 2009.TLC: Usnic acid	XI	JQ085337	JQ085300
9477-D	Canada, Manitoba, Spruce Woods Provincial Park. N49°39′39.1″; W99°16′6.5″; 2009. TLC: Usnic acid	X	JQ085329	JQ085308
9477-E	Canada, Manitoba, Spruce Woods Provincial Park. N49°39′39.1″; W99°16′6.5″; 2009. TLC: Usnic acid	V	JQ085330	JQ085299
9477-F	Canada, Manitoba, Spruce Woods Provincial Park. N49°39′39.1″; W99°16′6.5″; 2009. TLC: Usnic acid	VIII	JQ085332	JQ085309
477-G	Canada, Manitoba, Spruce Woods Provincial Park. N49°39′39.1″; W99°16′6.5″; 2009. TLC: Usnic acid	XI	JQ085331	JQ085311
5491-A	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	V	JQ085333	JQ085313
5491-B	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	XI	JQ085334	JQ085306
5491-C	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	XI	JQ085339	JQ085314
5491-D	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	IX	JQ085340	JQ085304
5491-E	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	–	–	JQ085316

TABLE 1. *Continued*

Specimen	Location of collection, source, and TLC results	Algal Haplotype	Algal ITS rDNA accession number	Fungal ITS rDNA accession number
5491-F	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	–	–	JQ085323
5491-G	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	XI	JQ085341	JQ085315
5491-H	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	XI	JQ085342	JQ085318
5491-I	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	XI	JQ085343	JQ085317
5491-J	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006.TLC: Usnic acid and fluorescent yellow in long wave; not visible after H+; Rf = 6.	IV	JQ085328	JQ085319
5491-K	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid	XII	JQ085345	JQ085322
5491-L	Canada, Manitoba, Whiteshell Provincial Park. On Hwy 307, on Ash. N50°08′43.8″; W95°48′24.3″; 2006. TLC: Usnic acid and fluorescent yellow in long wave; not visible after H+; Rf = 6.	VII	JQ085344	JQ085321
Trebouxia impressa (UTEX 892)	USA. Culture isolated from *Physcia stellaris* by V. Ahmadjian. Sequence from Piercey-Normore & DePriest 2001).	II	AF345891	ns
Trebouxia potteri (UTEX 900)	USA. Culture isolated from *Lecanora ubina* by V. Ahmadjian. Sequence from Kroken & Taylor (2000).	VI	AF242469	ns

Forest (SPF) is characterized by underlying sandy soil and open jack pine forest, alternating with low-lying black spruce wetlands. The Whiteshell Provincial Park (WPP) is characterized by open jack pine forest on exposed Precambrian shield and low-lying black spruce bogs, with large numbers of lakes and rivers. The absence of sand and predominance of granite in this area is notable. The thalli of *R. sinensis* and *R. americana* were collected from ash, poplar, oak, spruce, and pincherry shrubs. All vouchers are deposited in the cryptogam division of the Herbarium of the University of Manitoba (WIN). Three sequences of *R. sinensis* and two sequences of *R. americana* were retrieved from GenBank and added to the alignment.

Chemistry and DNA analysis

Thin-layer chromatography (TLC) was performed on Merck Silica coated glass plates (Fisher Scientific, Ottawa, Ontario, Canada), placed in 230 ml of toluene/dioxane/glacial acetic acid (180:45:5) solvent, following procedures described in Culberson (1972) and Orange *et al.* (2001).

Total DNA was extracted from 1–2 cm thallus lobes following the cetylmethylammonium bromide (CTAB) extraction protocol modified from Grube *et al.* (1995). DNA was resuspended in 50 µl warm sterile distilled water and stored at −20°C. The Internal Transcribed Spacer (ITS) of ribosomal DNA (rDNA) was amplified from each of the fungal and algal partners using taxon-specific primers, 1780A (Piercey-Normore & DePriest 2001) and a universal primer ITS4 (White *et al.* 1990) or Al1700F (Helms *et al.* 2001) to amplify the algal ITS rDNA. Fungal primers were ITS1F (Gardes & Bruns 1993) and ITS2 (White *et al.* 1990) for the ITS 1 spacer; and ITS3 (White *et al.* 1990) and LR15 (Vilgalys & Hester 1990) for the ITS2 spacer in the fungal DNA. Amplifications were performed in 20 µl reaction volumes in 1× buffer (200 mM Tris-HCl, 500 mM KCl) with 1·25 units GO Taq (Go Taq® Hot Start polymerase, Promega), 200 µM of each dNTP, 0·5 µM of each primer, and between 10–50 ng of DNA. Amplifications were performed in a thermal cycler (Biometra T-Gradient; Tampa, FL, USA) with the following conditions: initial denaturing at 94°C for 5 min, then denaturing at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min for 35 cycles.

Four replicates of 50 µl reaction volumes of PCR product were pooled for DNA sequencing. The pooled 200 µl PCR product was precipitated by adding 2·5 volumes of 100% ethanol and 0·2 volumes of 5M NaCl and centrifuged at 13 000 rpm for 10 min. The DNA pellet was resuspended in 20 µl sterile dH_2O, the resuspended DNA was gel purified by excising the band from 1·5% agarose gel and purified using the clean up Kit (Wizard® SV Gel and PCR Clean-Up System, Promega) following the manufacturer's instructions. The purified DNA was resuspended in 35 µl of sterile distilled water and stored at −20°C.

Cycle sequencing reaction volumes were 20 µl, containing *c.* 65 ng of purified DNA, 1 µl BigDye V3.1 (Applied Biosystem, Foster City, CA, USA) and the same PCR primers were used for sequencing. Post-reaction clean up followed the manufacturer's instructions for the EDTA and ethanol precipitation. The dried product was resuspended in 20µl formamide, denatured at 95°C for 5 min and immediately placed on ice and loaded into a 96-well plate for sequencing (3130 Genetic Analyser, Applied Biosystems, Foster City, CA, USA).

Data analysis

Nucleotide sequences were compiled in the program Sequencher 4.6 (Gene Codes Corp.) and manually aligned in Se-Al v 1.0 (Rambaut 2001). The aligned sequences were used to estimate algal ITS rDNA haplotypes using TCS 1.21 (Clement *et al.* 2000) with 90% connection limit and gaps recorded as missing data. Aligned ITS rDNA regions in each of the algal and fungal partners were also used to generate algal and fungal phylogenetic trees using the program PAUP 4.0 (Swofford 2003). Phylogenetic determinations were based on Maximum Parsimony (MP) and Bayesian Maximum Likelihood (ML) analyses. The option for MP analyses was tree bisection and reconnection (TBR) branch swapping. Heuristic searches were conducted using 1000 random addition replicates and bootstrap searches of 500 resamplings (Felsenstein 1985). Bootstrap was carried out using the MP option in PAUP and values ≥70 are reported in the phylogenies. Modeltest version 3.7 (Posada & Crandall 1998) was performed to determine the best model to use in MrBayes version 3.1.2 (Huelsenbeck *et al.* 2001; Ronquist & Huelsenbeck 2003). The six parameter GTR model with a gamma-shaped parameter and proportion of invariable sites uniformly distributed, was chosen for both algal and fungal analyses. Bayesian analyses were performed using 2 000 000 generations with random starting trees. Both runs converged on similar likelihood values. For the fungal partner, 750 burnin trees were discarded; 350 burnin trees were discarded for the algal partner. Posterior probability values ≥95 are reported on the phylogenies.

Results

The fungal ITS rDNA alignment contains 879 characters with 68 parsimony informative sites. The fungal phylogeny contains 303 changes and the topology of the tree is in agreement with the Bayesian tree. The algal ITS rDNA alignment contains 503 total characters with 24 parsimony informative sites. The algal phylogeny contains 67 changes and the topology of the tree is in agreement with the Bayesian tree.

Fungal evolutionary relationships

The fungal phylogeny produced two highly supported clades (Fig. 1). Five samples of *R. americana* Hale formed one highly supported clade A with 98% bootstrap support and ≥95% posterior probability. Three samples of *R. americana* were collected in Manitoba and the other two samples were collected in New York and North Carolina. Three samples of *R. sinensis* that were retrieved from GenBank were basal to the second highly supported clade B with 89% bootstrap and ≥95% posterior probability that were all collected in Manitoba in this study within 400 km. The three samples from GenBank were collected in China at an unknown location. The high support but low levels of divergence among samples of *R. sinensis* from

Manitoba suggests they have a recent common ancestor but the three samples of *R. sinensis* outside the clade are more divergent in nucleotide polymorphism and in geographical origin.

Chemical analysis of *R. sinensis* showed that two samples of *R. sinensis* (5491J and 5491L) produced usnic acid in addition to a trace of an unknown compound that appeared fluorescent yellow in long wave ultraviolet light. The unknown compound was not visible after spraying with sulphuric acid and heating, and the Rf class was 6 in the solvent system outlined in the methods. One sample of *R. americana* (7328) produced usnic acid and a trace of an unknown compound which appeared as purple-yellow after spraying with sulphuric acid and heating, and the Rf class was 4 with the same solvent system. All other samples of *R. sinensis* and *R. americana* contained usnic acid only (Table 1).

Algal identity and distribution

Based on the ITS rDNA sequence comparison in the phylogenetic analysis, the algal partners associated with *R. sinensis* and *R. americana* are most similar to those of *Trebouxia impressa* and *T. potteri*, but insufficient resolution in the ITS rDNA region prevents separation of the two algal species. The algal phylogeny shows two highly supported clades with bootstrap analysis and five highly supported clades with posterior probability (Fig. 2). None of the clades segregate with host tree, collection location, or fungal species. The algae associated with *R. americana* are placed in two different clades and one clade also contains an alga from *R. sinensis* (Fig. 2).

Twelve algal haplotypes are reported from 23 samples (Table 1, Fig. 3) and they do not correspond with geographical location (Fig. 3). The most common haplotype XI comprises nine samples, and the least common, III, IV, VI, VII, VIII, IX, X, and XII are each represented by a single member. The remaining three haplotypes (I, II, and V) are represented by two or three members (Fig. 3).

Discussion

Both partners of *R. sinensis* show polymorphism in the phylogenetic trees (Figs 1 & 2). The absence of a visual relationship between fungal sequence variation and geographical location of *R. sinensis* within Manitoba (Fig. 1) is consistent with that reported for *R. menziesii* (Werth & Sork 2008) and *Evernia mesomorpha* (Piercey-Normore 2006). Low resolution of the Manitoba samples might be improved with a more variable gene or more distantly collected specimens. However, the phylogenetic position of three more broadly distributed individuals suggested that there may be a relationship between geographical distance and fungal diversity for individuals in larger geographical regions. Therefore, *R. sinensis* may undergo widespread dispersal by sexually produced ascospores. The presence of apothecia on all specimens of *R. sinensis* supports this interpretation. Local dispersal is also possible after gut passage of fragments or spores by mites (Boch *et al.* 2011) or snails (Meier *et al.* 2002).

The fungal phylogeny does not disagree with that from Stocker-Worgotter *et al.* (2004) or LaGreca (1999). *Ramalina americana* forms a well-supported clade in this study even though two specimens were collected from distant locations. It was included in this study because of its morphological and chemical similarity with specimens of *R. sinensis* and is present in the study areas. *Ramalina americana* produces few secondary metabolites, often only usnic acid (LaGreca 1999). In this study, *R. sinensis* produced usnic acid alone and *R. americana* produced usnic acid with traces of several unknown compounds (Table 1). The production of additional compounds in *R. americana* was interpreted as a display of classical chemical variation (LaGreca 1999). The presence of usnic acid alone differentiates these species from other closely related species such as *R. culbersoniorum*, which has multiple compounds present (LaGreca 1999). Although the morphological characters of *R. americana* may overlap

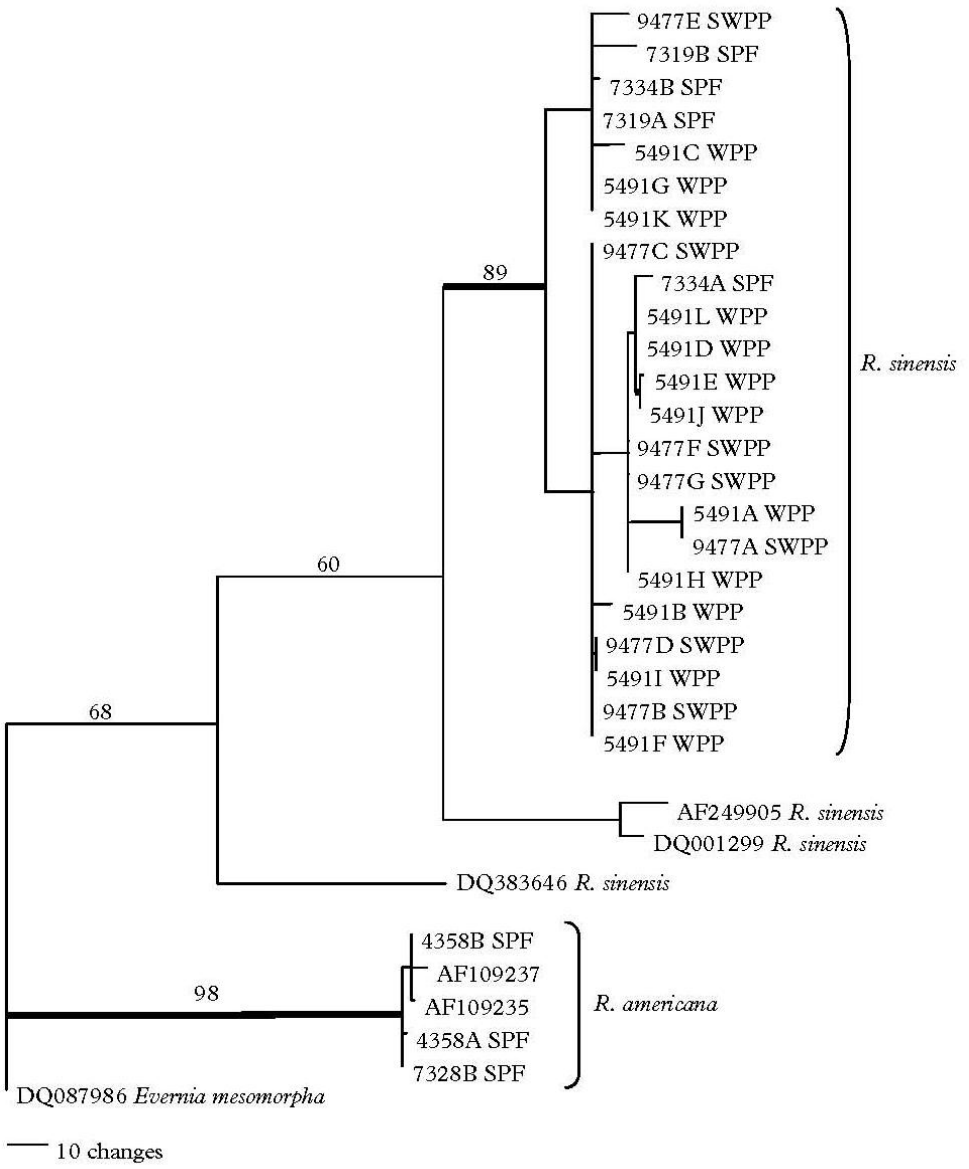

FIG. 1. One of 763 most parsimonious trees based on the nuclear ITS rDNA showing an evolutionary hypothesis of *Ramalina sinensis* collected from three localities in Manitoba with reference to three additional specimens retrieved from GenBank and five sequences amplified from *R. americana*. *Evernia mesomorpha* is assigned as the outgroup. Bootstrap proportions ≥70% are shown above the branches, and thickened branches represent posterior probabilities ≥95%. CI 0·9109 and RI 0·8393. The collection number refers to specimens that were collected from the same tree or neighbouring trees. The letter immediately following the number refers to different specimens.

with those of *R. sinensis*, making the two species difficult to separate, they were clearly distinguished by the ITS rDNA sequences in this study.

Photobiont identity: *T. potteri* and *T. impressa*

The photobiont ITS sequences of *R. sinensis* and *R. americana* are most similar to those of *Trebouxia impressa* and *T. potteri*

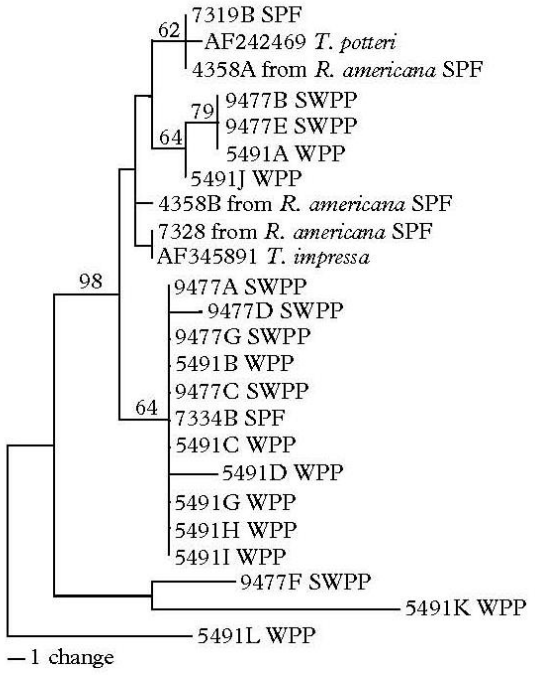

FIG. 2. One of 231 most parsimonious trees based on the nuclear ITS rDNA showing an evolutionary hypothesis of the algal partner associated with *Ramalina sinensis* and *R. americana* collected from three localities in Manitoba. Two sequences have been retrieved from Genbank that represent known species of *Trebouxia* for reference. The tree is unrooted. Bootstrap proportions ≥70% are shown above the branches, and thickened branches represent posterior probabilities ≥95%. CI 0·8889 and RI 0·8939.

(Fig. 2). *Trebouxia impressa* and *T. potteri* are sister species and were shown to form a highly-supported clade in Hauck *et al.* (2007) and Guzow-Krzeminska (2006), based on nucleotide sequence data. Morphological evidence placed them in two different groups (Takeshita 2001) based on types of life cycles. Takeshita (2001) showed that both species produced zoospores, aplanospores, and autospores, but in *T. impressa* the three types of reproductive cells were compartmentalized within 'sporangia complexes' surrounded by mother cell walls. *Trebouxia impressa* is known to associate with a large number of lichen fungi in the genera *Parmelia*, *Melanelia* (Kroken & Taylor 2000), and *Physcia* (Beck *et al.* 2002). Other species of *Ramalina* have been shown to associate with divergent species of *Trebouxia* where those from Brazil are more similar to *T. arboricola*, *T. galapagensis*, and *T. higginsiae* (Cordeiro *et al.* 2005). The photobiont associated with *R. menziesii* from California was determined to be *T. decolorans* (Werth & Sork 2010).

Photobiont diversity

The algae associated with *R. sinensis* did not cluster with geographical region in this study, suggesting that the photobiont is widely dispersed. The photobiont may disperse by symbiotic propagules from another lichen fungus (Ohmura *et al.* 2006) or as a free-living alga (Mukhtar *et al.* 1994; Friedl & Budel 2008), since *R. sinensis* does not produce any vegetative propagules. Knowledge of photobiont selection by the fungal partner in different geographical locations and habitats has been growing (Kroken & Taylor 2000; Piercey-Normore 2004; Yahr *et al.* 2006; Nelsen & Gargas 2008; Muggia *et al.* 2010; Peksa & Skaloud 2011), but little is known about the photobiont diversity within species of *Ramalina*. Werth & Sork (2010) showed that 72 samples of *R. menziesii* from California associated with the same species of alga, *T. decolorans*. It is not surprising that most *R. sinensis* individuals from a larger geographical region in Manitoba also associated with a single or closely related algal species. Additional diversity may be present as suggested by the more divergent samples, 9477F, 5491K, and 5491L (Fig. 2). The photobiont of *Tephromela atra* (Muggia *et al.* 2010) was shown to segregate with climatic differences between Mediterranean and temperate climates. A broader geographical investigation of *R. sinensis* may reveal a similar pattern.

This study covered sample sites approximately 400 km apart, a much larger geographical region than the studies with *R. menziesii* (2 km; Werth & Sork 2010) and *Evernia mesomorpha* (Piercey-Normore 2006), but less than the study of *T. atra* (Muggia *et al.* 2010). The study on *R. menziesii* showed more sequence polymorphism in the algal ITS than was present in this study. Photobionts associated with *R. menziesii* had 104 polymorphic

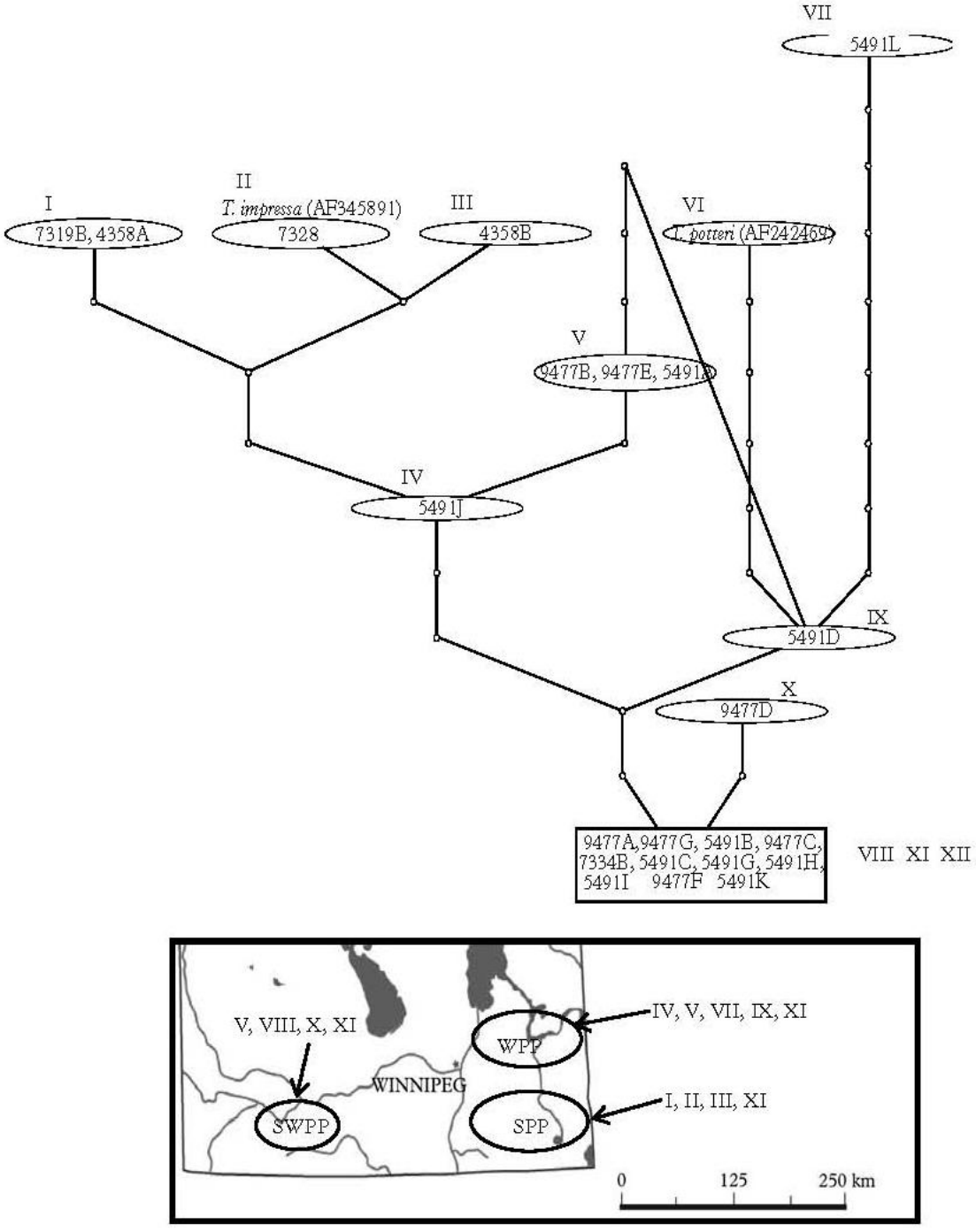

FIG. 3. Map of southern portion of Manitoba, Canada, showing location of collection sites, distance between sites with scale bar, and the algal haplotypes in roman numerals for each collection site. The insert above the map is a haplotype network of the 23 lichen samples, showing the similarity among algal haplotypes.

sites in 72 samples reported (1·4 polymorphisms per site in 2 km) (Werth & Sork 2010). The photobionts associated with *R. sinensis* had 63 polymorphic sites in 26 samples (2·4 polymorphisms per site in 400 km) in the same gene (ITS rDNA) (Fig. 2). In addition, the number of algal haplotypes reported for *R. menziesii* was 32 haplotypes in a total of 72 samples (44%) (Werth & Sork 2010), and for *R. sinensis* it was 12 haplotypes in a total of 23 samples (48%). The level of ITS variation, for polymorphic nucleotide sites and for haplotypes, is lower in the *R. menziesii* photobiont than in the *R. sinensis* photobiont, but the geographical distances and sample sizes were very different from one another, making comparison of studies difficult.

In conclusion, this study suggests that the diversity in each of the symbionts of *R. sinensis* would not be a limiting factor for adaptation to changing environmental conditions, despite the absence of vegetative propagules. The inferred photobiont identity was limited to two species but questions were raised concerning the geographical distribution of algal haptotypes. Sexual reproduction by the fungal partner, inferred from abundance of apothecia, may account for its widespread distribution. However, the absence of vegetative propagules of the lichen may account for small local population sizes and the apparent rarity of the species in local areas of Manitoba.

The authors thank Anete Pereira de Souza for the opportunity provided for the first author to visit Canada, and Manitoba Conservation for collection permits WPB20417 and WPB20429. Financial support was received from Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) Brazil, and the Natural Sciences and Engineering Research Council of Canada (NSERC).

References

Beck, A., Kasalicky, T. & Rambold, G. (2002) Mycophotobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida*. *New Phytologist* **153:** 317–326.

Boch, S., Prati, D., Werth, S., Rüetschi, J. & Fischer, M. (2011) Lichen endozoochory by snails. *PLoS ONE* **6:** e18770.

Brodo, I. M., Duran Sharnoff, S. & Sharnoff, S. (2001) *Lichens of North America*. New Haven & London: Yale University Press.

Clement, M., Posada, D. & Crandall, K. A. (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9:** 1657–1660.

Cordeiro, L. M. C., Reis, R. A., Cruz, L. M., Stocker-Wörgötter, E., Grube, M. & Iacomini, M. (2005) Molecular studies of photobionts of selected lichens from coastal vegetation of Brazil. *FEMS Microbiology Ecology* **54:** 381–390.

Culberson, C. (1972) Improved conditions and new data for the identification of lichen products by a standardized thin layer chromatographic method. *Journal of Chromatography* 72: 113–125.

Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39:** 783–791.

Friedl, T. & Büdel, B. (2008) Photobionts. In *Lichen Biology. Second Edition.* (T. H. Nash III, ed.): 7–26. Cambridge: Cambridge University Press.

Gardes, M. & Bruns, T. D. (1993) ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2:** 113–118.

Goffinet, B. & Bayer, R. J. (1997) Characterization of mycobionts of photomorph pairs in the Peltigeraceae (lichenized Ascomycetes) based on internal transcribed spacer sequences of the nucler ribosomal DNA. *Fungal Genetics and Biology* **21:** 228–237.

Grube, M., DePriest, P. T., Gargas, A. & Hafellner, J. (1995) DNA isolation from lichen ascomata. *Mycological Research* **99:** 1321–1324.

Guzow-Krzeminska, B. (2006) Photobiont flexibility in the lichen *Protoparmeliopsis muralis* as revealed by ITS rDNA analyses. *Lichenologist* **38:** 469–476.

Handa, S., Ohmura, Y., Nakano, T. & Nakahara-Tsubota, M. (2007) Airbourne green microalgae (Chlorophyta) in snowfall. *Hikobia* **15:** 109–120.

Hauck, M., Helms, G. & Friedl, T. (2007) Photobiont selectivity in the lichens *Hypogymnia physodes* and *Lecanora conizeaoides*. *Lichenologist* **39:** 195–204.

Hedenås, H., Lundin, K. & Ericson, L. (2006) Interaction between a lichen and a fungal parasite in a successional community: implications for conservation. *Journal of Vegetation Science* **17:** 207–216.

Helms, G., Friedl, T., Rambold, G. & Mayrhofer, H. (2001) Identification of photobionts from the lichen family *Physciaceae* using algal-specific ITS rDNA sequencing. *Lichenologist* **33:** 73–86.

Hermansson, J. & Kudryatseva, D. (1995) Notes on the lichens of the Pechoro-Ilych Zapovednik, Komi Republic, Russia. *Graphis Scripta* **7:** 67–78.

Honegger, R. (1996) Morphogenesis. In *Lichen Biology* (T. H. Nash III, ed.): 65–87. Cambridge: Cambridge University Press.

Huelsenbeck, J. P., Ronquist, F., Nielsen, R. & Bollback, J. P. (2001) Bayesian inference of phylogeny and its impact on evolutionary biology. *Science* **294:** 2310–2314.

Joneson, S. (2003) *Studies in* Ramalina *(Ascomycotina, Lecanorales) with emphasis on the* R. almquistii *species complex*. M.Sc. thesis, University of Washington.

Kroken, S. & Taylor, J. W. (2000) Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. *Bryologist* **103:** 645–660.

LaGreca, S. (1999) A phylogenetic evaluation of the *Ramalina americana* chemotype complex (lichenized Ascomycota, Ramalinaceae) based on rDNA ITS sequence data. *Bryologist* **102:** 602–618.

Li, W. C., Guo, S. Y. & Guo, L. D. (2007) Endophytic fungi VII. Three new records from lichens in China. *Mycosystema* **26:** 32–35.

Lücking, R., Lawrey, J. D., Sikaroodi, M., Gillevet, P. M., Chaves, J. L., Sipman, H. J. M. & Bungartz, F. (2009) Do lichens domesticate photobionts like farmers domesticate crops? Evidence from a previously unrecognized lineage of filamentous cyanobacteria. *American Journal of Botany* **96:** 1409–1418.

Meier, F. A., Scherrer, S. & Honegger, R. (2002) Faecal pellets of lichenivorous mites contain viable cells of the lichen-forming ascomycete *Xanthoria parietina* and its green algal photobiont, *Trebouxia arboricola*. *Biological Journal of the Linnean Society* **76:** 259–268.

Muggia, L., Zellnig, G., Rabensteiner, J. & Grube, M. (2010) Morphological and phylogenetic study of algal partners associated with the lichen-forming fungus *Tephromela atra* from the Mediterranean region. *Symbiosis* **51:** 149–160.

Mukhtar, A., Garty, J. & Galun, M. (1994) Does the lichen alga *Trebouxia* occur free-living in nature: further immunological evidence. *Symbiosis* **17:** 247–253.

Nelsen, M. P. & Gargas, A. (2008) Dissociation and horizontal transmission of codispersing lichen symbionts in the genus *Lepraria* (Lecanorales: Stereocaulaceae). *New Phytologist* **177:** 264–275.

Ohmura, Y., Kawachi, M., Kasai, F. & Watanabe, M. (2006) Genetic combinations of symbionts in a vegetatively reproducing lichen, *Parmotrema tinctorum*, based on ITS rDNA sequences. *Bryologist* **109:** 43–59.

Orange, A., James, P. W. & White, F. J. (2001) *Microchemical Methods for the Identification of Lichens*. London: British Lichen Society.

Otálora, M. A. G., Martínez, I., O'Brien, H., Molina, M. C., Aragón, G. & Lutzoni, F. (2010) Multiple origins of high reciprocal symbiotic specificity at an intercontinental spatial scale among gelatinous lichens (Collemataceae, Lecanoromycetes). *Molecular Phylogenetics and Evolution*. **56:** 1089–1095.

Peksa, O. J. & Skaloud, P. (2011) Do photobionts influence the ecology of lichens? A case study of environmental preferences in symbiotic green alga *Asterochloris* (Trebouxiophyceae). *Molecular Ecology* **20:** 3936–3948.

Piercey-Normore, M. D. (2004) Selection of algal genotypes by three species of lichen fungi in the genus *Cladonia*. *Canadian Journal of Botany* **82:** 947–961.

Piercey-Normore, M. D. (2006) The lichen-forming ascomycete *Evernia mesomorpha* associates with multiple genotypes of *Trebouxia jamesii*. *New Phytologist* **169:** 331–344.

Piercey-Normore, M. D. & DePriest, P. T. (2001) Algal switching among lichen symbioses. *American Journal of Botany* **88:** 1490–1498.

Posada, D. & Crandall, K. A. (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

Rambaut, A. (2001) *Se-Al: Sequence Alignment Editor V2.0*. Oxford: University of Oxford.

Ronquist, F. & Huelsenbeck, J. P. (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19:** 1572–1574.

Stocker-Worgotter, E., Elix, J. A. & Grube, M. (2004) Secondary chemistry of lichen-forming fungi: chemosyndromic variation and DNA analyses of cultures and chemotypes in the *Ramalina farinacea* complex. *Bryologist* **107:** 152–162.

Summerfield, T. C., Galloway, D. J. & Eaton-Rye, J. J. (2002) Species of cyanolichens from *Pseudocyphellaria* with indistinguishable ITS sequences have different photobionts. *New Phytologist* **155:** 121–129.

Swofford, D. L. (2003) *PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods)*, version 4.0. Sunderland, Massachusetts: Sinauer Associates.

Takeshita, S. (2001) A taxonomic revision of the genus *Trebouxia* (Trebouxiophyceae, Chlorophyta). *Hikobia* **13:** 425–455.

Vilgalys, R. & Hester, M. (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172:** 4238–4246.

Wagner, H. H., Holderegger, R., Werth, S., Gugerli, F., Hoebee, S. E. & Scheidegger, C. (2005) Variogram analysis of the spatial genetic structure of continuous populations using multilocus microsatellite data. *Genetics* **169:** 1739–1752.

Werth, S. & Sork, V. L. (2008) Local genetic structure in a North American epiphytic lichen, *Ramalina menziesii* (Ramalinaceae). *American Journal of Botany* **95:** 568–576.

Werth, S. & Sork, V. L. (2010) Identity and genetic structure of the photobiont of the epiphytic lichen *Ramalina menziesii* on three oak species in Southern California. *American Journal of Botany* **97:** 821–830.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications* (M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White, eds.): 315–322. New York: Academic Press.

Yahr, R., Vilgalys, R. & DePriest, P. T. (2006) Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. *New Phytologist* **171:** 847–860.

ANEXO III

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação de Mestrado/tese de Doutorado intitulada " Caracterização de populações naturais de Rhizophora mangle, Rhizophora racemosa e de Rhizophora harrisonii (Rhizophoraceae) de manguezais do litoral brasileiro e análise de zona de hibridação utilizando marcadores microssatélites" :

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

(X) CIBio – Comissão Interna de Biossegurança , projeto No14/2003, Instituição: Instituto de Biologia, UNICAMP;

( ) CEUA – Comissão de Ética no Uso de Animais , projeto No. _____, Instituição: _____.

( ) CEP - Comissão de Ética em Pesquisa, protocolo No. _____, Instituição: _____

* *Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Patricia M. Francisco

Aluno: Patrícia Mara Francisco

Orientador: Anete Pereira de Souza

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Carimbo e assinatura

Profa. Dra. Edi Lúcia Sartorato
Matrícula: 6384-3
CBMEG - UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

Carimbo e assinatura

Cidade Universitária "Zeferino Vaz",
05 de janeiro de 2015.

Projeto CIBio: 14/2003

Identificação:
Doutorado: Patrícia Mara Francisco, CPG-GBM UNICAMP
Projeto: "Caracterização de populações naturais de espécies do gênero *Rhizophora* spp. (Rhizophoraceae) de manguezais do litoral brasileiro e análise de zona de hibridação utilizando marcadores microssatélites"

Parecer:
Projeto aprovado pela CIBio/CBMEG em 03/02/2003 sob número 14/2003 (em andamento)
Coordenador: Profa. Dra. Anete Pereira de Souza

Profa. Dra. Edi Lúcia Sartorato
Presidente da CIBio/CBMEG - UNICAMP

UNIVERSIDADE ESTADUAL DE CAMPINAS - CENTRO DE BIOLOGIA MOLECULAR E ENGENHARIA GENÉTICA
CIDADE UNIVERSITÁRIA "ZEFERINO VAZ" - BARÃO GERALDO, CX.P.: 6010 - CEP 13083-875 - CAMPINAS - SP - BRASIL
FONE: (55-0xx19) 3521.1147, 3521.1130 ou 3521.1131 - FAX: (55-0xx19) 3788.1089