Laís Leite Barreto

Isolamento Reprodutivo e Diversidade Genética em Erythroxylum (ERYTHROXYLACEAE) P. BROWNE

Recife, Agosto de 2018

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Trabalho de tese apresentado ao Programa de Pós-graduação em Botânica da Universidade Federal Rural de Pernambuco, como parte dos requisitos para obtenção do título de Doutor.

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SUMÁRIO

Resumo Geral

A família Erythroxylaceae Kunth é bem representada na flora Brasileira, com 9 seções do gênero distribuidas na Amazônia, Caatinga, Cerrado e Mata Atlântica. A região com maior diversidade é o Nordeste (77), onde o estado com maior riqueza é a Bahia (56). Considerando este cenário nos podemos afirmar que estudos envolvendo populações do gênero *Erythroxylum*, onde os indivíduos apresentam morfologia intermediária, são essenciais para avaliar a intensidade de barreiras reprodutivas entre espécies, especialmente porque a grande maioria delas ocorre em simpatria. Baseado nestas informações usamos como modelo uma área de floresta montana do nordeste do Brasil onde ocorrem três espécies distílicas do *Erythroxylum* (*Erythroxylum citrifolium, E. pauferrense e E. simonis*) que apresentam sobreposição de períodos de floração e semelhanças nas características florais. O isolamento reprodutivo entre tais espécies foi investigado por meio dos estudos de fenologia, biologia reprodutiva e molecular, os quais, respectivamente, indicaram os níveis de sobreposição das épocas de floração, fluxo gênico intra e inter específico, detalhamento dos grupos de polinizadores e estrutura genética populacional. O estudo de campo foi realizado no Parque Estadual Mata do Pau-ferro (6°58'12'S e 35°42'15'W), onde coletamos dados fenológicos de floração, morfometria e biologia floral, polinização, sistema reprodutivo. Para a avaliação da diversidade genética e estrutura populacional, foram realizadas coletas aleatórias de folhas frescas em alguns estados Brasil, onde obtivemos amostras de 26 populações de diferentes espécies. Nenhuma das espécies estudadas apresentou reciprocidade entre morfos florais ou isopletia. Essa variação pode estar ligada ao fato das espécies serem autocompatíveis, devido a uma quebra no mecanismo da heterostilia. Os tratamentos de polinização controlada mostraram que há compatibilidade entre os diferentes morfos e compatibilidade interespecífica entre *E. pauferrense e E. simonis* como doador ou receptor de pólen, provando que as barreiras reprodutivas pré-zigóticas são fracas e favorecem o processo de hibridização entre elas. *Erythroxylum citrifolium*, embora não apresente hercogamia recíproca entre os morfos, também não mostrou compatibilidade entre morfos iguais ou em cruzamentos interespecíficos.A partir de amostras coletadas foram extraídas amostras de Dna para sequenciamento e amplificação, utilizando a técnica de PCR. Além disso foi construída uma biblioteca de primers, através da plataforma Iluminna II, dos quais 2 novos primers foram testados e otimizados, mostrando-se capaz de amplificar-se em diferentes espécies do gênero, contribuindo assim para o melhor entendimento da estrutura populacional de diversas espécies de *Erythroxylum*. Além dos primers desenvolvidos neste estudo, foram testados primers desenvolvidos a partir de espécies Australianas de *Erythroxylum*, dos quais 4 apresentaram

sucesso na amplificação. A partir dos dados obtidos foram construídas matrizes individuais para as regiões ITS e trnL e cada uma foi analisada individualmente usando análise bayesiana e de parcimônia. A filogenia foi utilizada para comparar as relações entre as espécies de *Erythroxylum*, abrangendo a distribuição geográfica do gênero. Uma árvore de Máxima Parcimônia revelou tanto o particionamento geográfico quanto o taxonômico em clados representando espécies da África, Ásia-Pacífico e Novo Mundo (Américas Tropicais). Nossos resultados mostram que: em todas as análises o gênero *Erythroxylum* é parafilético e as seções são também parafiléticas, o que sugere uma relação evolutiva independente e não linear entre as espécies de *Erythroxylum*, as análises identificaram grupos genéticos significativamente diferentes em *Erythroxylum* sugerindo que a atual classificação intragenérica de esse gênero precisa ser revisada.

1. Introdução

Espécies sincronopátricas, que compartilham os mesmos atributos florais tendem a ser visitadas pelos mesmos grupos de polinizadores, resultando em partilha e/ou competição além de, muitas vezes resultar em polinizações interespecíficas (CAMPBELL; MOTTEN 1985; COYNE; ORR, 2004; NADIA *et al*., 2007). Portanto, estudar as barreiras reprodutivas (isolamento reprodutivo) entre espécies que se encontram em tais condições promove o entendimento de processos que norteiam a especiação, já que a convergência dos caracteres florais em espécies sincronopátricas, especialmente do mesmo gênero, pode ser resultado da pressão seletiva exercida pelos polinizadores (SCHEMSKE, 1981) promovendo cruzamentos entre espécies, resultando em hibridização, introgressão (COYNE; ORR, 2004) e, consequentemente especiação.

A especiação é uma das implicações evolutivas da hibridização, pois a combinação de genomas de espécies distintas possibilita ao híbrido a ocupação de um nicho diferente em relação as espécies parentais, favorecendo a origem de novas espécies (RIESEBERG *et al*., 1997; ARNOLD, 2006). A especiação por hibridização pode ser consequência de uma rápida evolução cromossômica, especialmente por meio da poliploidia que regulariza a segregação dissômica e restaura a fertilidade.

A intensidade do isolamento reprodutivo entre espécies pode ser investigada por meio dos estudos de fenologia, biologia reprodutiva e biologia molecular. Dados de fenologia da floração indicam exatamente o nível de sobreposição das épocas de floração e, consequentemente, o nível de sobreposição de polinizadores (VOGEL; MACHADO, 1991). Investigações relacionadas à biologia reprodutiva e ecologia de polinização detalham os grupos de polinizadores partilhados tendo como base a estrutura e o funcionamento da flor, as frequências de visitação e o comportamento dos polinizadores nas flores (MACHADO *et al*., 2010, FRANCO *et al*., 2012). O uso de dados moleculares, tanto de espécies animais como vegetais, tem sido útil na resolução de problemas de delimitação de espécies e para a compreensão do papel das diferentes barreiras de isolamento reprodutivo (LORENZ–LEMKE *et al.*, 2005; KOEHLER-SANTOS *et al.*, 2006; GRAZZIOTIN *et al*., 2006; PELLEGRINO *et al*., 2008; PINHEIRO *et al*., 2010; PALMA-SILVA *et al*., 2011).

Em áreas de floresta montana na região do Brejo Paraibano ocorrem três espécies distílicas do gênero *Erythroxylum* que apresentam sobreposição de períodos de floração e fortes semelhanças nas características florais. Uma das espécies, *E. pauferrense*, é considerada rara e endêmica dessa região, da qual provém o espécime tipo (LOIOLA *et al*., 2007; PLOWMAN, 1987). A sincronopatria e o compartilhamento dos atributos florais entre

as três espécies, a distribuição extremamente restrita e o registro de caracteres morfológicos pouco definidos sugerem que ocorra fluxo gênico entre elas, resultante do compartilhamento de polinizadores. Baseando-se nesses indícios, esta tese se propôs a investigar os mecanismos de isolamento reprodutivo das espécies sincronopátricas do gênero *Erythroxylum*, a fim de responder os seguintes questionamentos: a) Qual o nível de sobreposição dos períodos de floração e de compartilhamento de polinizadores entre as espécies? b) Existe formação de frutos por polinização interespecífica c) Há isolamento reprodutivo entre as espécies? d) Há variabilidade genética intra e inter-populacional?

2. Revisão bibliográfica

2.1. Brarreiras reprodutivas e hibridização em espécies vegetais

A interação com os polinizadores proporciona as plantas, uma maior eficiência na polinização cruzada, possibilitando novas combinações genéticas e potencializando o sucesso reprodutivo (BARRETT, 2010). Essas interações promovidas pela polinização biótica, podem acarretar em adaptações florais a diferentes sistemas de polinização e elevado fluxo de pólen, influencindo as taxas de especiação e extinção (RICKLEFS; RENNER, 1994). Vários autores têm enfatizado que a eficiente transferência de pólen a longa distância por animais garante a troca de pólen entre populações esparsas e reduz a probabilidade de extinção em tais populações (por exemplo, REGAL, 1977).

Em populações simpátricas com fluxo gênico dentro e entre espécies dependentes da qualidade e da quantidade de fluxo de pólen, os animais funcionam como vetores e o seu comportamento desempenha um papel crucial no fluxo gênico através principalmente do fluxo de pólen, mediado pela coleta e deposição do mesmo no corpo dos polinizadores, além disso eles podem sofrer influência de fatores como: territorialidade, especificidade ao recurso, disponibilidade de recurso, sendo estes fatores determinantes no processo de dispersão do pólen (CAMPBELL, 1991; LEVIN; ANDERSON, 1970; CAMPBELL, 1985; WESSELINGH; ARNOLD, 2004; HIGA; SILVA, 2016).

O grau em que uma população pode ser delimitada de outras, depende da intensidade do fluxo gênico entre elas (FUTUYMA, 1992). O surgimento de barreiras de isolamento reprodutivo entre espécies é primordialmente importante tanto para a especiação de plantas quanto para animais (SOBEL *et al*., 2010), onde os mecanismos de isolamento reprodutivo possuem um papel muito importante na evolução (RIESEBERG; CARNEY, 1998; WIDMER *et al*., 2009; SOBEL *et al*., 2010). O isolamento reprodutivo na maior parte das plantas é consequência de um grande número de barreiras, classificadas em pre-zigóticas e pós-zigóticas" (RIESERBERG; WILLIS, 2007). As pré-zigóticas incluem fatores ecológicos que

atuam antes da formação do zigoto, tais como diferenças em épocas de floração, sistemas genéticos de incompatibilidade, competição gamética e modificações na estrutura floral que alteram o comportamento do polinizador (JUDD *et al*. 2009). As pós-zigóticas ocorrem após a formação do zigoto e incluem a inviabilidade e a esterilidade e/ou a redução da performance reprodutiva dos híbridos (LEVIN, 1971). Muitos estudos examinaram essas barreiras e as suas contribuições (CHARI; WILSON, 2001; HUSBAND; SABARA, 2003; RAMSEY *et al*., 2003, KAY, 2006, NOSIL *et al*., 2006, MARTIN; WILLIS, 2007), revelando que barreiras pré-zigóticas tendem a contribuir mais para reduzir a hibridização.

A hibridização é um fenômeno comum em plantas do mundo inteiro, amplamente difundido em populações naturais (KOROPACHINSKII; MILUTIN, 2006; MAHÉ *et al*., 2007; MORENO *et al*., 2015). Híbridos ocorrem em 40% das famílias e 16% de gêneros, com uma frequência global de 0,09 híbridos por espécies não híbridas (WHITNEY *et al*., 2010). A hibridização desempenha um papel fundamental na evolução das plantas, possibilitando um aumento da variação genética como consequência de novas combinações genômicas, favorecendo a diversificação vegetal e especiação (ARNOLD, 1992, RIESEBERG, 1998, SOLTIS; SOLTIS, 2009). O processo de hibridação, além de facilitar o fluxo gênico entre espécies previamente isoladas, pode dar origem a novas linhagens de espécies (RIESEBERG, 1997) por meio de radiação adaptativa (ELLSTRAND; ELAM, 1993; WHITHAM *et al*., 1994; RIESEBERG, 1997; MILNE; ABBOTT, 2008).

A maioria dos trabalhos sobre as barreiras reprodutivas concentrou-se em isolar mecanismos que limitam a formação de híbridos ou reduzem sua aptidão intrínseca (ARNOLD, 2000; BURKE; ARNOLD, 2001; MILNE *et al*., 2003). O estudo da hibridação natural tem sido altamente favorecido pelo desenvolvimento de métodos moleculares, pois combinando as duas seqüências de parentais herdados (o DNA nuclear e DNA do cloroplasto) através de analises Bayesianas ou de Parsimonia por exemplo, é possível distinguir os parentais paternos e maternos dos híbridos em angiospermas (KING *et al*., 2001; BAUMEL *et al*., 2002; ZHANG *et al*., 2007ª; ZHA *et al*., 2008, 2010).

Estes mecanismos de isolamento reprodutivo nem sempre são absolutos (WU, 2001). A permeabilidade das barreiras pré- e pós-zigóticas são altamente variáveis entre as espécies, e o cruzamento interespecífico é comum, o que gera a ocorrência de hibridização (COYNE; ORR, 2004). Algumas das conseqüências potenciais da hibridização são: reforço de mecanismos de isolamento reprodutivo, que podem englobar desde mudanças no período de floração para evitar a sobreposição até deslocamento de caracteres morfológicos; formação de um complexo híbrido, e quando estes não são estéreis, introgressão (incorporação permanente de genes de uma espécie em outra espécie), criação de diversidade genética por meio do

processo de especiação e adaptação a ambientes que propiciem seu desenvolvimento (LEVIN, 1993).

A hibridização entre espécies simpátricas vem sendo observada principalmente em espécies cujas barreiras reprodutivas são fracas (MEDAN, *et al*., 2012). Muitos trabalhos revelam padrões de hibridização variáveis entre espécies congêneres que ocorrem em áreas de simpatria, como os relatados em *Populus* (LEXER *et al*., 2005, VAN LOO *et al*., 2008), *Quercus* spp. (PETIT *et al*., 2003), *Helianthus* spp. (YATABE *et al*., 2007) e *Mimulus* spp. (MARTIN; WILLIS, 2007). No entanto, Mallet (2005) sugere que a formação e o estabelecimento de híbridos muitas vezes são facilitados por alterações ambientais, principalmente pela antropização.

O uso de dados moleculares, tanto de espécies animais como vegetais, tem sido útil na resolução de problemas de delimitação de espécies e para a compreensão do papel das diferentes barreiras de isolamento reprodutivo (LORENZ–LEMKE *et al*., 2005; KOEHLER-SANTOS *et al*. 2006; GRAZZIOTIN *et al*., 2006, PELLEGRINO *et al*., 2008; PINHEIRO *et al*., 2010; PALMA-SILVA *et al*., 2011). Em Bromeliaceae, o uso de marcadores do genoma nuclear e do cloroplasto possibilitaram a detecão de hibridização e introgressão entre as espécies *Pitcairnia albifos* e *P. staminea* cujas barreiras reprodutivas são fortes, apesar de permeáveis ao fluxo gênico (PALMA-SILVA *et al*., 2011). Neste caso, a integridade das espécies é mantida pela ação simultânea de barreiras pré-zigóticas, incluindo períodos de floração, incompatibilidade e sistema reprodutivo diferenciado (autofertilização *versus* fertilização cruzada) (PALMA-SILVA *et al*., 2011).

Recentemente, interações entre espécies invasoras e nativas tornaram-se outra importante área de pesquisa de hibridização, já que algumas espécies introduzidas parecem promover o deslocamento de espécies nativas (LIU *et al*., 2007; KANBE *et al*., 2008). Neste contexto, o estudo do processo de hibridização permite detectar eventos de fluxo gênico, podendo discriminar padrões genéticos causados por eventos de fluxo gênico atual ou ancestral, já que tais informações são essenciais para distinguir processos populacionais, tais como o isolamento reprodutivo, colonização e expansão populacional e restrição espacial do fluxo gênico (SCHAAL *et al*., 1998).

2.1. Marcadores moleculares e seu uso na genética de populações

Marcador genético é toda e qualquer característica fenotípica ou qualquer segmento de Dna que sejam herdadas de parentais e permitam a análise de similaridade e/ou diversidade genética entre indivíduos e estes são amplamente utilizados nos estudos de estenção e

distribuição da variação entre espécies, como também para elucidar questões taxonômicas e evolutivas (FERREIRA; GRATTAPAGLIA, 1998).

Diversas técnicas surgiram com o advento da PCR, tais como o AFLP (*Amplified Fragment Length Polymorphism*), os ISSRs (*Inter Simple Sequence Repeat*), os RAPDs (*Randomly Ampliflied Polymorphic DNA*), os microssatélites (SSR *Simple Sequence Repeat*) e os SNPs (*Single Nucleotide Polymorphism*), todas variando quanto aos custos, material incial necessário e reprodutibilidade (ALLENDORF *et al*., 2013). Além disso, os diversos marcadores moleculares possuem diferentes taxas de substituição/evolução e, por isso, cada um é mais apropriado para responder um determinado tipo de questão (PETIT *et al*., 2005; SOLÉ-CAVA; CUNHA, 2012; SCOTTI-SAINTAGNE *et al*., 2013). Por exemplo, para análises a nível populacional, marcadores de evolução rápida são mais indicados, como mtDNA para animais e os SSR para animais e plantas (SUNNUCKS, 2000; ZALAPA *et al*., 2012). Os marcadores também variam de acordo com a quantidade de polimorfismo (e consequentemente de informação) que geram.

Em geral, isoenzimas são menos variáveis que marcadores do tipo RAPD ou RFLP, os quais por sua vez são menos variáveis que os micro e minissatélites (OUBORG *et al*., 1999). Dentre os marcadores codominantes, destacam-se os marcadores do tipo SSR, os quais são baseados em PCR e apresentam altos níveis de polimorfismo intraespecífico, além de possuírem uma distribuição aleatória no genoma (FERREIRA; GRATTAPAGLIA, 1996; ZANE *et al*., 2002; KALIA *et al*., 2011). Os SSR são encontrados tanto em eucariotos como em procariotos, e são sequências de DNA de um a seis nucleotídeos repetidos em *tandem* de 5 a 100 vezes, por exemplo, (ATG)n, com produtos que variam em geral de 75 a 300 pb, dependendo do loco e da localização dos *primers* (NYBOM, 2004; ALLENDORF *et al*., 2013).

Entre os diferentes indivíduos da população, o número de repetições dessas unidades pode variar, gerando um grande potencial de polimorfismo, na faixa de 10-3 ou 10-4 por geração, colocando os SSR dentre os mais polimórficos disponíveis atualmente e, portanto, ideais para análises populacionais (FERREIRA; GRATTAPAGLIA, 1996; ELLEGREN, 2004; RAKOCZY-TROJANOWSKA; BOLIBOK, 2004; GUICHOUX *et al*., 2011; ALLENDORF *et al*., 2013). Para análise de polimorfismo de SSRs são usados *primers* flanqueadores da região do microssatélite, sendo, portanto, necessário um conhecimento prévio da sequência de DNA do organismo a ser estudado, tornando essa técnica inicialmente inacessível a muitos taxa (SQUIRRELL *et al*., 2003; NYBOM, 2004).

Os microssatélites, constituem uma das classes mais polimórficas de marcadores moleculares disponíveis atualmente, pois apresentam maior taxa de mutação e

consequentemente de variação, sendo os mais indicados nos estudos de genética de populações, diversidade genética, sistemas de cruzamento e mapas de ligação (NASS, 2007). Os microsssatélites, possuem algumas vantagens sobre os métodos clássicos de genética populacional, podemos citar alguns exemplos como: elevado conteúdo de informação de polimorfismo, a herança das características detectadas pode ser facilmente identificada, a maioria dos locos são codominantes e facilmente reproduzíveis onde é possível calcular as frequências alélicas sem a necessidade de cruzamentos, as estimativas de variabilidade podem entre populações ou espécies ser comparadas diretamente (FERREIRA; GRATTAPLAGLIA, 1998; BORÉM; CAIXETA, 2006).

Com o advento de técnicas mais simples para construção de bibliotecas enriquecidas em microssatélites e, posteriormente, com o sequenciamento de nova geração, entretanto, o uso desses locos para análises populacionais se tornou mais viável, sendo utilizada para um número cada vez maior de espécies (ZANE *et al*., 2002; ANGELONI *et al*., 2011; GUICHOUX *et al*., 2011; WÖHRMANN *et al*., 2013). Diferentes marcadores moleculares podem ser utilizados para avaliar a diversidade genética e a estruturação inter e intrapopulacional em plantas. Marcadores baseados em sequências de cloroplasto têm se mostrado úteis na descrição de padrões filogeográficos e para inferir as rotas de migração de espécies (AVISE, 2000; PETIT *et al*., 2003, 2005; RAMOS *et al.*, 2007; COLLEVATTI *et al.*, 2009). Os marcadores nucleares fornecem informações quanto ao fluxo gênico através de sementes e pólen (COLLEVATTI *et al.*, 2010), variabilidade e estruturação espacial da espécie (PALMA-SILVA *et al.*, 2009), processos de hibridação natural (FJELLHEIM *et al.*, 2009) e parentesco de indivíduos (BACLES; ENNOS, 2008). Juntos, marcadores plastidiais e nucleares geram uma ampla visão dos padrões evolutivos de cada espécie e são fundamentais na elaboração de planos de manejo (MARTINS *et al.*, 2006).

O sequenciamento de nova geração (NGS) possui tecnologias de rendimento extremamente elevado e produzem milhares ou milhões de sequências de uma só vez a uma fração do custo do método tradicional de Sanger (SHENDURE; JI, 2008; EKBLOM; GALINDO, 2011). Uma das aplicações atuais e emergentes da NGS é a descoberta de polimorfismos genéticos (SHENDURE; JI, 2008; EKBLOM; GALINDO, 2011), além de possibilitar a identificação rápida de repetições de sequências simples (SSR) ou microssatélites, que são amplamente utilizados em estudos envolvendo populações de plantas. A aplicabilidade desta técnica é ampla, podendo ser utilizada para o desenvolvimento de mapas de ligação (linkage), identificação de loci de características quantitativas (QTL), mapeamento, seleção assistida por marcadores moleculares, análise de parentesco, DNA

fingerprinting, estudos de diversidade genética e evolução (CAVAGNARO *et al*., 2010; ZHU *et al*., 2011).

A restrição para a aplicação desta tecnologia na análise genética está na grande quantidade de trabalho desenvolvido, necessitando de equipamento sofisticado além do alto custo para o desenvolvimento de primers específicos, quando não estão disponíveis para as espécies a serem estudadas (FERREIRA; GRATTAPLAGLIA, 1998; FALEIRO, 2007). No entanto, atualmente com o desenvolvimento das pesquisas relacionadas aos primers denominados "heterólogos", é possível utilizar primers que foram desenvolvidos para uma espécie e emprega-lo em espécies do mesmo gênero (FERREIRA; GRATTAPLAGLIA, 1998).

2.3. *Erythroxylum*: Biologia reprodutiva e heterostilia

Erythroxylaceae compreende quatro gêneros e cerca 240 espécies com distribuição pantropical, tendo seus principais centros de diversidade e endemismo na Venezuela, Brasil e Madagascar (DALY, 2004). A maioria das espécies (ca. 230) pertence ao gênero *Erythroxylum* P. Browne, que apresenta distribuição ampla encontrado nos quatro continentes, principalmente na América tropical (PLOWMAN, 2001). *Erythroxylum* é um gênero que apresenta alcaloides tropânicos, dentre os quais estão à cocaína, um alcaloide natural produzido por *E. coca*, empregado como anestésico local em pequenas cirurgias (EMCHE *et al*., 2011) e Catuaba (SILVA, 2005), e a partir dessa descoberta o interesse se intensificou devido ao grande potencial farmacológico do gênero, e a partir de então as plantas deste gênero começaram a ser amplamente utilizadas na medicina tradicional no tratamento de infecções bacterianas e/ou virais da pele, amenorreia, hemorragia, distúrbios renais e respiratórios, gripes, sinusite, dores de estômago, para combater a fadiga e a sensação de fome e como estimulante (SILVA *et al*., 2001; RODEIRO *et al*., 2008).

As flores das espécies de *Erythroxylum* são geralmente brancas, monóclinas com cinco sépalas e cinco pétalas que se alternam, dez estames de filetes unidos na base, formando um tubo que circunda o pistilo. O gênero apresenta heterostilia do tipo distilia (GANDERS, 1979; BERRY *et al*., 1991), isto é, flores com estiletes longos (longistilas) e flores com estiletes curtos (brevistilas), ambas com filetes de comprimentos correspondentes. As flores longistilas apresentam estames em dois níveis, enquanto que as flores brevistilas possuem estames em alturas iguais (LOIOLA *et al*., 2007). O ovário é súpero, tricarpelar e trilocular, mas possui apenas um lóculo fértil com um óvulo (SOUZA; LORENZI, 2012). A razão pólen/óvulo (P/O) é alta, pois as espécies de *Erythroxylum* só possuem um óvulo (BARROS, 1998), chegando a ser maior que o proposto por Cruden (1977) para espécies xenogâmicas. O fruto é

vermelho do tipo drupa (LOIOLA *et al*., 2007) e ornitocórico (FAEGRI; VAN DER PIJL, 1979).

As flores são melitófilas e visitadas principalmente por vespas, abelhas e moscas (BARROS, 1998, SILVA *et al.*, 2007). As vespas são consideradas os polinizadores principais de algumas espécies, por manter constante o número de visitas, permanecerem tempo suficiente na flor e por contatarem as estruturas reprodutivas (BARROS, 1998, SILVA *et al*. 2007).

Além de Eythroxylaceae, existem aproximadamente 25 famílias e 164 gêneros que apresentam heterostilia (GANDERS, 1979, LLOYD; WEBB, 1992), A heterostilia é um polimorfismo floral geneticamente controlado e interpretado como um mecanismo que promove a polinização cruzada (GANDERS, 1979, BARRETT *et al*., 2000, LI; JOHNSTON, 2001). Espécies heterostílicas apresentam flores com hercogamia recíproca e um sistema de incompatibilidade no qual apenas cruzamentos intermorfos resultam em formação de frutos (GANDERS, 1979, BAWA; BEACH, 1983, KOHN; BARRETT, 1992, THOMPSON; DOMMÉE, 2000). Essa reciprocidade manifesta-se na apresentação de morfos florais que são diferenciados pela altura do estigma e posicionamento recíproco das anteras, podendo ocorrer em condições distílicas e tristílicas (GANDERS, 1979). Estas podem ser típicas ou atípicas; típicas são aquelas que apresentam o sistema de incompatibilidade e os morfos brevistilo e longistilo com hercogamia recíproca exata (BARRETT, 1992, KOHN; BARRETT, 1992, FAIVRE; MCDADE, 2001). Espécies com distilia atípica apresentam variações nos polimorfismos florais (BARRETT, 1988). Dentre as variações encontradas, pode ocorrer dimorfismo estilar sem a presença de sistema de incompatibilidade dialético como observado em algumas espécies (LLOYD *et al*., 1990).

As variações podem ocorrer tanto entre gêneros quanto entre indivíduos ou populações, dentro de espécies tipicamente distílicas, além de poderem estar presentes entre populações distílicas separadas por barreiras geográficas (LI; JOHNSTON, 2001). Essas variações podem ocorrer por vários motivos, tais como situações desfavoráveis à polinização cruzada por meio de ineficiência ou ausência de polinizadores, ou ainda, por reprodução clonal, perturbações ambientais e espécies com algum grau de autocompatibilidade (SOBREVILA, *et al*., 1983).

A heterostilia em *Erythroxylum* foi observada pela primeira vez por Darwin (1877), que associou este mecanismo à polinização cruzada, efetivada por insetos. Posteriormente, Ganders (1979) documentou distilia em *E. coca* e a associou a um mecanismo de proteção à auto-fecundação e a flores polipétalas e gamopétalas, nas quais existe uma porção tubular como ocorre em *Erythroxylum*, *Lythrum* e *Jepsonia*.

Análises do sistema reprodutivo em espécies simpátricas de *Erythroxylum* P. Br. (Erythroxylaceae) do Brasil (*E. campestre*, *E. suberosum* e *E. tortuosum*) mostram que existe hercogamia recíproca entre os morfos (BARROS, 1998). Também se observa HR entre *Erythroxylum sideroxyloides* e *E. hypericifolium* (RICHARDS; KOPTUR, 1993). Apesar do gênero apresentar heterostilia, ocorre uma pequena compatibilidade intramorfo em *E. campestre* (BARROS, 1998), o que também foi constatado para *E.* cf *macrophylum* (SILVA *et al*., 2007) e *E. laurifolium* (PAILLER *et al*., 1998), sugerindo que isso ocorre para manter o sucesso reprodutivo das espécie (SILVA *et al*., 2007). Foi observada a ocorrência de apomixia em *E. undulatum* (BERRY, 1991), demonstrando que o gênero possui vários tipos de estratégias reprodutivas.

Para o gênero *Erytroxylum* ainda não existe nenhum estudo sobre distilia atípica. No entanto, a ocorrência de dois comprimentos de estames/estiletes somente em flores longistilas foi observada em nove espécies de *Erythroxylum* (AMARAL JR. 1980), bem como em flores longistilas e brevistilas para *E. coca* e *E. novogranatense*. A única espécie registrada como tristilica foi *E. cuneatum*, que possui quatro tipos de flores que diferem em relação ao comprimento do estilete e à separação das anteras (GANDERS, 1979).

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CHAPTER I

INTERSPECIFIC POLEN FLOW IN *ERYTHROXYLUM* P.BROWNE (ERYTHROXYLACEAE)

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Interspecific polen flow in *Erythroxylum* P.Browne (Erythroxylaceae)

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Abstract

Synchronopatric plant species that have morphologically similar flowers tend to be pollinated by the same groups of pollinators, facilitating hybridization. This study aimed to evaluate reproductive isolation and potential hybridization between synchronopatric species that share floral features and pollinators, using three heterostylous *Erythroxylum* species as a model system. We evaluated mechanisms of pre-zygotic (flowering synchrony, floral biology, and floral visitors) and post-zygotic (seed formation after controlled pollination experiments) reproductive isolation. Field data were collected in the State Park Mata do Pau-Ferro, NE Brazil (6°58'12'S; 35° 42'15'W). The three species have high P/O ratios, meaning that they are all xenogamous. None of the species showed reciprocity between floral morphs or isoplethy. This variation may be linked to the fact that the species are self-compatible, due to a breakdown in the mechanism of heterostyly. Controlled pollination treatments showed that there is compatibility between equal and different morphs and interspecific compatibility between *E. pauferrense* and *E. simonis* as pollen donor or recipient, proving that pre-zygotic reproductive barriers are weak and favor the process of hybridization between them. *Erythroxylum citrifolium*, although showing no reciprocal herkogamy between morphs, also did not show compatibility between equal morphs or in interspecific crosses.

Key words: gene flow - hybridization – speciation - heterostyly.

Introduction

Floral convergence in synchronopatric species, especially members of the same genus, can be the result of selective pressure by pollinators (SCHEMSKE, 1981) and result in hybridization, introgression and, consequently, speciation (RIESEBERG, 1995; LEVIN, 2000; COYNE; ORR, 2004). The combination of genomic elements from distinct species can increase the ecological range and diversity of niches which a hybrid can occupy, thus giving rise to new species (ARNOLD, 2006). Speciation by hybridization may result from rapid chromosomal evolution, and/or the occurrence of favorable habitat for establishment and subsequent development of the hybrids (RIESEBERG et al., 1997). Studying reproductive barriers between synchronopatric species with similar flowers will improve our understanding of processes that guide this speciation (DOBZHANSKI, 1970).

The mechanisms that promote reproductive isolation between sympatric groups are known as pre- and post-zygotic barriers (ELLSTRAND et al., 1996; SCOPECE et al., 2010). Pre-zygotic barriers include differences in flowering times, non-sharing of pollinating agents (due to differences in floral characteristics), deposition of pollen on different sites of the body of shared pollinators (FRANCESCHINELLI, 2005) and interspecific incompatibility systems, acting on the germination of pollen (BRADSHAW et al., 1995), in the syngamy (LEXER et al. 2005) and in the formation of fruits and/or seeds. Post-zygotic barriers include non-viability, sterility and/or reduced reproductive performance of hybrids (LEVIN, 1971; JUDD et al., 2009, JOHNSON, 2010; GREINER et al., 2011).

Phenological data are important because they can indicate the level of overlapping flowering times and, consequently, if the species can use the same pollinators (VOGEL; MACHADO, 1991). Studies of pollination ecology (identification of pollinators, frequency of visits and behavior) and floral and reproductive biology provide details of shared pollinator groups and identify possibilities for interspecific gene flow (WASER, 1978; MOELLER, 2004; MACHADO et al., 2010; FRANCO et al., 2012).

Most papers on reproductive barriers in plants have focused on investigating mechanisms of hybrid formation and/or their reproductive fitness (ARNOLD, 2000; BURKE; ARNOLD 2001; MILNE et al., 2003). Some of these studies have revealed that pre-zygotic barriers are more efficient in reducing hybridization and often influenced by the behavior of pollinators (CAMPBELL et al., 2002; MARQUES et al., 2007). Other studies, however, have shown that pre-zygotic isolation mechanisms may be inefficient (WENDT et al., 2001; 2008). The efficiency levels of pre- and post-zygotic barriers cannot be considered as absolute (WU, 2001), and these can be variable among species (CHARI; WILSON, 2001; HUSBAND;

SABARA, 2003; RAMSEY et al., 2003; COYNE; ORR, 2004; KAY, 2006, NOSIL et al., 2006, MARTIN; WILLIS, 2007).

In a montane forest in Northeast Brazil there are three distylous species of *Erythroxylum* P.Br (*E. citrifolium* A.St.-Hil., *E. pauferrense* Plowman and *E. simonis* Plowman) which have strong similarities in floral attributes. Of these, *E. pauferrense* is classified as a rare and endemic species of this region (LOIOLA et al., 2007; PLOWMAN, 1987).

Darwin (1877), who associated heterostyly with cross-pollination, carried out by insects, observed heterostyly in *Erythroxylum* for the first time. Later, Ganders (1979) documented distyly in *E. coca* Lam. and associated it with a mechanism against self-fertilization in polypetalous and gamopetalous flowers, in which there is a tubular portion as in *Erythroxylum*, *Lythrum* L. and *Jepsonia* Small. Analyses of the reproductive system in sympatric species of *Erythroxylum* (Erythroxylaceae) from Brazil (*E. campestre* A.St.-Hil., *E. suberosum* A.St.-Hil. and *E. tortuosum* Mart.) show that there is reciprocal herkogamy among the morphs (BARROS, 1998).

These results corroborate those of Richards and Koptur (1993), who found reciprocal herkogamy among morphs of *E. sideroxyloides* Lam. and *E. hypericifolium* Lam. However, although the genus shows heterostyly, Barros (1998) found in *E. campestre* there is a low level of intra-morphic compatibility, and this has also been found in *E.* cf *macrophyllum* Cav. (SILVA et al., 2007) and *E. laurifolium* Lam. (PAILLER et al., 1998), suggesting that this occurs as a mechanism to ensure reproductive success (SILVA et al., 2007). Apomixis has been observed in *E. undulatum* Plowman (BERRY, 1991), demonstrating that the genus has several types of reproductive strategies.

Based on these observations, we investigated the mechanisms of reproductive isolation in the three species in order to answer the following questions. i) What is the period of flowering overlap and how this can interfere with the reproductive sucess? ii) Are pollinators shared between species? iii) Is there floral and reproductive similarity between species? iv) Is there a breakdown in the interspecific and interspecific incompatibility systems?

Material and Methods

Study area and species studied: We conducted the study in natural populations of *E. citrifolium*, *E. pauferrense* and *E. simonis* in Parque Estadual Mata do Pau-Ferro, (6°58’12’S; 35°42’15’W), Paraíba, Northeast Brazil. The park is approximately 600 ha, and the predominant vegetation type is open ombrophylous forest (BARBOSA et al., 2004). The

populations are comprised of shrubs to small trees, occurring in the sub-forest and are distributed throughout the study area. The species have wide distributions in most of Northeast Brazil, except *E. pauferrense*, which is endemic to Paraíba (PLOWMAN; HENSOLD, 2004).

Phenology: To evaluate the overlap in flowering periods, we monitored 20 clusters of marked individuals of the three species each month for three years (2014 to 2016), recording the presence of buds and flowers using the Activity Index (BENCKE; MORELLATO, 2002).

Flower visitors: We conducted direct observations of floral visitors in focal plants from 5:00h to 18:00h, on different days, totaling 36 observation hours per species. During the observations we recorded frequency (by direct counting of visitors to flowers every hour) and behavior (contact with the floral reproductive structures, collected floral resource and time of visit) of floral visitors. We classified floral visitors as effective pollinators (PE; contacts anther and stigma often ≥ 10 visits.hour^{-1}), occasional pollinators (PO; contacts anther and stigma often < 10 visits.hour^{-1}) or robbers (PI; uses the floral resource without contact with reproductive structures).

Floral and reproductive biology: Flower samples and buds ($n = 20$) of 10 individuals of each morph (brevistylous and longistylous) of each species for the analysis of the morphometry of the reproductive structures (CASTRO; OLIVEIRA, 2004) and estimation of the pollen/ovule ratio (CRUDEN, 1977) were fixed in 70% ethanol. To investigate isoplethy, the scanning method was performed along a sample line of approximately 1000m, along which we annotated the morph of all individuals of *E. pauferrense*, *E. simonis* and *E. citrifolium*. To describe anthesis, we marked 20 buds on 20 individuals, by morph, by species, which were monitored from opening to the flower senescence. In another 10 flowers, per morph, in 10 individuals for each species, we tested the period of stigmatic receptivity in the early morning, at midday and late afternoon, using hydrogen peroxide (DAFNI et al., 2005).

We studied the reproductive system of *E. pauferrense*, *E. simonis* and *E. citrifolium* through controlled pollination experiments and natural pollination (control), following the protocol adopted by BAWA and BEACH (1983). We marked 60 buds in pre-anthesis, distributed in 10 individuals, in each morph per species, and applied the following treatments: (1) spontaneous self-pollination, (2) manual self-pollination, (3) intra-morphic cross-pollination and (4) cross-pollination intermorph. In treatments 3 and 4, the pre-anthesis buds were emasculated, and all the flowers treatments were kepted bagged during anthesis to avoid contact of visitors with flowers. We marked and followed 50 flowers of 10 individuals of each

morph of each species to evaluate fruit production by natural pollination (control). In all treatments, we counted the number of mature fruits.

We conducted intermorph crossings on three species to test the possibility of hybridization between them. Each hybridization test between two species included four treatments: brevistylous pollen of species 1 on the longistylous stigma of species 2, longistylous pollen of species 1 on the brevistylous stigma of species 2, brevistylous pollen of species 2 on the longistylous stigma of species 1, longistylous pollen of species 2 on the brevistylous stigma of species 1.

Statistical analysis: We compared the morphometric data among the floral morphs of each species (to evaluate the occurrence of reciprocal herkogamy, RICHARDS; KOPTUR, 1993). This provides two values: one for reciprocity of high whorls and one for the low, allowing us to verify if both morphs show similar values of reciprocity, using the value of 0,0 as a band of perfection (numbers between 0.05 and -0.05 are acceptable). To evaluate the ratio of morphs and compare the formation of fruit among the treatments performed the chi-square test (Zar 2010), in Biostat software 5.0 (AYRES et al., 2007).

Results

Phenology: The species shown variation in the period of flowering between years (Figure 1). In the first year, they did not flower, in the second year they only flowered from April to May and in the third year from February to April. *Erythroxylum citrifolium* showed the lowest flowering intensity, but it maintained an overlap with the other species.

Flower visitors: The bees *Apis mellifera, Trigona spinipes* and *Tetragona* sp. were observed visiting both floral morphs of the three species looking for nectar and pollen. The visits occurred in all open flowers of the inflorescence and lasted about 40 seconds per inflorescence. *Tetragona* sp. and *Trigona spinipes* had the highest visit rates in both morphs for all species and the entire flowering period (Figure 2). All visitors were classified as effective pollinators (EP), since they contacted the reproductive structures (anthers and stigmas), carrying pollen all over the body. We observed that pollinators had no preference among plant species, visiting all simultaneously.

Floral and reproductive biology: The three species share the same characteristics of floral attributes, including flowers white, nectariferous, hermaphrodite, pentamerous, actinomorphic and of similar sizes, sepals conate at the base, with 10 stamens in two positions: one opposite the sepals and one opposite the petals. The stamens reach two levels in longistylous flowers of *E. citrifolium* and *E. pauferrense*, but only one level in *E. simonis*. The brevistylous flowers

reach the same level for all species (Table 1). *Erythroxylum pauferrense* and *E. simonis* have dehiscent anthers in both morphs, and stigmas of longistylous morphs are arranged in a downwards direction, touching the anthers in the bud phase.

All species show daytime anthesis, occuring between 6:00am to 09:00am. Stigmatic receptivity occurs approximately one hour after opening of the floral bud. The flowers remain receptive for a day; on the second day, the petals become beige and fall. The only floral feature that sets *E. pauferrense* apart from the other species is that the styles are fused, whereas in the others they are free. The fruits are drupes and red when mature.

According to the proportion of morphs, *E. pauferrense* (χ^2 = 9.3; p = 0.0022) and *E. simonis* (χ^2 = 5.3; p = 0.0209) were considered anisoplethic, where as *E. citrifolium* was isoplethic. The estimated values of Richards and Koptur (1993) showed that the species showed a low degree of reciprocal herkogamy. In addition, the longistylous morph was reciprocal compared to the brevistylous morph, with the exception of *E. citrifolium* brevistylous flowers of which were considered more reciprocal (0.41) in relation to the opposite morph (0.9).

Reproductive system: *Erythroxylum simonis* formed fruits in all treatments. *Erythroxylum pauferrense* formed fruits in all treatments except manual self-pollination in the longistylous morph, and *E. citrifolium* only formed fruits in natural pollination treatments and intermorphs (Table 2).

Fruit formation by intermorphic crosses was higher than for intra-morphs for all species and morphs, except for the brevistylous morph of *E. simonis* which was lower (Table 2). The formation of self-pollinating fruits from intra-morphs crosses was only equal among the brevistylous morphs of *E. simonis*. In relation to interspecific crosses, there was fruit formation after intermorphic pollinations in both morphs only in *E. pauferrense* and *E. simonis* (Table 3).

Discussion

The flowering overlap found here has been observed in other species of the genus (BARROS, 1998), and the sharing of floral characteristics by the species means that they attract the same guild of pollinators, which may be important for reproductive success by promoting facilitation (MORAGUES; TRAVESET, 2005; LOPEZARAIZA-MIKEL et al., 2007; LIAO et al., 2011; MCKINNEY; GOODELL, 2011), since visitation rates are maximized due to greater floral attractiveness (MOELLER, 2004; GHAZOUL, 2006; SIEBER et al., 2011). However, in this case we did not observe any increase in reproductive

success promoted by shared pollinators; even when two or more species share the same group of pollinators, the patterns of visitation may promote neutral results, where sharing does not influence reproduction.

The fact that the stigma is curved and the anthers dehiscent still in the pre-anthesis bud phase should contribute to spontaneous self-pollination events, especially in longistylous flowers. Spontaneous self-pollination has already been recorded for other *Erythroxylum* species, as observed by Barros (1998). The high ratio pollen/ovule (P/O) classifies species as facultative xenogamous, which are usually self-compatible, but favoring xenogamic crosses through morphological and/or physiological adaptation occurs, with self-pollination restricted to absence or in addition to pollination (CRUDEN, 1977). According to Barros (1998), this high ratio P/O is favored by the presence of a single ovule and high number of pollen in *Erythroxylum* species.

Although seed formation was observed in all the treatments performed, the reproductive success obtained in the spontaneous self-pollination experiment was significantly lower than natural and manual cross-pollination. These results suggest the predominance of self-incompatibility and, consequently, the importance of pollinators. According to Proctor, Yeo and Lack (1996), all systems of self-incompatibility present "failures", because it is possible for self-pollination to occur, justifying the occurrence of lower levels of fruit formation, in the self-pollination experiment.

Like some authors (BARROS, 1998; RICHARDS; KOPTUR 1993), we demonstrated that some species did not show reciprocal herkogamy or isoplethy, and these have been identified as some of the necessary conditions for distyly to be characterized as typical and sufficient to determine minimum levels of legitimate pollination (LLOYD; WEBB, 1992b; CESARO; THOMPSON, 2004). Reciprocity is the morphological key that distinguishes distyly from other dimorphisms like style polymorphism (BARRETT et al., 2000; FERRERO et al., 2011a e b). If these characteristics are not present in the population, then atypical distyly can be characterized (HAMILTON, 1990), in which the absence of mutual herkogamy can be accompanied by self-compatibility and anisoplethy, as recorded for the studied species, monomorphic and homostylous populations. Therefore, *E. pauferrense* and *E. simonis* can be characterized as atypical and the behavior of the pollinators should induce the breakdown of the mechanism. This is due to the strong similarity of floral characters, resulting in the pollinators being unable to distinguish the species, automatically generating genetic recombination between different species. Among the several factors that may influence the atypical distyly, we can highlight the inefficiency or absence of pollinators (FAIVRE;

McDADE, 2001; KULBABA; WORLEY, 2008; VALOIS-CUESTA et al., 2012), mutations and genetic recombination (BARRETT; RICHARDS, 1990), environmental disturbance (BARRETT, 1988; AGREN, 1996), reduced population sizes (JENNERSTEN, 1988; KÉRY, et al. 2000) and geographical isolation (SOBREVILA, et al. 1983; WASHITANI, et al. 1994; MATSUMURA; WASHITANI, 2000; BRAMOW, et al. 2012); these all act on the selective force for distyly, which may cause breakage of the mechanism.

The self-compatibility intra-morph observed here has been recorded in other *Erythroxylum* species (BARROS, 1998; PAILLER, et al., 1998; SILVA et al. 2007), and is yet another fact that corroborates our hypothesis of a break in the distylous system in *Erythroxylum*. This feature may have arisen by loss or relaxation of the growth inhibitory capacity of the pollen tube and the regulation of the incompatibility system becoming independent (BAKER, 1966; NICHOLLS, 1985).

Although the species studied do not provide a perfect reciprocal herkogamy, they showed high levels of fruit formation, which may indicate an efficiency in pollen transport and deposition by pollinators, i.e. the absence of perfect reciprocity cannot affect the reproductive success of the species studied. Some studies show that the lack of a perfect reciprocity does not prevent pollen flow (FAIVRE; MCDADE, 2001; CASTRO; ARAÚJO, 2004; TEIXEIRA; MACHADO, 2004; CONSOLARO, 2008; SAMPSON; KREBS, 2012), whereas others suggest that variations in the degrees of herkogamy alter the deposition of pollen among the morphs, so that the smaller the distance between the reciprocal reproductive structures, the higher the deposition of legitimate pollen (GUZMÁN et al., 2012; BAENA-DÍAZ et al., 2012; THOMPSON et al., 2012; BRAMOW et al., 2012).

The data show, in general, that species have a typical distylous reproductive system, in which the formation of intermorph fruit is more efficient than intra-morph. Thus, variations in the levels of herkogamy observed among the morphs did not affect the formation of fruits in natural pollinations in any of the species. Fruit formation by intra-morph crosses and self-pollination showed that, as in most distylous species, some degree of incompatibility and compatibility are possible between morphs.

The overlap in flowering phenology, floral attributes and pollinators and the formation of fruits after interspecific pollination, indicate that *E. pauferrense* and *E. simonis* are inter-compatible, although the literature does not indicate any clear evidence of interspecific hybridization in species of the genus.

Hybridization among sympatric species has been observed mainly in species where the reproductive barriers are weak (MEDAN et al., 2012) and some authors have shown hybridization patterns ranging from congeners occurring in sympatric areas, e.g. in *Populus* L. (LEXER et al., 2005, VAN LOO et al., 2008), *Quercus* L. (PETIT et al., 2003), *Helianthus* L. (YATABE et al. 2007) and *Mimulus* L. (MARTIN; WILLIS, 2007). However, Mallet (2005) suggested that training and the establishment of hybrids are often facilitated by environmental changes, especially by human disturbance.

Thus, although the species show heterostyly, a mechanism that includes self-incompatibility and intra-morphological incompatibility and consequently favors inter-morphic cross-pollination (GANDERS, 1979, BARRETT, 1992, THOMPSON; DOMMÉE, 2000), ecological and genetic conditions may render this mechanism less efficient.

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Table 1. Morphometry and pollen/ovule ratio of *Erythroxylum* (Erythroxylaceae) syncronopathric species in rain forest in Northeast Brazil.

Species	Morph	Floral Diameter (mm)	Pistil length (mm)	Stamen length (mm)	Stamen length (cm)	Pollen/ovule ratio
E. pauferrense	Brevistylous	4 - 5	3,5 - 5	1,5 - 6		2.800:1
	Longistylous	3,5 - 5	9 - 10	5 - 8	10 - 14	4.000:1
E. simonis	Brevistylous	4 - 5	1 - 3	2 - 6		3.500:1
	Longistylous	4 - 5	2 - 6	8 – 10		3.800:1
E. citrifolium	Brevistylous	4 - 6	3 - 4	8 - 12		5.700:1
	Longistylous	4 - 7	15 - 20	3 - 4	12 - 16	8:600:1

1

2

Table 2. Manual and natural pollination experiments (% fruits formed) on three sympatric species of *Erythroxylum* (Erythroxylaceae) in rainforest in Northeast Brazil. NFF: Natural formation of fruits; BXL: brevistylous pollen in the longistylous stigma; LXB: longistylous pollen in the brevistylous stigma; LXL: longistylous intra-morphic pollination; BXB: brevistylous intra-morphic pollination. BSP: brevistylous self-pollination; LSP: longistylous self-pollination.

Species	NFF B (50)	NFF L (50)	BXL (60)	LXB (60)	LXL (60)	BXB (60)	BSP (60)	LSP (60)
E. pauferrense	82	54	73,3	43,3	1,7	6,7	5	0
E. simonis	94	68	90	5	10	6,7	6,7	3,3
E. citrifolium	56	24	33,3	81,7	0	0	0	0

Table 3. Interspecific cross-pollination experiments among three sympatric species of *Erythroxylum* (Erythroxylaceae) in rainforest in Northeast Brazil. B1/L1= *Erythroxylum pauferrense;* B2/L2= *Erythroxylum simonis;* B3/L3= *Erythroxylum citrifolium*, where B = brevistylous morph and L= longistylous morph.

B1XL2 (n=60)	**B1XL3** (n=60)	**B2XL1** (n=60)	**B2XL3** (n=60)	**B3XL1** (n=60)	**B3XL2** (n=60)	**L1XB2** (n=60)	**L1XB3** (n=60)	**L2XB1** (n=60)	**L2XB3** (n=60)	**L3XB1** (n=60)	**L3XB2** (n=60)
8	0	3	0	0	0	13	0	6	0	0	0

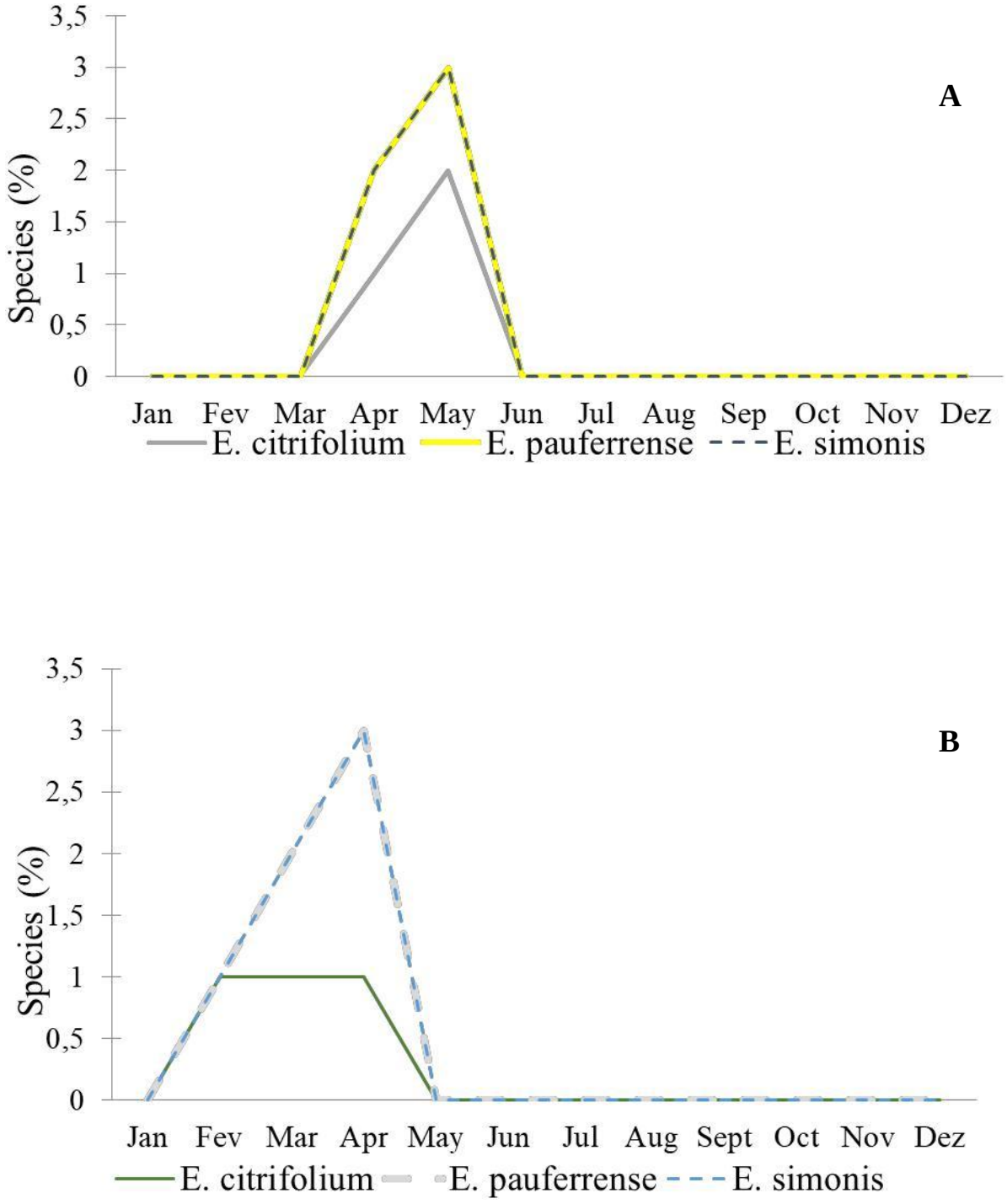

Figure 1. Flowering period of three syncronopathric species of *Erythroxylum* (Erythroxylaceae) in rainforest in Northeast Brazil in 2015 (A) and 2016 (B).

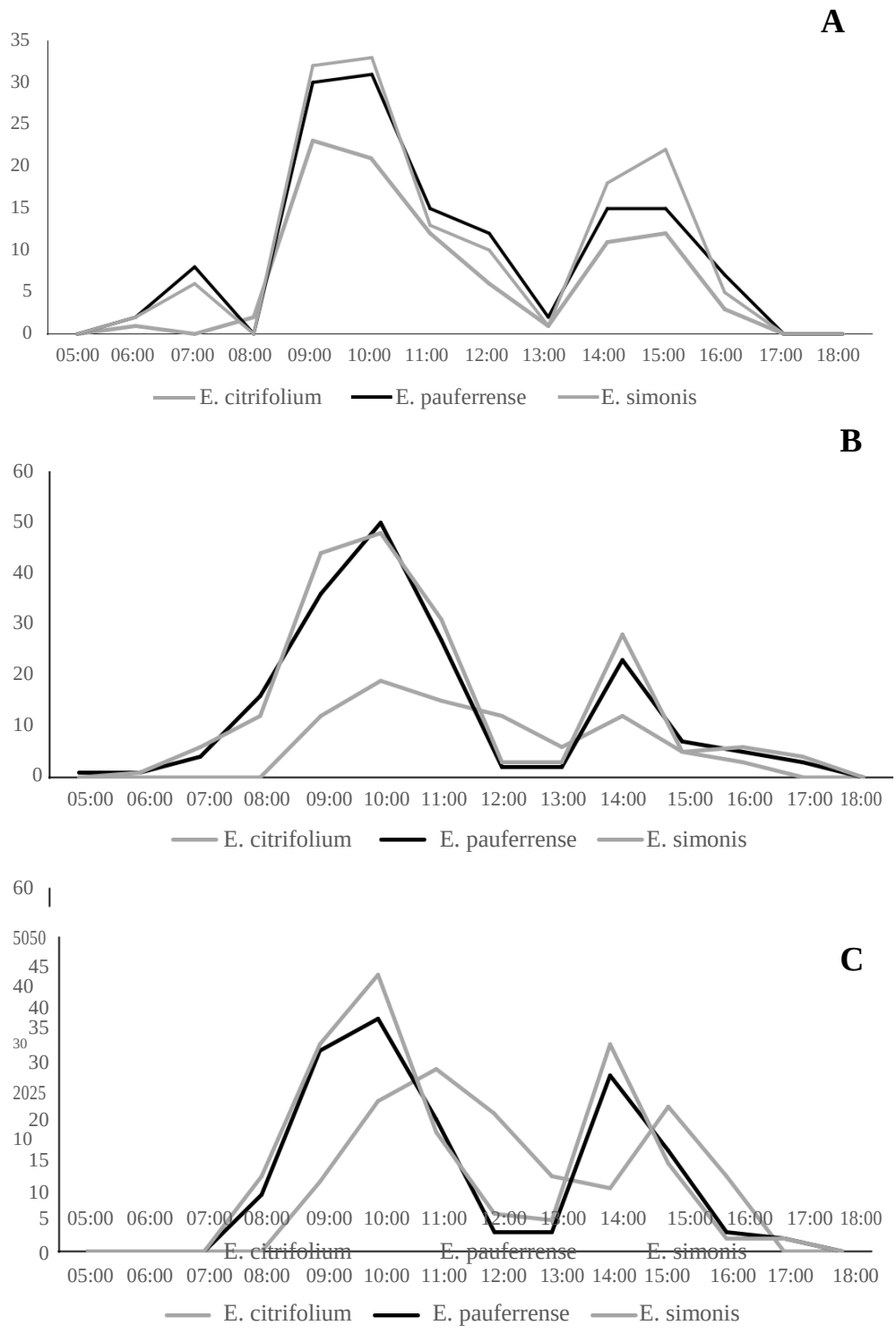

Figure 2. Frequency of pollinators on flowers of the species of *Erythroxylum pauferrense*, *E. simonis* and *E. citrifolium* during the day in rainforest in Northeast Brazil. *Apis melífera* (A), *Trigona spinipes* (B) and *Tetragona* sp. (C).

Chapter II

Genetic diversity in *Erythroxylum* species in Northeast Brazil

Manuscript to be submitted to Biotropica

Genetic diversity in *Erythroxylum* species in Northeast Brazil

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Abstract

The genus *Erythroxylum* is the largest in Erythroxylaceae, and species in this genus often occur in sympatry and have similar reproductive structures which may favour gene flow between species and consequently hybridization. Identification, mainly using characters such as stipules and cataphylls, is extremely difficult because species share similar vegetative and reproductive structures. There is little information on genomic variation and most studies on *Erythroxylum* lack resolution. Thus, the use of genomic tools, such as molecular markers (e.g. ITS, *trnL* and microsatellites), will be important for understanding genetic mechanisms and clarifying relationships among and within *Erythroxylum* species. The aim of this study was to characterize genetic diversity and differentiation in *Erythroxylum* populations. Towards this goal, an ITS sequence matrix was constructed and used for tree construction. Phylogenetic analysis (using maximum parsimony) was used to compare the relationships among *Erythroxylum* species, focusing on those from North East Brazil. . Our sampling included multiple samples of sections *Archerythroxylum* and *Rhabdophyllum*, and phylogenetic analysis showed both of these sections to be polyphyletic, suggesting that the current intrageneric classification of the genus needs be revised. Diversity at six microsatellite loci for 26 populations was also investigated. Genetic diversity in terms of heterozygosity, allelic diversity and patterns of differentiation were not strictly correlated with taxonomy or geography. Our analyses identified genetic groups within *Erythroxylum* that do not reflect current taxonomic delimitation of some species, notably *E. revolutum*. Further work will be necessary to resolve species delimitation in *Erythroxylum* from North East Brazil.

Key words: phylogeny, gene flow, outcrossing, ribosomal DNA, SSR markers, .

Introduction

Erythroxylaceae are well represented in the Brazilian flora. Worldwide, they comprise four genera and *c*. 230 species, in Africa, Asia, Australia and the Americas, from North America (Mexico), reaching Central America and West Indies, to South America (Daly, 2004). *Aneulophus* Benth., *Nectaropetalum* Engl. and *Pinacopodium* Exell & Mendonça, have few species and are African (Plowman and Berry 1999), whereas *Erythroxylum* is more globally distributed. Erythroxylaceae have been placed in Malpighiales and are sister to Rhizophoraceae (Chase et al.1993; Schwarzbach and Ricklefs 2000). Wurdack and Davis (2009) demonstrated weak to strong support, depending on the method of phylogenetic reconstruction, for the placement of the monogeneric family Ctenolophonaceae as sister to Rhizophoraceae and Erythroxylaceae.

Members of all three families have 'opposite leaves with sheathing, interpetiolar stipules' (Wurdack and Davis 2009). The current pattern of distribution of Erythroxylaceae across the Old and New World tropics is consistent with a possible origin in the biota of northern Tropical Gondwana, and this break-up of this northern tropical region of Gondwana, beginning 130 Mya, may account for the current distribution of *Erythroxylum*; however, several recent biogeographical studies of plants with similar distributions to *Erythroxylum* support an alternate hypothesis to the classic vicariant model (see review by Sanmartín and Ronquist 2004).

Centres of endemism for *Erythroxylum* are Brazil, Venezuela and Madagascar. It is estimated that in the Neotropics there are > 190 species occurring mainly in humid forests (Amazonian and Atlantic) and in the cerrado domain, with fewer species in deciduous and semideciduous forests of the caatinga and carrasco vegetation (Loiola 2001; Plowman and Hensold 2004; Loiola and Costa-Lima 2018).

The most relevant study for understanding the genus was the review elaborated by Schulz (1907), who recognized 19 sections in the genus, of which nine include exclusively tropical species; however, according to some authors, these do not form monophyletic groups (Payens 1958; Rury 1982; Plowman and Rivier 1983; Loiola 2001; Emche et al. 2011). Considering that it is a well-represented group in the flora of Brazil (with *c*. 50% of the species occurring in Brazil), the difficult taxonomic delimitation due to polymorphism and the pharmacological value [some species are grown for the extraction of alkaloids such as cocaine (Emche et al. 2011) and catuaba

(da Silva 2005), there are surprisingly few papers that help in our understanding of the richness and diversity of *Erythroxylum*.

In Brazil there are representatives of nine sections of the genus occurring in Amazonia, caatinga, cerrado and the Atlantic Forest, but the region with the greatest diversity is the Northeast (77), and the State with the highest richness is Bahia (56). According to Cordeiro et al. (2013) the genus has a fragmented and discontinuous distribution, and most of the endemic species occur in environments with a marked climate seasonality; richness decreases towards the tropics, and the endemics are more frequent in older lands with exposed and shallow soils.

Considering the scenario described above, studies involving synchronopatric populations of *Erythroxylum*, with the occurrence of individuals of intermediate morphology, are essential for the evaluation of the intensity of the reproductive barriers among species. Research related to the association between genotypes and habitats has been aided by the use of species-specific molecular markers from different genomic compartments, more usually the nuclear and plastid genomes in the case of plants (Pinheiro et al. 2010). Despite the high diversity of the genus in Brazil, *Erythroxylum* has been little studied in relation to aspects of reproductive biology and molecular variation.

In this study, we explored the variation and genetic composition of species and populations, looking to clarify evolutionary routes between the species and to further our knowledge of the structure and dynamics of populations in order to enhance our understanding of the relationships between species and to elucidate the evolutionary mechanisms related to their diversification. In addition to presenting a broad analysis of *Erythroxylum*, we also conduct a range of molecular analyses of nuclear and plastid DNA sequences. The use of microsatellites provides valuable information related to gene flow via seeds and pollen (Collevatti et al. 2010), variability and spatial structure of the species (Palma-Silva et al. 2009), and relationships between individuals (Bacles and Ennos 2008).

With the collection of genetic data in this study, we intends to increase our knowledge of genetic diversity in *Erythroxylum* throughout its geographical distribution in North East Brazil. This study constitutes the first molecular study on a national scale for *Erythroxylum* and will allow the identification of patterns of evolution of the genus, as well as the investigation of the role of gene flow in the diversity of the group.

Material and Methods

Sampling - Leaves from 98 individuals of *Erythroxylum* species, were collected and stored in silica (Table 1). We prepared herbarium vouchers according to standard procedures (Mori et al., 1989), and these have been incorporated into the Jaime Coelho de Morais Herbarium (EAN) of the Universidade Federal da Paraíba. In addition, samples from the DNA bank at the Royal Botanic Gardens, Kew, were incorporated into this work. Most species were identified by Iracema Loiola, the specialist on the genus in Brazil; some samples are still being identified.

Table 1. Geographical information for the sampled populations of *Erythroxylum* species.

Species name	Pop nº	Section	Country	Sample Size
Erythroxylum barbatum	1	*Pogonophorum*	Fortaleza/Ceara	2
Erythroxylum citrifolium	1	*Rhabdophyllum*	Areia/Paraiba	10
Erythroxylum citrifolium	2	*Rhabdophyllum*	Bonito/Pernambuco	1
Erythroxylum confusum	1	*Archerythoxylum*	Florida (K DNA Bank)	1
Erythroxylum hypericifolium	1		Mauritius (K DNA Bank)	1
Erythroxylum lanceum	1		Indian Ocean (K DNA Bank)	1
Erythroxylum mucronatum	1	*Rhabdophyllum*	Bonito/Pernambuco	1
Erythroxylum nummularia	1	*Archerythoxylum*	Catimbau/Pernambuco	1
Erythroxylum nummularia	2	*Archerythoxylum*	Catimbau/Pernambuco	1
Erythroxylum pauferrense	1	*Rhabdophyllum*	Areia/Paraiba	10
Erythroxylum pulchrum	1	*Leptogramme*	Areia/Paraiba	1
Erythroxylum pulchrum	2	*Leptogramme*	Areia/Paraiba	1
Erythroxylum revolutum	1	*Rhabdophyllum*	Fortaleza/Ceara	2
Erythroxylum revolutum	2	*Rhabdophyllum*	Crato/Ceara	3
Erythroxylum revolutum	3	*Rhabdophyllum*	Fortaleza/Ceara	3
Erythroxylum revolutum	4	*Rhabdophyllum*	Bahia	4
Erythroxylum revolutum	5	*Rhabdophyllum*	Guarabira/Paraiba	1
Erythroxylum revolutum	6	*Rhabdophyllum*	Areia/Paraiba	5
Erythroxylum revolutum	7	*Rhabdophyllum*	Pesqueira/Pernambuco	2
Erythroxylum revolutum	8	*Rhabdophyllum*	São Caetano/Pernambuco	1
Erythroxylum revolutum	9	*Rhabdophyllum*	Pesqueira/Pernambuco	3
Erythroxylum simonis	1	*Rhabdophyllum*	Areia/Paraiba	10
Erythroxylum sp.	1		Minas Gerais	1
Erythroxylum sp.	2		Minas Gerais	3
Erythroxylum sp.	3		Minas Gerais	5
Erythroxylum sp.	4		Minas Gerais	3
Erythroxylum sp.	1		Pesqueira/Pernambuco	3
Erythroxylum sp.	1		Capão da Volta/Bahia	1
Erythroxylum nummularia	1	*Archerythoxylum*	Serra St. Bento/ R. G. Norte	10
Erythroxylum nummularia	2	*Archerythoxylum*	Serra St. Bento/ R. G. Norte	10
Erythroxylum subrotundum	1	*Archerythoxylum*	Catimbau/Pernambuco	1
Nectaropetlum zuluense	1	Outgroup	South Africa (K DNA Bank)	1

DNA extraction - Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA, USA) and after purification, the DNA integrity was checked on a 1.5% agarose gel. Concentrations was measured using 2 μL of extracted DNA on the NanoDrop microvolume sample retention system (Thermo Scientific NanoDrop Products).

PCR and sequencing - Genetic variation among the accessions was assessed by employing the nuclear internal transcribed spacers (ITS1 and ITS2; collectively ITS) using the primer pairs described by Sun et al. (1994) and White et al. (1990). Amplifications were carried out in a final volume of 25μL, with 12.5 μL 2× DreamTaq PCR Master Mix (Thermo Scientific), 0.5 μL 0.4% (w/v) bovine serum albumin, 0.5 μL DMSO for ITS amplification, 0.5 μL of forward and reverse primers and 9.5 μL sterile deionized water, in a GeneAmp PCR System 9700 (Applied Biosystems).Thermal profile as the following: 94 °C for 2 min, followed by 28 cycles of the following profile: 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min with a final hold of 72 °C for 7 min. PCR products were purified using QUIAquick PCR purification kit (QUIAGEN) and run on an ABI3730 DNA Analyzer (Applied Biosystems).

Sequence editing and analyses - News sequences were edited and aligned in GENEIOUS v8.1.7 (Kearse et al. 2012) using standard settings. The alignment was then refined manually. The parsimony analyses were conducted in PAUP* v.4.02b (Swofford 2002) on CIPRES Science Gateway V. 3.3 (http://www.phylo.org/).

Microsatellite development - For the development of the microsatellite library, extracted DNA was used for a library preparation with a NEBnext Library Prep Kit for Illumina and sequencing was performed on a MiSeq Benchtop sequencer (Illumina, Inc., San Diego, California, USA). This method is currently the most widely used NGS platform and is used (http://www.illumina.com). This library, was generated from one sample of *E. pauferrense* and one sample of a species that still being identified. Microsatellite loci were detected in 5,645 reads from *E. pauferrense* and 2,929 from the other species. The reads obtained were converted into a single FASTA format file and screened for perfect microsatellites (di-, tri-, and tetranucleotides) with at least eight repeats using MSATCOMMANDER version 0.8.1 (Faircloth 2008). This software has an inbuilt workflow that enables the simultaneous detection of repeat motifs and the

design of PCR primers to amplify these repeats when flanking regions enable it. If the microsatellite detected is too close to the extremity of the read, the sequence is automatically discarded.

Microsatellite amplification – We had success in amplifying four microsatellite loci with primers previously developed for Australian species of *Erythroxylum* (Van der Merwe 2009), and we developed two from our library (six in total; Table 2). Polymerase chain reactions were carried out in a final volume of 10 μL, with 10 ng genomic DNA, 0.5 μL reverse and FAM- or JOE-labeled forward primers (Eurofins Genomics), 6 μL 2× DreamTaq PCR Master Mix (Thermo Scientific), 0.5 μL 0.4% (w/v) bovine serum albumin and 1.5 μL sterile deionized water, in a GeneAmp PCR System 9700 (Applied Biosystems). The thermal profile for Ery_2 was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec with a final hold of 72 °C for 10 min. The thermal profile for Ery_3 was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 57 °C for 30 sec and 72 °C for 45 sec with a final hold of 72 °C for 10 min. The thermal profile for Ery_7 was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 45 sec with a final hold of 72 °C for 10 min The thermal profile for Ery_8 was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 45 sec with a final hold of 72 °C for 10 min. The thermal profile for E_5 was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec with a final hold of 72 °C for 10 min. The thermal profile for E_6 was 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 sec, 56 °C for 30 sec and 72 °C for 45 sec with a final hold of 72 °C for 10 min. Amplification products (1μL) were combined with 10μL of HiDiTM formamide (Applied Biosystems) and 0.15μL GeneScan 500 ROX Size Standard (Applied Biosystems). Capillary electrophoresis was conducted on an ABI3730 DNA Analyzer (Applied Biosystems).

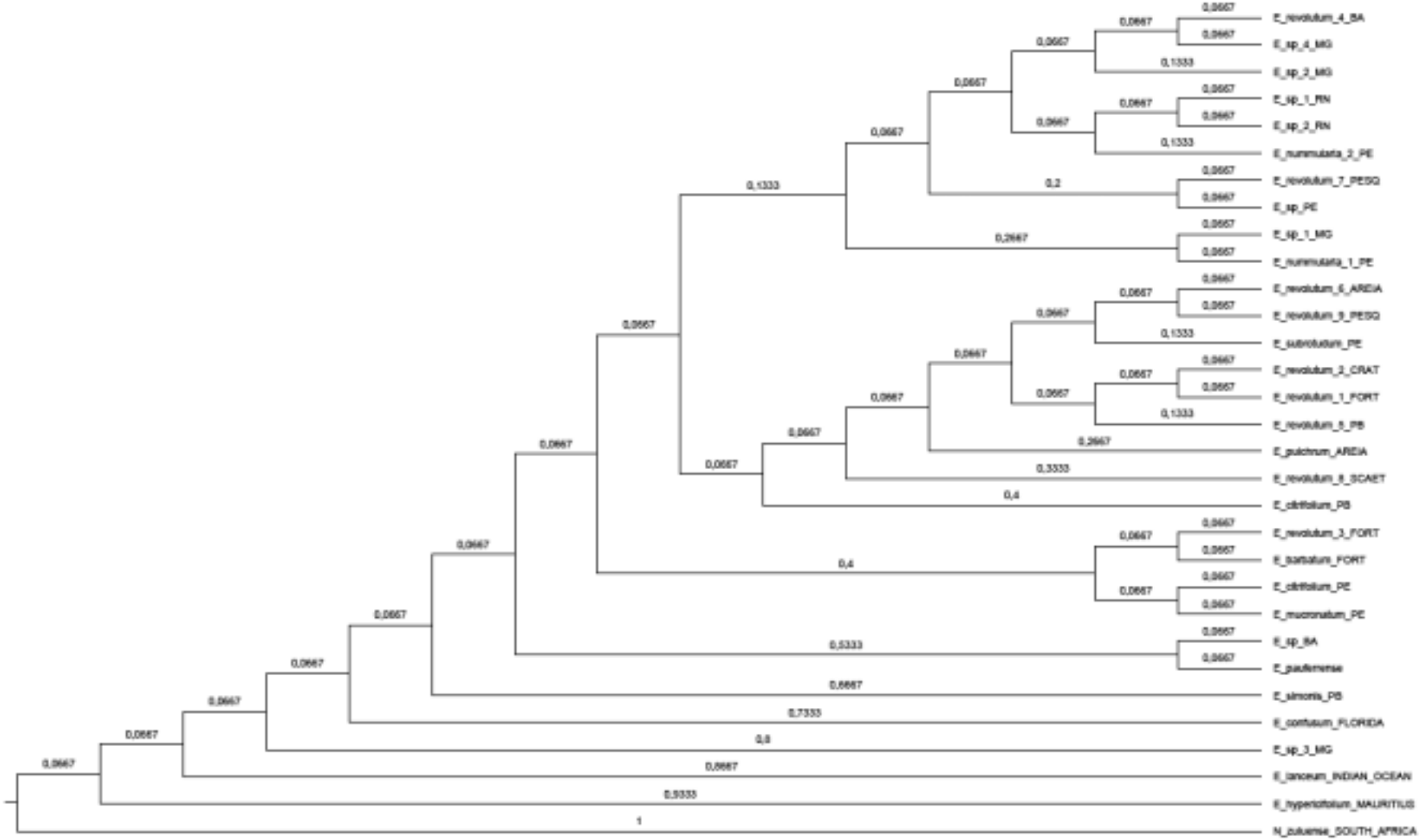
Microsatellite scoring and analyses - Electropherograms were automatically scored with GeneMapper version v5 (Applied Biosystems) and manually corrected. GenAlEx v6.5 (Peakall and Smouse, 2006, 2012) was used to compute the genetic following diversity parameters: observed and (unbiased) expected heterozygosities (*H*O and u*H*E, respectively) and total (*N*A) and private allele (*N*P) numbers. Distance between the individual SSR genotypes was calculated and visualized with a principal coordinate

analysis (PCoA) based on the covariance standardized distance matrix was performed. To investigate population structure, we performed a Bayesian analysis implemented in the software Structure version 2.3.4 (Pritchard, Stephens and Donnelly, 2000). We ran Structure for K = 1-12 with ten replicates each and a model based on admixture and correlated allelic frequencies, without taking into account information regarding sampling localities. Each run had 1 000 000 iterations with a burn-in of 10 000. The optimum K value was determined according to the model values (DK) based on the second- order rate of change, with respect to K of the likelihood function (Evanno, Regnaut and Goudet, 2005). ΔK was calculated in Structure Harvester version 0.6.94 (Earl & von Holdt, 2012).

Results

ITS analysis - The ITS aligned matrix comprised 847 characters, of which 509 are constant and 152 potentially informative for parsimony. The relationships are not well resolved, as we can see in Figure 1, and the species *E. revolutum* and *E. nummularia* appear in different places in the tree. Overall, accessions from geographically close locations tend to be more closely related with those in the same taxonomic section. Sympatric species are more closely related to each other based on their considerable morphological similarities.

Figure 1. Strict consensus tree inferred by maximum parsimony analysis of nuclear DNA sequences (ITS) for *Erythroxylum* species.



Genetic diversity: We tested ten primers from the library, of which two amplify all species studied (Table 2). All primers tested were based on regions with dinucleotide repeats and, in total, 65 alleles were detected at the six loci (four loci from the previous study on Australian taxa, two new loci developed in this study) assessed among 98 samples of 26 *Erythroxylum* populations. The number of alleles per locus ranged from four (Ery7) to 15 (Ery3). The primers proved to be useful in revealing levels of diversity within populations and for exploring the genetic structure of the genus across its current geographical range in north-eastern Brazil. The species with the highest levels of heterozygosity were *E. revolutum* followed by *E. barbatum* (Table 3).

Table 2. Characterization of two polymorphic microsatellite loci for *Erythroxylum*, including locus name, primer sequences, motif and (N) number of individuals.

Locus	**Primer sequence**	**motif**	**Size range (bp)**	**N**
E_5	F: **5´**TGGATGCTTGTTTGTTTAGACC R: **5´**GCTTTGCTTGTTTGATTCTCC	$(AG)_{14}$	180 - 220	98
E_6	F: **5´**GGGTTCATCATCATGCCTTC R: **5´**GATGAACTCCGTCGCAGC	$(AG)_{12}$	200 - 250	98

Table 3. Average value of genetic diversity in populations of *Erythroxylum* species investigated in this study, including sample size (*N*), number of alleles (*Na*), Number of effective alleles (*Ne*), observed and expected heterozygosities (*Ho*, *He*), inbreeding coefficient (*F*).

Specie (Population)		N	Na	Ne	I	Ho	He	uHe	F
E. revolutum (Fortaleza)	**Mean**	1,833	1,833	1,722	0,462	0,250	0,292	0,389	0,200
	SE	0,167	0,401	0,338	0,213	0,171	0,132	0,176	0,327
E. revolutum (Crato)	**Mean**	3,000	2,333	2,129	0,718	0,444	0,454	0,544	0,088
	SE	0,000	0,422	0,359	0,182	0,205	0,101	0,121	0,375
E. revolutum (Areia)	**Mean**	2,667	1,500	1,433	0,328	0,167	0,231	0,278	0,333
	SE	0,333	0,224	0,196	0,147	0,167	0,104	0,125	0,471
E. revolutum (Bahia)	**Mean**	3,833	1,667	1,667	0,414	0,500	0,278	0,324	-0,833
	SE	0,167	0,333	0,333	0,195	0,224	0,127	0,149	0,118
E. revolutum (Guarabira)	**Mean**	1,000	1,333	1,333	0,231	0,333	0,167	0,333	-1,000
	SE	0,000	0,211	0,211	0,146	0,211	0,105	0,211	0,000
E. revolutum (Pernambuco)	**Mean**	4,667	2,000	1,778	0,603	0,500	0,419	0,470	-0,130
	SE	0,211	0,000	0,127	0,059	0,198	0,052	0,058	0,381
E. revolutum (Pesqueira)	**Mean**	2,000	1,000	1,000	0,000	0,000	0,000	0,000	
	SE	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
E. revolutum (São Caetano)	**Mean**	0,500	0,500	0,500	0,000	0,000	0,000	0,000	
	SE	0,224	0,224	0,224	0,000	0,000	0,000	0,000	0,000
E. pulchrum (Paraíba)	**Mean**	1,500	2,000	1,933	0,671	0,583	0,438	0,639	-0,333
	SE	0,342	0,516	0,521	0,180	0,201	0,101	0,152	0,333
Erythroxulum sp_1_MG	**Mean**	3,333	2,333	2,119	0,710	0,431	0,437	0,502	0,074
	SE	0,494	0,333	0,362	0,183	0,151	0,110	0,125	0,212
E. numullaria (Paraíba)	**Mean**	2,000	2,000	2,000	0,578	0,667	0,375	0,500	-0,833
	SE	0,000	0,447	0,447	0,213	0,211	0,125	0,167	0,136

E.citrifolium (Paraíba)	**Mean**	9,000	1,667	1,371	0,306	0,138	0,196	0,207	0,410
	SE	0,683	0,333	0,207	0,154	0,114	0,100	0,106	0,339
E.citrifolium (Pernambuco)	**Mean**	1,000	1,000	1,000	0,000	0,000	0,000	0,000	0,000
	SE	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
Erythroxylum sp_2_MG	**Mean**	4,500	1,667	1,533	0,386	0,167	0,257	0,285	0,333
	SE	0,224	0,333	0,243	0,177	0,167	0,115	0,128	0,471
E. simonis (Paraíba)	**Mean**	9,333	2,167	1,535	0,511	0,213	0,316	0,334	0,211
	SE	0,333	0,401	0,141	0,123	0,083	0,072	0,076	0,257
E.barbatum (Fortaleza)	**Mean**	1,667	1,500	1,500	0,347	0,167	0,250	0,333	0,333
	SE	0,211	0,224	0,224	0,155	0,167	0,112	0,149	0,471
Erythroxylum sp_PE	**Mean**	6,333	1,833	1,431	0,358	0,185	0,211	0,231	0,328
	SE	0,494	0,401	0,255	0,178	0,141	0,105	0,115	0,286
E.pauferrense (Paraíba)	**Mean**	8,833	1,667	1,476	0,342	0,174	0,216	0,233	0,319
	SE	0,654	0,333	0,283	0,178	0,143	0,113	0,123	0,391
Erythroxylum sp_RN	**Mean**	18,167	3,167	2,293	0,840	0,284	0,488	0,502	0,423
	SE	0,833	0,477	0,427	0,173	0,118	0,090	0,092	0,218
E.mucronatum (Paraíba)	**Mean**	0,667	0,833	0,833	0,116	0,167	0,083	0,167	-1,000
	SE	0,211	0,307	0,307	0,116	0,167	0,083	0,167	0,167
Erythroxylum sp_3_MG	**Mean**	2,500	1,500	1,333	0,376	0,389	0,250	0,300	-0,556
	SE	0,500	0,428	0,333	0,170	0,200	0,112	0,134	0,314

PcoA analysis - The principal coordinate analysis produced a partial separation of the individuals and most of the individuals occupying an intermediate position (i.e. occuring in more then one group) were assigned to mixed genetic clusters in the Bayesian analysis (Figure 2). The first and second principal coordinate axes expressed 21.7% of the total variance in the Jaccard dissimilarity matrix analysis revealed some groups; they match the major groups revealed by parsimony. *Erythroxylum nummularia* and *Erythroxylum* sp. (new species from Pesqueira/PE currently being described) are more isolated than the other populations in the scatter plot. We can see the groups below and confirm the match in Figures 2 and Table 4.

Figure 2. Results of the principal coordinates analysis of six microsatellite loci in 26 populations of *Erythroxylum* species

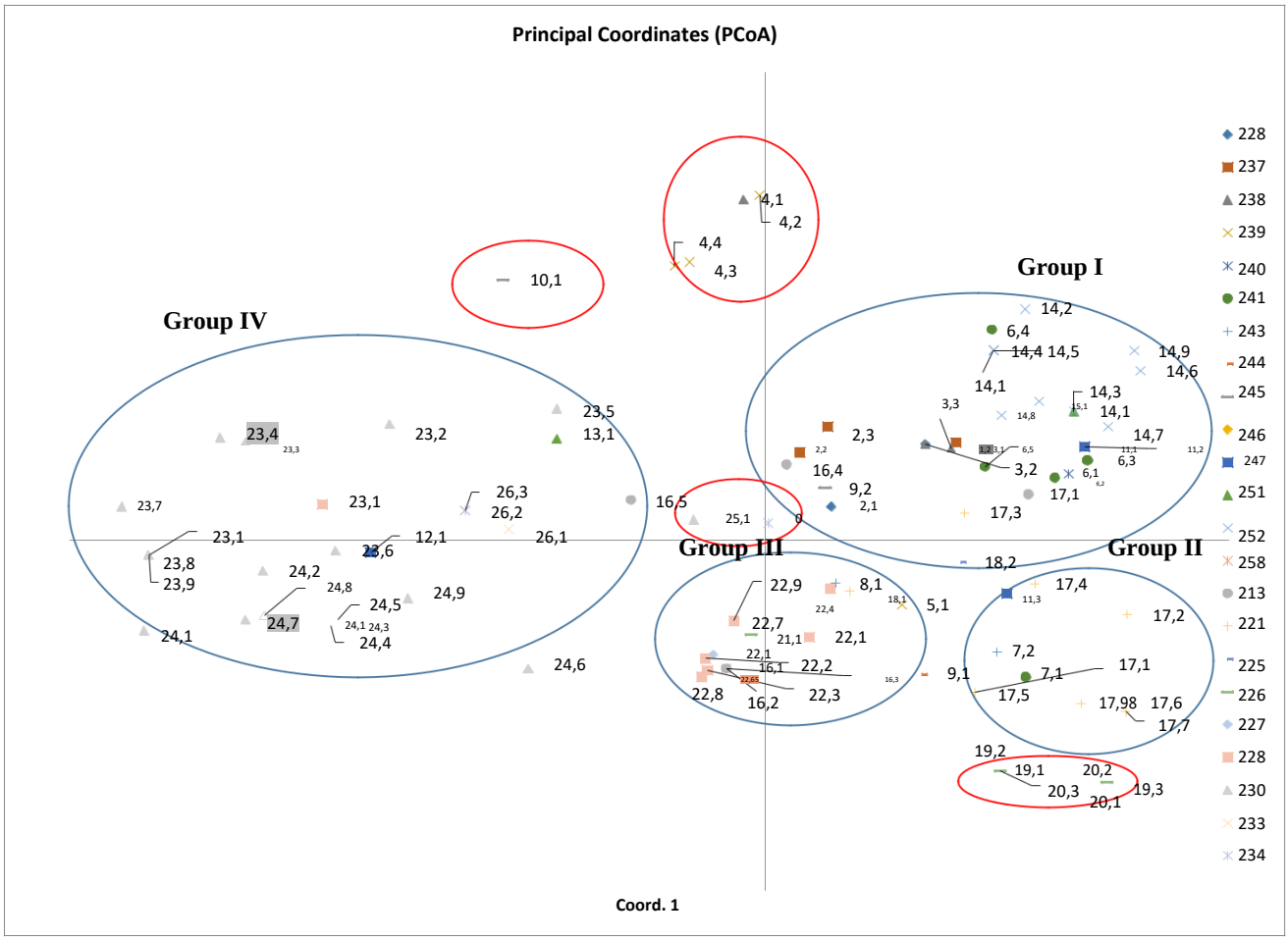
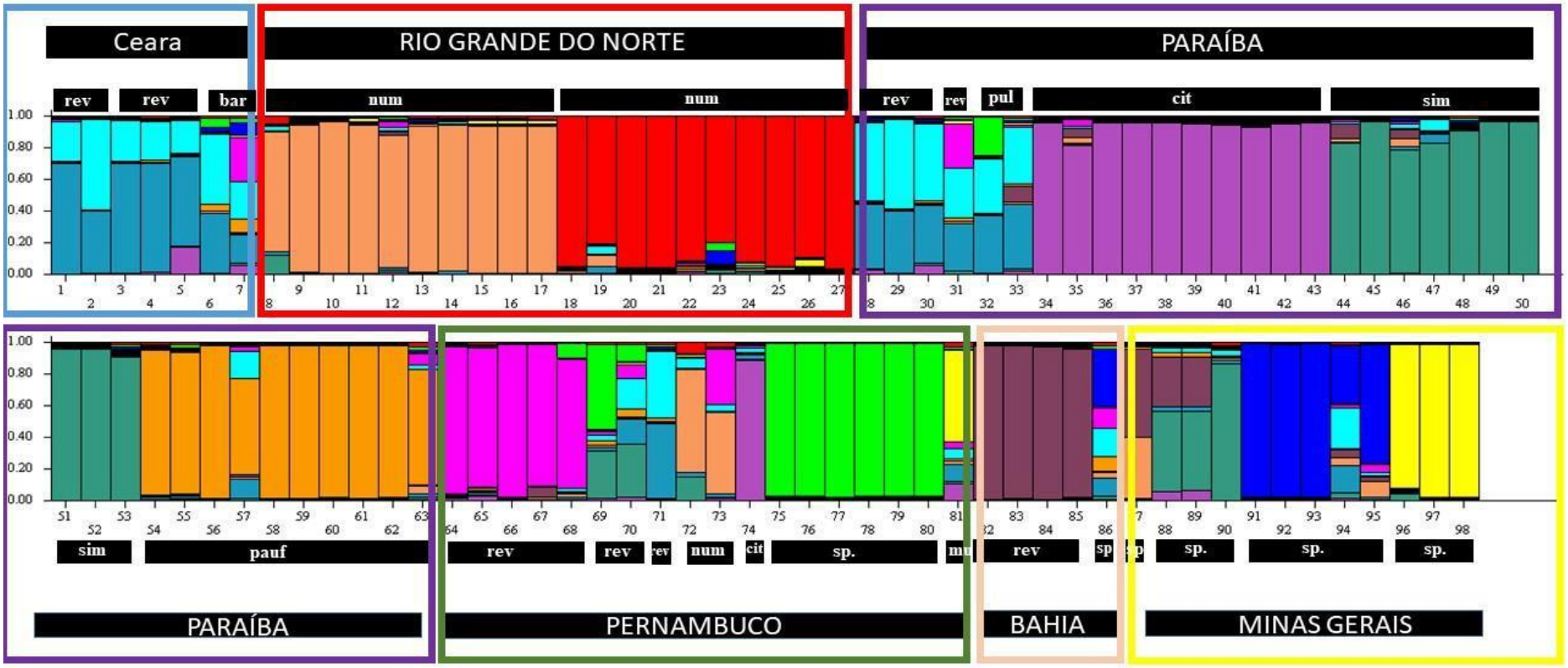


Table 4. PcoA groups made by genetic diversity analysis.

GROUP I	GROUP II	GROUP III	GROUP IV	ISOLATED SPECIES
E. revolutum PE	*Erythroxylum* sp. MG (2)	*E. revolutum* S. CAET/PE	*E. nummularia* PE	*Erythroxylum* sp. (new specie, problably will be called *E. xucuruensis*) Pesqueira/PE
E. revolutum FORT/CE	*E. revolutum* PESQU/PE	*E. revolutum* GUARAB/PB	*E. nummularia* RN	*E. revolutum* BA
E. revolutum CRAT/CE	*E. simonis* AREIA/PB	*Erythroxylum* sp. BA		*Erythroxylum* sp. MG
E. revolutum AREIA/PB		*E. pauferrense* AREIA/PB		
Erythroxylum sp. MG (1, 2)		*E. pulchrum* AREIA/PB		
E. citrifolum PB		*E. barbatum* FORT/CE		
E. citrifolum PE		*Erythroxylum* sp. MG		
E. simonis PB		*Erythroxylum* sp. MG		
E. barbatum CE				

Structure analysis – Our results showed that most of species are related by geographic location, and sympatric species share some genetic data, Hybrid zones probably occur in some areas, as can be seen between *E. revolutum* and *E. barbatum* in Ceará populations and between *E. revolutum* and *E. pulchrum* in Paraíba populations (Figure 3). This can also be observed in the PCOA results (Table 4), corroborating the hypothesis that the sympatry could be promoting gene flow between species.

Figure 3. Results of the Structure analysis of six microsatellite loci in 26 populations of *Erythroxylum* species. Abbreviations:rev=*Erythroxylum revolutum;* bar=*Erythroxylum barbatum*; num=*Erythroxylum nummularia*; pul=*Erythroxylum pulchrum*; cit=*Erythroxylum citrifolium*; sim=*Erythroxylum simonis*; pauf= *Erythroxylum pauferrense*; sp=*Erythroxylum* sp.



Discussion

This study represents the first approach that comprehensively investigates the genetic diversity in different collections of *Erythroxylum* species using ITS and microsatellite markers. The hypothesis that sympatry probably favours gene flow between different species seems to have been corroborated since samples were mostly grouped by their sympatry group.

Our analyses do not support linear evolution, and all results show that the sampled sections of *Erythroxylum* are para- or polyphyletic and suggest an independent, non-linear evolutionary relationship between *Erythroxylum* species, and despite the incongruence at the level of section and species, the analyses identified significantly different genetic groups within *Erythroxylum.* These groups do not agree well with the morphological sections defined by Schulz (1907), and they show stronger geographical patterns, suggesting that the current intrageneric classification of this genus needs be revised. Although, modern day Ghana and Northern Brazil were once united as part of the supercontinent Gondwanaland, the relationship between *Erythroxylum* sp._3 from Minas Gerais and *E. lanceum* from the Indian Ocean is far more likely to reflect long-distance dispersal or to be an artefact of inadequate sampling than an Gondwanan pattern, as the taxa involved are much younger than the split of Gondwana. Much more thorough investigation, with greater sampling, would be needed.

PCoA and parsimony analysis revealed a clear separation of the accessions into four major groups. *Erythroxylum revolutum* showed the greatest level of variation, suggesting that there are probably subspecies or that the species is not correctly circumscribed. This analysis provides the first overview of phylogenetic relationships in *Erythroxylum*, but the lack of resolution in various parts of phylogenetic trees suggests the need to incorporate more characters into the analysis to achieve a clearer picture of the boundaries and relationships of the species involved.

In the microsatellite analysis, the observed and expected heterozygosities ranged from 0.083 to 0.667 and from 0.072 to 0.488, respectively (Table 3). The genetic diversity indexes found in the present study can be attributed, at least partially, to the fact that some species show interspecific gene flow mediated by sympatry. These results may be connected to the results presented in a first paper that is part of this work, showing how the influence of pollinator displacement could result in the exchange of pollen between sites, promoting crossing between related individuals. The parameters of genetic diversity and structure estimated in this study indicate that populations of *Erythroxylum* present genetic diversity indexes and moderate gene flow among populations. There is also a high level of

morphological variation, meaning that it is not possible to identity each population. Microsatellite markers proved to be effective for evaluating genetic relationships between genetic diversity and geographic origins. Despite the fact that the studied populations did not include the same number of sampled individuals, we demonstrated in our study the existence of some diversification pools and high diversity levels and this data can be used to optimize and clarify the currently classification of *Erythroxylum*.

Conclusion

These are the first microsatellites, of which we are aware of, developed within Brazilian *Erythroxylum* species, a genus more well known for *E. coca* from which cocaine is extracted. These primers showed a relatively high level of polymorphism, and therefore they should be useful for population studies; these loci will be used to study population genetic diversity, gene flow, and mating systems, combined with the previously isolated loci. The markers will facilitate the further investigation of parentage analyses and kinships between the planted and natural populations of *Erythroxylum*, contributing to our knowledge of diversification processes and conservation in Brazilian *Erythroxylum* species.

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ANEXOS

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