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DIVERSIDADE GENÉTICA E ECOLÓGICA DE ABELHAS DAS ORQUÍDEAS EM DIFERENTES FORMAÇÕES VEGETAIS BRASILEIRAS

DENILSON COSTA MARTINS

São Luís - MA
2022

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE, na Universidade Federal do Maranhão, como requisito parcial para a obtenção do Título de Doutor em Biodiversidade e Biotecnologia

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quem perdi no decorrer do caminho (2019).
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Amo você!

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“Sonhei que estava caminhando na praia juntamente com Deus.
E revi, espelhado no céu, todos os dias da minha vida.
E em cada dia vivido, apareciam na areia, duas pegadas:
as minhas e as d’Ele.
No entanto, de quando em quando, vi que havia apenas as minhas pegadas, e isso precisamente nos dias mais difíceis da minha vida.
Então perguntei a Deus:
"Senhor, eu quis seguir-Te, e Tu prometeste ficar sempre comigo.
Porque deixaste-me sozinho, logo nos momentos mais difíceis?
Ao que Ele respondeu:
"Meu filho, Eu te amo e nunca te abandonei.
Os dias em que viste só um par de pegadas na areia são precisamente aqueles em que Eu te levei nos meus braços."

(Pegadas na Areia, Margaret Fishback Powers)

MARTINS, Denilson Costa. **Diversidade Genética e Ecológica de Abelhas das Orquídeas em Diferentes Formações Vegetais Brasileiras**, 2022, 145f. Tese (Doutorado em Biodiversidade e Biotecnologia – Rede BIONORTE) – Universidade Federal do Maranhão, São Luís, 2022.

RESUMO

As abelhas da tribo Euglossini (Apidae) desempenham um importante papel na manutenção das florestas neotropicais, pois são polinizadoras de várias famílias de plantas. O aspecto mais notório das abelhas das orquídeas é a busca por compostos químicos presentes nas plantas por parte dos machos desta tribo. O Brasil possui as duas formações de florestas tropicais úmida com maior diversidade de espécies do mundo, a Floresta Amazônica e a Mata Atlântica, que intercaladas pelo Cerrado reúnem a maior biodiversidade da América do Sul. A distribuição amostral dos levantamentos da fauna de abelhas das orquídeas não é homogênea, a maioria das áreas sequer são amostradas. Além do número insuficiente de inventários, há uma incipiência com relação ao conhecimento ecológico e sobre a diversidade genética das populações do grupo. Assim, o presente estudo tem como finalidade investigar com base em marcadores moleculares mitocondriais (*16S* e *COI*) e nucleares (*Opsina*), a diversidade e estrutura genética das populações de duas espécies que possuem uma íntima relação de parasita-hospedeiro, no caso *Eulaema nigrita* Lepeletier, 1841 (hospedeira) e *Exaerete smaragdina* (Guérin, 1844) (parasita) ao longo da Mata Atlântica e outras formações brasileiras. Em conjunto com os dados genéticos, avaliar a riqueza de espécies, composição e abundância das comunidades de abelhas Euglossini em remanescentes de Cerrado e Floresta Amazônica presentes na área de transição do nordeste (TN), área em que se encontra o estado do Maranhão. Os resultados das análises de Bayesian Skyline Plot (BSP), as redes de haplótipos, testes de neutralidade e análises de diversidade genéticas baseado no gene *COI* indicam expansão populacional de *El. nigrita* e *Ex. smaragdina* durante o Pleistoceno. Nossos dados revelaram áreas com elevada diversidade genética consideradas potenciais refúgios durante as oscilações climáticas do Pleistoceno na Mata Atlântica, em Pernambuco para *Ex. smaragdina* (Hd=0,750±0,139) e Espírito Santo para *El. nigrita* (Hd=1±0,272). Para esta última espécie os padrões mais elevados de diversidade genética (Hd=0,873) foram encontrados na região TN, e corroborando os dados da literatura esta área foi associada a um potencial refúgio para as abelhas Euglossini durante as oscilações climáticas no Pleistoceno. Alinhados a estes dados, foram encontrados elevados valores de riqueza de espécies no Cerrado (24 espécies) e Floresta Amazônica (42 sp) presentes na região TN. Com destaque para Área de Proteção Ambiental das Reentrâncias Maranhense em que foram encontrados um dos mais altos valores de riqueza em estudos com abelhas das orquídeas do Brasil. Tanto os dados genéticos (gene *COI*), quanto dados de estrutura de comunidades indicam que a área de transição do nordeste teve papel importante na história evolutiva das abelhas das orquídeas.

Palavras-chave: Euglossini; cleptoparasitismo; variabilidade genética; Pleistoceno; riqueza de espécies; conservação de abelhas.

MARTINS, Denilson Costa. **Genetic and Ecological Diversity of Orchid Bees in Different Brazilian Vegetal Formations**, 2022, 145f. Thesis (PhD in Biodiversity and Biotechnology – BIONORTE) - Federal University of Maranhão, São Luís, MA-Brazil, 2022.

ABSTRACT

The bees of the tribe Euglossini (Apidae) perform an important role in the maintenance of Neotropical forests, as they pollinate several families of plants. The most notable aspect of orchid bees is the search for chemical compounds present in plants, by the males of this tribe. Brazil has the two humid tropical forest formations with the highest diversity of species in the world, the Amazon forest and the Atlantic forest, which, interspersed with the Cerrado domain, contain the highest biodiversity in South America. The sampling distribution of the surveys of the orchid bee fauna is not homogeneous, most areas are not even sampled. Besides the insufficient number of inventories, there is an incipiency regarding the ecological knowledge and the genetic diversity of the populations of the group. Thus, the present study aimed to investigate, based on mitochondrial (*16S* and *COI*) and nuclear (*Opsin*) molecular markers, the genetic diversity and structure of populations of two species that have an intimate host-parasite relationship, in this case *Eulaema nigrita* Lepeletier (host) and *Exaerete smaragdina* (Guérin) (parasite) throughout the Atlantic forest and other Brazilian formations. In association with the genetic data, assess the species richness, composition and abundance of the Euglossini bee communities in Cerrado and Amazon forest remnants present in the northeastern transition area (TN), an area in which the state of Maranhão is located. The results of Bayesian Skyline Plot (BSP) analyses, haplotype networks, neutrality tests and genetic diversity analyses based on *COI* gene indicate population expansion of *El. nigrita* and *Ex. smaragdina* during the Pleistocene. Our data revealed areas with high genetic diversity considered potential refugia during Pleistocene climatic ocillations in the Atlantic forest, in Pernambuco for *Ex. smaragdina* (Hd=0.750±0.139) and Espírito Santo for *El. nigrita* (Hd=1±0.272). For the latter species the highest diversity patterns (Hd=0.873) were found in the TN region and corroborating with literature data this area was associated with a potential refuge for Euglossini bees during climatic oscillations in the Pleistocene. Aligned with these data, high species richness values were found in the Cerrado (24 species) and Amazon forest (42 sp) present in the TN region. With highlights for the *Reentrâncias Maranhense* Environmental Protection Area of where one of the highest richness values in studies with orchid bees in Brazil was found. Both genetic (*COI* gene) and community structure data indicate that the northeastern transition area played an important role in the evolutionary history of orchid bees.

Keywords: tribe Euglossini; cleptoparasitism; genetic variability; Pleistocene; species richness; bee conservation.

LISTA DE FIGURAS

REFERENCIAL TEÓRICO

Capítulo I

Material Suplementar

Capítulo II

Capítulo III

LISTA DE TABELAS
Capítulo I

Capítulo II

LISTA DE ABREVIATURAS

- **AM –** Floresta Amazônica
- **AMOVA –** Análise de variância molecular
- **AF –** *Atlantic forest* **ou** Mata Atlântica
- **ArgK –** Arginina Kinase
- ***COXI* - S**ubunidade I do gene mitocondrial citocromo c oxidase
- ***Cyt b*** - Citocromo oxidase b
- **DNA –** Ácidos desoxirribonucleico
- **DNA *barcode*** – Ou códigos de barra de DNA são sequências curtas de DNA, amplificadas por PCR e sequenciadas. Elas podem ser utilizadas na distinção e identificação de espécies
- **ESU –** do inglês "*Evolutionarily Significant Units***"** a unidade evolutiva significativa é uma linhagem demonstrativamente com fluxo gênico altamente restrito de outras linhagens também incluídas no nível organizacional mais elevado das espécies
- **MSP -** Mirador State Park, Parque Estadual do Mirador
- **mtDNA –** DNA mitocondrial
- **nuDNA –** DNA nuclear
- **EF1-α –** Fator de Alongamento 1 alfa
- **PCR -** Reação em Cadeia da Polimerase
- ***Pol-II* –** RNA polimerase II
- **UHF – *"****uncorrelated frequency model"*, modelo não correlacionado as frequências haplotipicas do programa Geneland
- ***Opsin* –** gene Opsina
- **RNA –** Ácido ribonucleico
- ***16*S -** O gene da subunidade maior do rRNA
- **rRNA –** RNA ribossômico
- **TN –** Áreas de Transição no Nordeste Brasileiro, aqui considerados os pontos nos estados do Maranhão e Ceará.
- **tRNA** – RNA transportador
- **UCEs –** "*Ultraconserved elements*", elementos ultraconservados do genoma que são regiões altamente conservadas de genomas dos organismos compartilhados entre táxons evolutivos distantes
- **UPGMA** – "*unweighted pair group method with arithmetic mean"*, é um método de agrupamento hierárquico aglomerativo simples

SUMÁRIO

1 INTRODUÇÃO GERAL

As abelhas são organismos essenciais para a existência das plantas com flores em todo o mundo, devido ao papel indispensável dos serviços de polinização, manutenção e desenvolvimento das florestas (SILVEIRA, ALMEIDA; MELO 2002; ROUBIK; HANSON, 2004). Dentre estes elementos mantenedores das florestas úmidas neotropicais, destacam-se as abelhas das orquídeas (Hymenoptera: Apidae) como são chamadas popularmente as espécies de abelhas da tribo Euglossini.

A tribo Euglossini agrupa 250 espécies divididas em cinco gêneros viventes: *Aglae* Lepeletier & Serville, 1825 (*Ag.*); *Eufriesea* Cockerell, 1908 (*Ef.*); *Euglossa* Latreille, 1802 (*Eg.*); *Eulaema* Lepeletier, 1841 (*El.*); *Exaerete* Hoffmannsegg, 1817 (*Ex.*) e um gênero fóssil encontrado na República Dominicana chamado *Paleoeuglossa* Poinar, 1998 (MOURE; MELO, 2022). Características particulares como o longo comprimento da glossa, alinhados ao tegumento metálico brilhante, porte médio a grande (8,5mm a 29 mm) e uma série de estruturas morfológicas relacionadas a coleta de compostos químicos (MICHENER 2007), chama a atenção de pesquisadores e naturalistas de todo o mundo (DODSON *et al.* 1969).

As abelhas das orquídeas são exímias polinizadoras de longa distância, aspecto garantido pelo seu aparato morfológico que permite aos euglossine explorar mais de 60 famílias de plantas (GIANNINI *et al.*, 2015; RAMÍREZ; DRESSLER; OSPINA, 2002). Além disto sua grande capacidade de voo relatada na literatura como de aproximadamente 40 km no caso dos machos e de 23 km para fêmeas (JANZEN 1971, PORKONY *et al.* 2015), torna-se vital no carreamento de pólen entre espécimes de plantas distantes em grandes blocos florestais remanescentes (ROUBIK; HANSON, 2004).

Embora o grupo seja bem estudado com relação a taxonomia e sistemática, ainda pouco se sabe sobre as interações ecológicas existentes entre as abelhas das orquídeas. Além disto, dados básicos como a estrutura das comunidades em formações como a Floresta Amazônica, centro de origem do grupo (RAMÍREZ *et al.*, 2010), domínio de Cerrado, por exemplo, ainda são bastante escassos. Em relação a estrutura genética das populações destes organismos nestes ambientes, os dados são ainda mais limitados.

Informações sobre a variabilidade genética associadas as relações ecológicas de cleptoparasitismo são muito pouco exploradas na literatura, principalmente entre as espécies de Euglossini (SILVA *et al.* 2009). Um exemplo disso é a interação de cleptoparasitismo entre as espécies *El. nigrita* Lepeletier, 1841 (hospedeira) e *Ex. smaragdina* (Guérin, 1844) (cleptoparasita), que são fortalecidas por informações sobre a dinâmica populacional, a diversidade e estrutura genética destas espécies coevolutivamente relacionadas. Portanto, pouco

se sabe sobre estas espécies quanto ao comportamento, interações e diversidade genética. Um exemplo disso é a dúvida sobre quais espécies de Euglossini são parasitadas por *Ex. smaragdina*, pontos que tornam os estudos sobre abelhas das orquídeas interessantes em diversos aspectos.

De forma geral, o presente estudo propõe-se a entender a dinâmica de algumas espécies de abelhas das orquídeas que habitam os domínios florestais de Cerrado, Floresta Amazônica (do inglês *Amazon forest*: AM) e Mata Atlântica (do inglês *Atlantic forest*: AF). Neste contexto, por situar-se em uma zona de transição, o estado do Maranhão constitui uma área interessante para estudos abordando a diversidade genética e ecológica de abelhas. A reunião de diferentes formações vegetais como o Cerrado, Floresta Amazônica e a Caatinga nordestina possibilita a superposição de elementos da fauna destas formações promovendo uma elevada variabilidade genética e ainda uma alta diversidade de espécies.

Para tanto, dividimos o trabalho em três capítulos. No **Capítulo I** investigou-se, com uso de marcadores mitocondriais (genes *16S* e *COI*) e nucleares (gene *Opsina*), a diversidade e estrutura genética de duas espécies de abelhas Euglossini em interação ecológica de cleptoparasitismo, neste caso *El. nigrita* (hospedeira) e *Ex. smaragdina* (cleptoparasita). A variabilidade genética destas espécies foi estudada em área de domínios florestais da Amazonia, Cerrado e Caatinga, e principalmente ao longo da Mata Atlântica que foi o domínio mais bem representado neste estudo. Neste capítulo foi identificada na área de Transição do Nordeste (TN) brasileiro, em que se encontra os estados do Maranhão e Ceará, uma elevada diversidade genética para *Eulaema nigrita* possivelmente relacionada à presença de uma região de refúgio pleistocênico, como relatado por López-Uribe *et al.* (2014)

No **Capítulo II**, utilizando de dados ecológicos como riqueza de espécie, composição e abundância buscamos caracterizar a fauna de abelhas Euglossini de duas fitofisionomias de Cerrado: Cerrado *stricto sensu* (Css) e floresta de galeria (GF, do inglês *gallery forest*) presentes em um remanescente de cerrado localizado no Parque Estadual do Mirador no estado do Maranhão (MSP, Mirador State Park). Analisamos a diversidade e composição de espécies, além da adequação das amostragens aos dois ambientes estudados, e verificamos a preferência dos machos de Euglossini por determinadas iscas.

Ainda com base em dados de diversidade de espécies de Euglossini do Maranhão, no **Capítulo III**, identificamos na região da costa leste do Maranhão, na Área de Proteção das Reentrâncias Maranhenses no município de Cururupu e Mirinzal, uma área com a maior riqueza de espécies de Euglossini já registrada no Brasil (42 espécies). Esta mesma área se sobrepõe a área identificada no Capítulo I como um possível refúgio durante as oscilações climáticas do Pleistoceno. Para este capítulo, buscou-se investigar a possível variação na riqueza de espécies,

composição e abundância entre dois métodos de coleta (armadilhas odoríferas e rede entomológica), baseados no perfil de diversidade de Hill (*Hill numbers*), verificando ainda o efeito da flutuação na abundância das abelhas das orquídeas durante o ano.

Esperamos com essa tese, uma melhor compreensão das interações ecológicas entre *Eulaema nigrita* e *Exaerete smaragdina* nos ambientes de Floresta Amazônica e Mata Atlântica, assim como a identificação de área com elevada diversidade genética para estas espécies, interessantes para elaboração de estratégias de conservação destas abelhas. Espera-se ainda o reconhecimento da diversidade e composição de espécies para os remanescentes florestais encontrados no Cerrado e Floresta Amazônica do Maranhão, áreas importantes para a permanência de várias espécies associadas a ambientes mais preservados como as abelhas das orquídeas.

2. REFERENCIAL TEÓRICO

2.1 ABELHAS CORBICULADAS

No mundo existem mais de 20.000 espécies de abelhas descritas (ASCHER; PICKERING 2020), as quais integram sete famílias da superfamília Apoidea (Hymenoptera, subgrupo Apiformes), são estas: Andrenidae Latreille (49 gêneros ou subgêneros), Apidae Latreille (85), Colletidae Lepeletier (37), Halictidae Thomson (61), Megachilidae Latreille (75), Melittidae Schenck (68) e Stenotritidae Cockerell (36), isto segundo classificação de Michener (2007). Para a Região Neotropical existem aproximadamente 15.200 espécies registradas em cinco destas sete famílias, estando ausentes as famílias Stenotritidae e Melittidae (FREITAS *et al.*, 2009).

Em relação a origem e diversificação das abelhas, estimou-se que o ancestral comum a todas as abelhas deve ter se originado há aproximadamente 123 milhões de anos (m.a), no Cretáceo inferior, já os grupos conhecidos atualmente, provavelmente se diversificam no período de transição entre o Cretáceo e Paleogeno (~65,5 m.a), sendo mais recente que a origem das Angiospermas, Médio-Cretáceo ~143 m.a (CARDINAL; DANFORTH, 2013). A época estimada para a origem das abelhas é congruente com o período de maior diversificação das eudicotiledóneas, durante o Cretáceo-Médio (CREPET, 2008). Antes disto, os registros fósseis para Angiosperma indicam a existência de flores com características semelhantes as polinizadas por Coleoptera, Diptera e Lepidoptera.

Entre as famílias de abelhas, Apidae é a mais bem estudada e destaca-se por ser o grupo mais diversificado, englobando o maior número de gêneros, cerca de 85 deles, com distintos aspectos morfológicos e comportamentais (PEREIRA; GONÇALVES; RAMOS, 2021). Michener (1999) subdividiu os Apinae em Apini, Bombini, Euglossini e Meliponini, com base na presença da corbícula, uma estrutura especializada para o transporte de pólen, presentes no terceiro par de pernas das fêmeas. Por isso as quatro tribos são denominandas como abelhas corbiculadas (MICHENER, 1999). Esta classificação tem um contexto histórico e econômico, uma vez que os corbiculados incluem espécies de relevância econômica como as abelhas do mel ou melíferas (*honey bees*, Apini), as *bumble bees* (Bombini) e as abelhas sem ferrão (Meliponini), usadas como fonte de mel, própolis, geoprópolis, apitoxina, polén, geleia real ou na polinização, através da criação racional, em diversos cultivos (MICHENER, 2007).

Embora os estudos filogenéticos concordem na monofilia dos corbiculados, as relações entre estas quatro tribos de abelhas ainda são controversas. Alguns estudos com base em caracteres morfológicos como comprimento da língua, morfologia das pernas posteriores, aliados a dados moleculares (*e.g. 16S, 28S, Cyt b* e *Opsin)* não apresentavam uma relação clara

quanto a posição dos Euglossini como grupo irmão dos demais corbiculados (CAMERON, 2004; CAMERON; MARDULYN, 2001), mas apontam diferentes posições para os Euglossini como grupo irmão dos demais corbiculados, ora próximos dos Bombini, ora próximos dos Apini (Figura 1.).

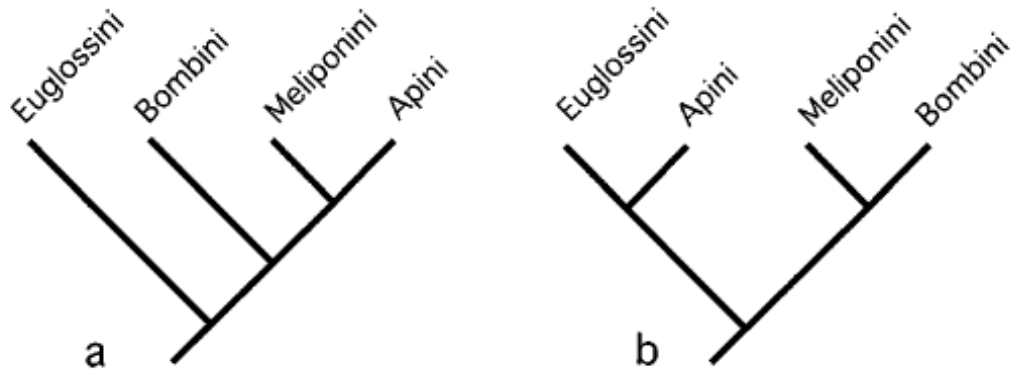

Figura 1 - Relações filogenéticas entre as tribos de corbiculados (a) baseada em caracteres morfológicos e (b) a partir de sequencias de genes como *16S, 28S, citocromo oxidase c*, e *Opsina* (CAMERON, 2004).

2.2 NÍVEIS DE ORGANIZAÇÃO SOCIAL NOS APIDAE CORBICULADOS

Dentre os Apidae corbiculados, Apini e Meliponini incluem as espécies eussociais, Bombini as espécies sociais e em Euglossini estão as espécies que possuem comportamento tanto social quanto solitário. Augusto e Garófalo (2007) destacam que os Euglossini podem exibir dois níveis de organização sociais: (i) primitivamente social – em que a fêmea fundadora ovoposita em uma quantidade de células que podem chegar até 14 no total, após isto ela interrompe a postura até a emergência das filhas (associação matrifilial). Este comportamento estabelece uma relação de dominância reprodutiva, em que a fêmea fundadora se alimenta das posturas realizadas pelas filhas (oofagia), permanecendo somente os seus ovos no ninho. Isto é comum em espécies como: *Eg. cordata* Linnaeus, 1758 e *Eg. townsendi* Cockerell, 1904; e (ii) comunal - duas ou mais fêmeas podem dividir o ninho, contudo não há divisão de trabalho, pois estas atuam como se fosse ninhos independentes (AUGUSTO e GARÓFALO 2007; AUGUSTO; GARÓFALO, 2011; FREIRIA; GARÓFALO; DEL LAMA, 2017), exemplo: *Eg. annectans* Dressler, 1982 e *El. nigrita* (ZUCCHI; SAKAGAMI; CAMARGO, 1969).

Nas espécies eussociais (Apini e Meliponini), desde poucas a milhares de fêmeas adultas convivem em um único ninho, compartilhando tarefas em sua manutenção, havendo uma divisão de trabalho reprodutivo entre as fêmeas (operarias e rainha) e sobreposição de gerações (MICHENER, 2017). Em colônias como de Apini e Meliponini, grupos que exibem comportamento altamente eussocial, as operárias ficam com a responsabilidade de cuidar,

guardar e alimentar a prole gerada pelas rainhas que é morfologicamente distinta das demais abelhas corbiculadas (MICHENER, 2007).

De forma geral, a maioria das abelhas apresenta comportamento solitário, cujas fêmeas constroem seus próprios ninhos, fornecendo abrigo e alimentação a suas crias (BAWA, 1990; MICHENER, 2007). Uma variação a este comportamento é o comportamento comunal, em que espécies solitárias compartilham um mesmo ninho (MICHENER, 2007). Contudo, como já destacado acima, níveis mais elaborados de organização social podem estar presentes em Euglossini, isto torna os Euglossini grupo chave para o entendimento do surgimento do comportamento eussocial em abelhas (RAMÍREZ; DRESSLER; OSPINA, 2002).

2.3 CLEPTOPARASITISMO EM ABELHAS CORBICULADAS

Assim como em outros grupos de abelhas, o cleptoparasitismo é um comportamento também presente entre as abelhas corbiculadas (MICHENER, 2017). Na relação de cleptoparasitismo, as fêmeas invadem o ninho de outras espécies e colocam seus próprios ovos nas células de cria das abelhas hospedeiras, muitas vezes matando a cria destas e substituindo-as pelos próprios ovos (GARÓFALO; ROZEN, 2001; WCISLO, 1987). Até então apenas três estudos têm reportado observações diretas do comportamento cleptoparasita em ninhos de abelhas das orquídeas (ZUCCHI *et al.* 1969; BENNETT, 1972; GARÓFALO; ROZEN , 2001), sendo assim um tema pouco explorado

O comportamento cleptoparasita evoluiu a partir de taxa evolutivamente relacionados, os quais se tornaram seus hospedeiros, ou seja, as espécies parasitas e hospedeiras tem origem a partir de um ancestral comum, portanto, espécies irmãs (regra de Emery) (WILSON 1971). Com isto, estas espécies incluídas nesta relação ecológica apresentam sobreposição parcial de nicho ecológico que possibilita a sobrevivência de ambas em determinados habitats, compartilhando assim aspectos como equivalência em seus tamanhos corpóreos, necessidades nutricionais semelhantes na fase larval, coabitação em um determinado habitat e outros recursos (ROUBIK *et al.* 2019). Além disto, para que as espécies cleptoparasitas tenham sucesso existe uma correlação dependente de densidade entre as frequências delas no ambiente, ou seja, em áreas em que o hospedeiro é raro ou ausente as abelhas cleptoparasitas não conseguem se estabelecer (WCISLO 1987).

Na primeira revisão sobre biologia do ninho de abelhas das orquídeas, Zucchi *et al.* (1969) destacaram que 50% das células de um ninho de *El. nigrita* (hospedeira) foram parasitadas por *Ex. smaragdina* (cleptoparasita), revelando uma alta habilidade das fêmeas desta última espécie em depositar seus ovos nas células de *El. nigrita*. Além destas observações, Bennett (1972) relata o ataque de *Ex. dentata* (Linnaeus, 1758) a ninhos de *Ef. surinamensis*

Linnaeus, 1758. E em um segundo estudo sobre a relação entre *El. nigrita* e *Ex. smaragdina*, Garófalo e Rozen (2001) trazem detalhes do desenvolvimento larval de *Ex. smaragdina* (Figura 2).

De forma geral, pouco se sabe sobre a biologia e comportamento de espécies parasitas do gênero *Exaerete* (MICHENER, 2007; ROUBIK, 2019). Ainda a respeito da relação de parasitismo entre estas duas espécies mencionadas acima, Nemésio e Silveira (2006) testaram a hipótese de associação parasita-hospedeiro entre as espécies mencionadas acima, com base na correlação de dados de frequências relativas das mesmas, e verificaram uma fraca correlação entre os dados de ambas. Fato que possivelmente está associado a limitação na amostragem de *El. nigrita* em ambientes de Floresta Amazônica (NEMÉSIO; SILVEIRA, 2006), o que possivelmente causou um viés em seus dados de frequência.

Estes mesmos autores destacam que *El. nigrita* está ausente em várias áreas da Bacia Amazônica, mesmo em áreas fragmentadas (POWELL; POWELL, 1987; BOTSCH *et al.*, 2017). Embora *El. nigrita* tenha uma distribuição mais ampla, esta é mais comum em áreas de latitudes mais altas no sul e sudoeste do Brasil, onde a pressão de cleptoparasitismo por parte de *Ex. smaragdina* é menor (NEMÉSIO; SILVEIRA, 2006), o que parece uma estratégia evolutiva do hospedeiro para evitar o parasitismo em áreas mais florestadas.

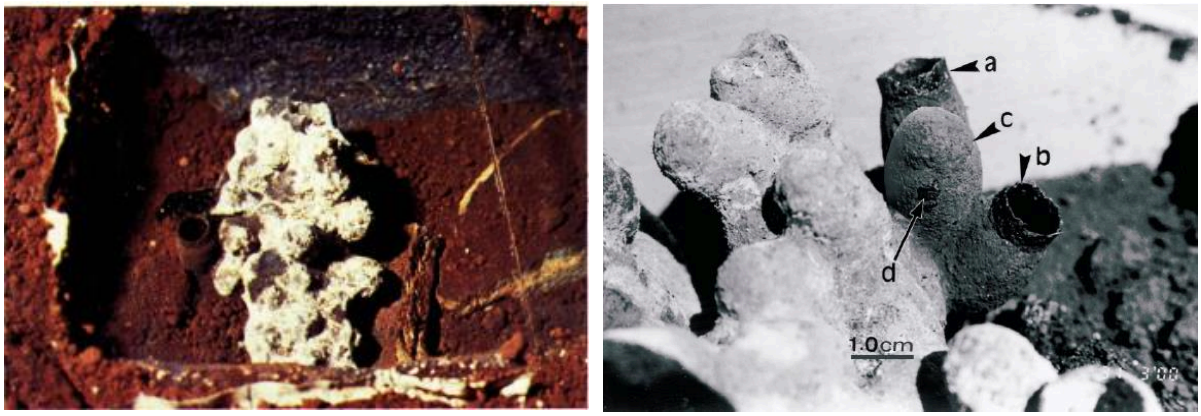

Figura 2 - Observações feitas por Zucchi *et al.* (1969) em ninhos de *Eulaema nigrita* sendo provisionadas (esquerda). Detalhes de um ninho parasitado por *Exaerete smaragdina* com algumas células abertas pelo parasita (a e b) e célula parasitadas já fechada (c), destacando-se a cicatriz do local de fechamento feita por *Exaerete smaragdina* (seta d) (direita). As novas células (células escuras) foram anexadas a um conjunto de células mais antigas (células claras) construído durante o primeiro processo de reutilização do ninho por *Eulaema nigrita* Adaptado de Garófalo e Rozen (2001).

Além disto, embora as espécies possuam uma estreita relação coevolutiva, ligada principalmente pelo comportamento cleptoparasítico, estas abelhas apresentam diferentes afinidades por ambientes florestais. Enquanto *El. nigrita* tem sido constantemente associada a ambientes mais abertos como os presentes nos domínios de Cerrado e Caatinga, sendo devido a isto considerada como bioindicadoras de áreas florestais degradadas (PERUQUETTI *et al.*,

1999; SILVA; DE MARCO, 2014), *Ex. smaragdina* é reportada como tendo maior afinidade por florestas (NEVES; VIANA, 2003; MARTINS *et al.*, 2018). Ambas as espécies podem ocorrer em áreas abertas ou florestais o que há é uma mudança na frequência em que são amostradas.

2.4 EUGLOSSINI: UM DOS GRUPOS DE ABELHAS NEOTROPICAIS MAIS BEM ESTUDADOS TAXONOMICAMENTE E POUCO CONHECIDO ECOLOGICAMENTE

A fauna brasileira de abelhas, há duas décadas contava com 1678 espécies catalogadas, distribuídas nas famílias Andrenidae (82 sp), Apidae (913 sp), Colletidae (104 sp), Halictidae (251 sp) e Megachilidae (328 sp) (SILVEIRA; ALMEIDA; MELO, 2002). Contudo, na mesma publicação, estes autores estimaram uma riqueza de aproximadamente 3.000 espécies de abelhas para o Brasil.

A tribo Euglossini abriga abelhas com características peculiares que em conjunto as tornam semelhantes a "joias vivas" (ROUBIK 2018 - *comunicação pessoal*). A morfologia do grupo inclui o tamanhos variando de moderado a grande (8mm – 28mm), longo comprimento da língua, coloração metálica exuberante e comportamento de coleta de compostos químicos pelos machos (DRESSLER, 1982). As fêmeas, diferentes dos machos que apresentam estruturas especializadas na coleta de compostos químicos precursores de feromônios sexuais (ELTZ; ROUBIK; WHITTEN, 2003), apresentam nas corbículas estruturas especializadas na coleta de pólen (Figura 3).

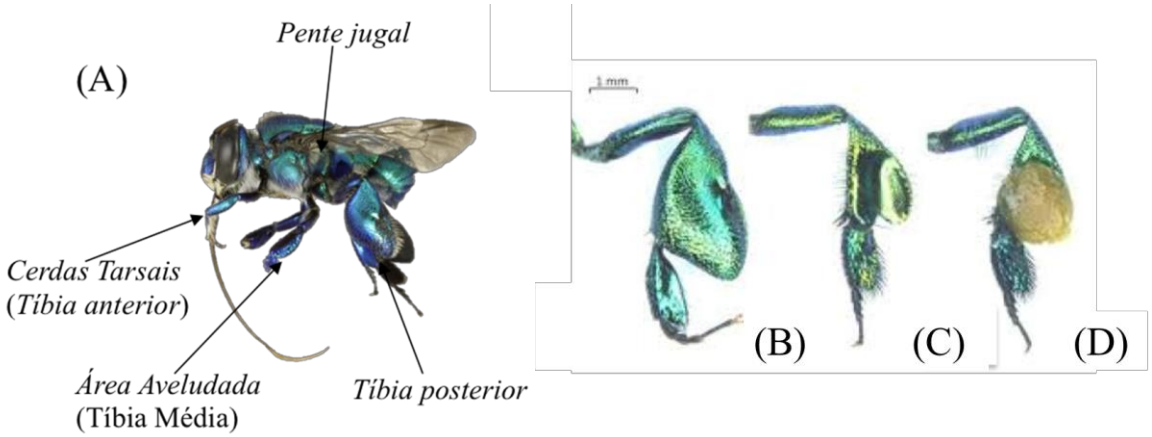

Figura 3 - Esquemas mostrando adaptações morfológicas envolvidas na coleta de compostos químicos e recursos florais por uma espécie de *Euglossa* sp. (A). Tíbia posterior dos machos (B) e corbículas das fêmeas (C) com pólen (D). Adaptado de MULLIS (2013).

Atualmente, Euglossini conta com 250 espécies reconhecidas (MOURE; MELO, 2022) distribuídas em cinco gêneros*: Aglae* (monotípico), *Eufriesea* (65 sp), *Euglossa* (146 sp)*, Eulaema* (30sp) e *Exaerete* (8 sp) (HINOJOSA-DÍAZ; NEMESIO; ENGEL, 2012; NEMÉSIO; RASMUSSEN], 2011; ROUBIK, 2004). Os gêneros, *Aglae* e *Exaerete* são compostos por

espécies parasitas de ninhos de *Eufriesea* e *Eulaema* (DRESSLER, 1982; ROUBIK, 2019). Fêmeas de *Aglae* e *Exaerete* perseguem as fêmeas de *Eufriesea* e *Eulaema,* e uma vez que encontram os ninhos destas espécies se alimentam dos ovos, ovopositam e fecham a célula de cria novamente. As crias ao nascerem utilizam os recursos alimentares do ninho parasitado (MICHENER, 2007).

Os Euglossini são exclusivamente encontrados na região Neotropical, com sua distribuição atual cobrindo parte sul dos Estados Unidos, nos estados do Arizona e Florida onde pode-se encontrar respectivamente as espécies *Eg. dilemmae* Bembé & Eltz, 2011 e *El polychroma* Mocsáry, 1988 possivelmente introduzidas (MINCKLEY; REYES, 1996; SKOV; WILEY, 2005; MOURE; MELO; FARIA JUNIOR, 2012; MULLIS, 2013), chegando até o sul do Brasil e norte da Argentina (DRESSER, 1982; SYDNEY; GONÇALVES; FARIA, 2010). Estas abelhas podem ser encontradas em todos os ecossistemas presentes no Brasil, inclusive áreas urbanas.

Em relação a capacidade de voo existe uma clara distinção no uso de recursos florais entre os sexos em Euglossini. Enquanto as fêmeas conseguem visitar plantas a cerca de 23 km de distância de seus ninhos em um mesmo dia (JANZEN, 1971), as quais são utilizadas como fontes de pólen, néctar e resina (REBÊLO, 2001), os machos percorrem distâncias até 45 km na busca por fontes reconhecidas de compostos químicos no ambiente, comportamento chamado de "*trapline"* (DRESSLER, 1982; POKORNY *et al.*, 2015). Para coletar e armazenar os compostos químicos e se alimentarem, os machos de Euglossini possuem estruturas morfológicas associadas especializadas que os possibilitam coletar néctar e os compostos disponíveis em diversas fontes, principalmente nas orquídeas (ELTZ; ROUBIK; WHITTEN, 2003). Os machos de Euglossini também adquirem compostos químicos de outras fontes não botânicas, como fungos, fezes de animais e até do inseticida DDT – diclorodifeniltricloroetano (DRESSLER, 1968; REBÊLO, 2001).

O comportamento de coleta de químicos foi descrito com detalhes somente a partir do estudo realizado por Dodson *et al.* (1969) com uso da técnica de cromatografia de gases. Estes autores conseguiram identificar através desta técnica cerca de 50 tipos diferentes de compostos disponíveis nas orquídeas, possivelmente como recompensas florais e verificaram a atração dos machos de Euglossini por alguns destes compostos. O estudo embasou a realização de vários outros em diferentes ecossistemas da região Neotropical, vários deles com uso de substâncias análogas as produzidas pelas orquídeas (NEMÉSIO, 2009; SYDNEY; GONÇALVES; FARIA, 2010).

Dodson *et al.* (1969) sugerem que estes compostos seriam químicos precursores de feromônios sexuais. Eles seriam assim utilizados pelos machos de Euglossini na forma de um

buquê (*tibial bouquet*) para as fêmeas durante o comportamento de *display,* no acasalamento (ELTZ; ROUBIK; WHITTEN, 2003). O avanço taxonômico sobre as abelhas das orquídeas e a facilidade na coleta dos machos do grupo melhoraram a avaliação dos impactos sofridos pelas espécies de Euglossini ao longo do tempo. Juntos esses fatores contribuíram para que as abelhas das orquídeas se tornassem o grupo de abelhas mais estudados na região Neotropical (PEREIRA; GONÇALVES; RAMOS, 2021).

As abelhas Euglossini são eficientes carreadores de pólen de mais de 60 famílias de plantas entre espécies nativas e cultiváveis (REBÊLO, 2001; ROUBIK; HANSON, 2004; RAMÍREZ; DRESSLER; OSPINA, 2002; ROCHA-FILHO *et al.* 2012; GIANNINI *et al.*, 2015). Os machos desse grupo possuem uma relação exclusiva com mais de 700 espécies de Orchidaceae neotropicais, das quais são consideradas como polinizadores (ACKERMAN, 1983; WILLIAMS; WHITTEN, 1983; RAMÍREZ, 2009), o que rendeu ao grupo a denominação de "abelhas das orquídeas". Similaridade quanto a morfologia de ambos os sexos, principalmente o longo comprimento da língua levam os cientistas a crer que machos e fêmeas visitam as mesmas plantas como fonte de néctar (RAMÍREZ; DRESSLER, 2002).

O acervo de plantas com que estes organismos interagem, os peculiares caráteres morfológicos que facilitam a identificação taxonômica de várias espécies e a facilidade de amostragem destas abelhas com uso de ninhos armadilhas, atratividade por iscas e outros, tornam as abelhas das orquídeas potenciais espécies guarda-chuva na preservação dos ecossistemas terrestre da região Neotropical, uma vez que funcionam como bioindicadores e permitam o monitoramento destas áreas (ALLEN *et al.*, 2019; MIRANDA *et al.*, 2019).

Recentemente, Añino e colaboradores (2019) discutiram o *status* dos Euglossini como bioindicadores de áreas alteradas com base em sete criterios apontados por Reyes-Novelo *et al.* (2009). Para Añino *et al.* (2019) estes organismos não atenderiam aos critérios de taxonômia conscientente e de reconhecimento da biologia de nidificação das espécies. Contudo, Gonçalves e Faria (2021) fizeram o contraponto a este estudo, e apontaram a existência de falhas na definição de espécie bioindicadora e adoção de critérios arbitrários utilizados por Añino *et al.* (2019). Entre os outros problemas apontados por Añino *et al.* (2019) menciona também a falta de segurança taxonômica devido a dificuldade de identificação das fêmeas das abelhas das orquídeas.

Adicionalmente, Gonçalves e Faria (2021) explicam que a facilidade de captura de machos de Euglossini com iscas aromáticas e a representatividade destes organismos nas coleções biologicas torna-os eficientes bioindicadores. Estes autores relatam ainda que o critério relativo a dificuldade de identificação de fêmeas pode ser contornado pela adoção da metodologia de ninhos armadilhas em que é possível a distinção das fêmeas na ocasião do

nascimento de machos dentro dos ninhos. Em sua revisão Gonçalves e Faria (2021) explicam ainda que, como um todo, os Euglossini estão intimamente ligados a ambientes mais preservados de florestas úmidas, com poucas espécies intimamente relacionadas a áreas perturbadas.

2.5 RELAÇÕES FILOGENÉTICAS ENTRE OS EUGLOSSINI

O ancestral comum mais recente entre os Euglossini surgiu a aproximadamente 42 milhões de anos, na região da Bacia Amazônica, local onde provavelmente ocorreu sua diversificação entre 15 e 20 m.a (RAMÍREZ *et al.,* 2010). Na tentativa de reconstruir a história evolutiva e filogenética dos Euglossini, Ramírez *et al.* (2010) utilizaram quatro *loci* de DNA, sendo um de mitocondrial o *COXI* e três de nucleares *EF1-α* (codifica o fator de alongamento 1 alfa que atua na síntese proteica na fase de alongamento, promovendo a ligação de aminoácidos), *Arg K* (arginina quinase: possui atividade de fosfotransferase, a qual catalisa a conversão entre fosfoarginina e ATP (PEREIRA *et al.*, 2011) e o *Pol-II* (RNA polimerase II: responsável por codificar a subunidade maior da RNA polímera que e responsável por sintetizar o RNA mensageiro em eucariontes (ALBERTS, 2017), Figura 4. Em seus resultados Ramírez *et al.* (2010) reporta a existência de sete áreas de endemismo para os Euglossini (Figura 5), e ainda diferentes arranjos das relações filogenéticas entre os gêneros, com destaque para as diferentes posições do clado composto pelas espécies de *Exaerete*.

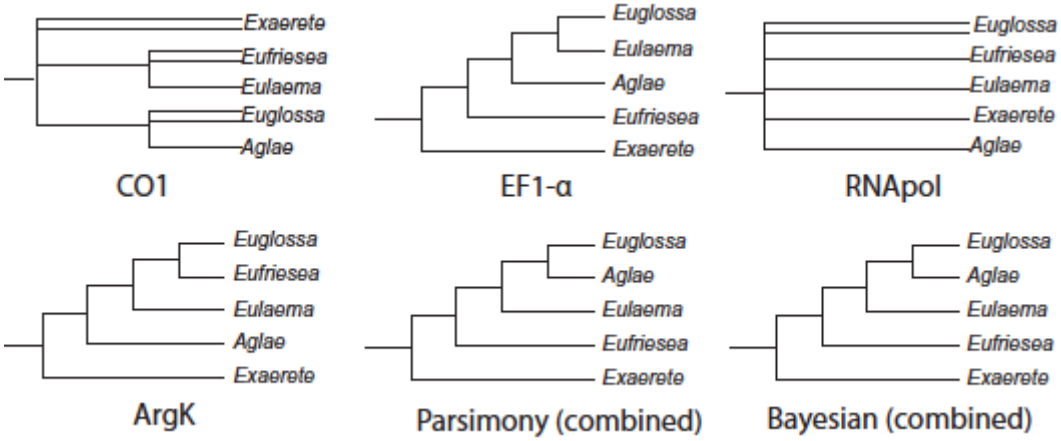

Figura 4 - Relações filogenéticas encontradas por Ramírez *et al* (2010) para os gêneros da tribo Euglossini

Algumas incertezas ainda permanecem quando se trata das relações filogenéticas entre os gêneros da tribo Euglossini, principalmente em relação a ancestralidade das espécies cleptoparasitas (CAMERON, 2004; RAMÍREZ *et al.*, 2010). Segundo a regra de Emery, o comportamento cleoptoparasítico evoluiu a partir de taxa intimamente relacionados que ao

longo da história evolutiva destes se tornaram seus hospedeiros (WILSON 1971), ou seja, os taxa cleptoparasitas têm origem posterior aos seus hospedeiros.

Em concordância a esta regra, Ramírez e colaboradores (2010) concluíram que devido a dependência de uma espécie hospedeira construtora de ninhos por parte de *Exaerete*, seria improvável que o ancestral comum mais recente para os Euglossini seja uma espécie parasita. O que os levou a concluir que o ancestral de Euglossini provavelmente foi uma espécie construtora de ninhos que foi extinta, a qual tinha como cleptoparasita uma espécie de *Exaerete* (RAMÍREZ *et al.*, 2010).

Figura 5 - Áreas de endemismo identificadas por Ramírez *et al.* (2010) para a Região Neotropical

Diante da inadequabilidade do conjunto de dados utilizado por Ramírez e colaboradores (2010), na tentativa de trazer clareza as relações entre os gêneros de Euglossini, verificada pelas incongruências na conformação de suas topologias, Bossert e colaboradores (2019) usaram dados genômicos para inferir as relações filogenéticas de Apidae. Os resultados encontrados por Bossert *et al.* (2019) apontam que os Euglossini são proximamente relacionados com Centridini. E diferente de Ramírez *et al.* (2010), estes autores revelaram inclusive uma ancestralidade comum entre os gêneros cleptoparasitas *Aglae* e *Exaerete* (Figura 6). Aspectos que vão sendo solucionados com os avanços de técnicas moleculares atuais, que têm agregado conhecimento ao estudo das abelhas.

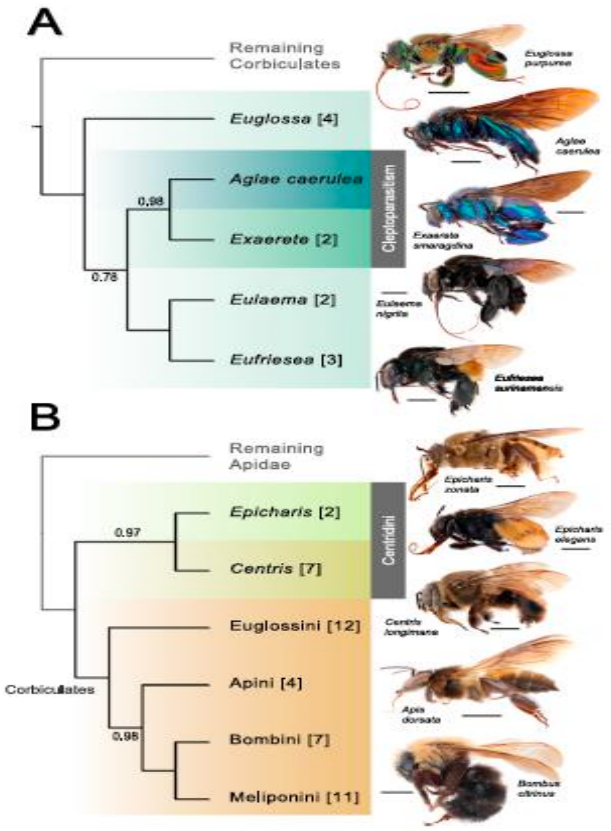

Figura 6 - Reconstrução filogenética mais atual para a família Apidae, segundo Bossert *et al.* (2019)

2.6 AVANÇOS NAS PESQUISAS COM AS ABELHAS DAS ORQUÍDEAS

Antes das técnicas moleculares aplicadas atualmente (ver seção 2.8), como o uso de marcadores genéticos, mitocondriais e nucleares, e utilização da modelagem climática de nicho ecológico (FRANTINE-SILVA *et al.*, 2017; GARRAFFONI; MOURA; LOURENÇO, 2017; MIRANDA *et al.*, 2019), o que se sabia a respeito das abelhas das orquídeas eram somente informações oriundas de estudos ecológicos realizados a partir da captura de machos por meio da atração por compostos sintéticos que eram disponibilizados em iscas aromáticas, frequentemente feitas com papel absorvente (papel filtro) e fixados nas ramagens das árvores (Figura 7). A partir da descoberta da atração dos machos de Euglossini por compostos químicos sintéticos, o número de pesquisas com o grupo aumentou (DODSON *et al.*, 1969; DRESSLER, 1982). Entre as características que chamaram a atenção dos pesquisadores estão a facilidade de coleta, a importância ecológica como polinizadores, a disponibilidade de chaves para a identificação taxonômica e a presença de espécies indicadoras de qualidade ambiental (PERUQUETTI *et al.*, 1999; ALLEN *et al.*, 2019).

Figura 7 - Métodos de capturas comumente utilizado nos estudos com abelhas das orquídeas. (A) Método passivo com uso de armadilhas odoríferas confeccionadas de garrafas PET (RAMALHO; GAGLIANONE; OLIVEIRA, 2009). (B) Método ativo com uso de rede entomológica (REBÊLO; GARÓFALO, 1997). (C) Machos de *Euglossa cordata* (Linnaeus) visitando iscas de eucaliptol, escala de 1 cm.

Durante os primeiros estudos realizados na Floresta tropical do Panamá (DODSON *et al.*, 1969; ACKERMAN, 1983; ROUBIK, 2019), área mais bem amostrada quanto a fauna de Euglossini da América Central (ROUBIK, 2019), as abelhas eram capturadas e levadas ao laboratório, contudo, os espécimes capturados ainda não eram identificados corretamente devido a dificuldades taxonômicas, pois o que se conhecia sobre o grupo era proveniente da captura de fêmeas em flores (DRESSLER, 1982).

No início da década de 1980, os pesquisadores perceberam que os espécimes atraídos eram machos de distintos taxa, fato que impulsionou os estudos taxonômicos (DRESSLER, 1982; NEMÉSIO, 2009), possibilitando ainda a identificação de novas espécies e agregando uma base forte para as pesquisas avançadas que se tem hoje. Em 1983, Williams e colaboradores já reconheciam os Euglossini como importantes objetos de estudos para pesquisas na Região Neotropical, sugerindo a realização de pesquisas acerca de estruturas envolvidas na coleta e armazenamento dos compostos químicos pelos seus machos (ELTZ; ROUBIK; WHITTEN, 2003; ELTZ *et al.*, 2007). O que estes pesquisadores não imaginavam era o quão longe alcançariam as pesquisas com este grupo de abelhas.

Os motivos mencionados acima levaram as espécies do grupo a se tornarem excelentes modelos para estudos sobre polinização (CARVALHO; WEBBER, 2000; CAVALCANTE *et al.*, 2018), biologia de nidificação e relações de cleptoparasitismo entre espécies (GARÓFALO; ROZEN, 2001; ROUBIK, 2019), estudos taxonômicos (KIMSEY, 1982; NEMEŚIO, 2009), químicos e fisiológicos para se identificar os compostos e compreender o uso dele pelas abelhas (WILLIAMS; WHITTEN, 1983; ELTZ *et al.*, 2007), interações abelhas-plantas (OSPINA-TORRES *et al.*, 2015) e até o uso de rádio telemetria de rastreamento para se verificar os padrões de dispersão dos Euglossini (WIKELSKI *et al.*, 2010).

2.7 MARCADORES MOLECULARES EM ESTUDOS SOBRE ABELHAS

As mitocôndrias são organelas que ocupam um espaço 25% do volume das células eucarióticas. Elas possuem a função de geração de energia através do processo conhecido como respiração celular, produção de moléculas carregadas de energia como o ATP (trifosfato de adenosina) utilizado pelas células (BALLARD; WHITLOCK, 2004). Este tipo de material genético tem origem protobacteriana e provavelmente surgiu a partir da endosimbiose desta última por uma célula pré-eucariotica. Ao longo da evolução eucariótica a maior parte do genoma citoplasmático foi perdido para o núcleo celular, assim o mtDNA é representado por 37 genes em células animais (LANG; GRAY; BURGER, 1999).

A partir da década de 1970 o mtDNA tornou-se uma ferramenta importante em estudos de estrutura populacional, filogenéticos e evolutivos, principalmente devido a suas características peculiares como: (i) estrutura circular em dupla fita pequena (16kb); (ii) herança exclusivamente materna e sem recombinação genica; (iii) possuir 13 genes codificadores de proteínas, 22 RNAs transportadores (tRNA) e duas subunidades ribossômicas, *12S* e *16S* rRNA); (iv) presença da região não codificadora *D-loop*, responsáveis pelo controle da replicação e transcrição do DNAmt; (v) raramente possui sequencias espaçadoras (*introns*) ou repetidas e nem pseudogenes; (vi) conteúdo conservado, gene sempre na mesma ordem; (vii) Taxa evolutiva elevada quando comparado ao DNA nuclear (AVISE *et al.*, 1987; AVISE, 2000; ARIAS *et al.*, 2008). Abaixo serão detabalhos apenas os genes utilizados na tese.

No caso das abelhas, estruturamente o mtDNA não mostra grandes diferenças em comparação aos outros animais, exceto na ordenação de alguns dos genes de RNAt e da região controle da replicação, em que as abelhas e outros insetos apresentam elevada taxa de repetições das bases AT (CROZIER; CROZIER, 1993). Assim, comparação duas especies de abelhas, neste caso *Apis mellifera* Linnaeus, 1758 e *Euglossa dilemma* Bembé & Eltz, 2011, percebe-se uma distribuição conservada dos genes mitocondriais, embora existam regiões ainda não congruentes (área hachuradas)

O gene *COI* (subunidade I da citocromo oxidase) é responsável por codificar parte do complexo multiproteico localizado na membrana das mitocôndrias, onde atua como enzima final na cadeia de transporte de elétrons (ALBERTS, 2017), Figura 8. Este gene tem sido empregado para a criação de um sistema universal de inventário de espécies se mostrado eficiente em análises taxonômicas e filogenéticas com divergência recente (HEBERT *et al.*, 2003; FRÉZAL; LEBLOIS, 2008).

O DNA *barcode* é um método rápido para avaliação da diversidade biológica. Ele consiste na utilização de um fragmento de DNA (*e.g.* do gene COXI) utilizado para o reconhecimento e separação de espécies (WAN *et al.*, 2004; HEBERT *et al.*, 2003, 2004). O

DNA *barcode* já tem sido utilizado na resolução de dúvidas taxonômicas em alguns grupos de abelhas (CAMERON *et al.*, 2011; SANTOS JÚNIOR; SANTOS; SILVEIRA, 2015; HURTADO-BURILLO *et al.*, 2013; KOCH, 2010). Para Françoso e Arias (2013) a conservação das abelhas nativas requer o uso de ferramentas genéticas rápidas e eficientes no delineamento de táxons, sendo o DNA *barcode* viável para este fim, uma vez que ele possibilita a correta identificação de espécies e ainda a descoberta de espécies crípticas.

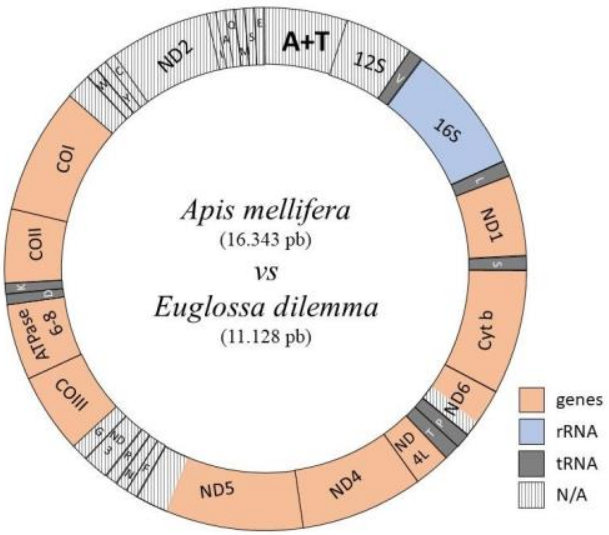

Figura 8 - Comparação do genoma mitocondrial de duas especeis de abelhas corbiculadas, *Apis mellifera* e *Euglossa dilema*. Cores representam tipos diferentes de sequências: N/A – região sem informação para *Eg. dilema*, A+T – origem de replicação do DNA mitocondrial adaptado de Frantine-Silva (2018).

Além do *COI*, o gene da subunidade maior do rRNA (*16S*) tem sido utilizado na busca para resoluções filogenéticas em diversos níveis, de populações ou espécies até tribo, subfamília e família (WHITFIELD; CAMERON, 1998; MICHEL-SALZAT; CAMERON; OLIVEIRA, 2004; FRANÇOSO *et al.*, 2016; FRANTINE-SILVA *et al.*, 2017). Outro gene bastante utilizado é o gene *Opsin* (*LWRh*) é responsável pela codificação de proteínas das membranas de fotorreceptoras em animais. A absorção de fótons pelos receptores tem como resultado a ativação de uma cascata bioquímica de eventos que resulta na transdução do sinal visual no sistema nervoso (CHANG *et al.*, 1995). *Opsin* foi identificado para a abelhas do mel *A. melllifera* (*honey bees*) por Chang *et al.* (1996) e tem sido aplicado em análises filogenéticas entre as tribos das abelhas corbículadas (MARDULYN; CAMERON, 1999).

A aplicação de ferramentas moleculares em estudos sobre populações de abelhas vem contribuir com dados importantes no delineamento de espécies crípticas (ELTZ *et al.,* 2011), variabilidade genética (SUNI; BROSI, 2012), e manejo das populações (LÓPEZ-URIBE; SORO; JHA, 2017). Os avanços neste campo têm possibilitado inferências sobre a filogeografia

de diferentes espécies (FRANÇOSO *et al.,* 2016; FRANTINE-SILVA *et al.,* 2017), a identificação de diploidia em espécies sob ameaça (SOUZA *et al.,* 2010), e a compreensão da dinâmica de subpopulações de espécies consideradas como ESU – *evolutionarily significant units* (PENHA *et al.,* 2015; FRANTINE-SILVA *et al.,* 2017), conhecimentos necessários para a tomada de decisões e elaboração de estratégias para a conservação das espécies.

Além destes aspectos, o uso de marcadores mitocondriais e nucleares nos estudos com os Euglossini tem se mostrado de grande relevância no entendimento de processos evolutivos que direcionam a especiação, extinção, demografia, colonização, migração e forrageio de espécies (CERÂNTOLA *et al.*, 2011; FREIRIA *et al.*, 2012; LÓPEZ-URIBE *et al.*, 2014; PENHA *et al.*, 2015). Estas pesquisas são úteis no entendimento dos efeitos da degradação ambiental sobre as espécies, e orientam as estratégias de conservação e tomadas de decisões (SOUZA *et al.*, 2010; SUNI; BROSI, 2012).

No Brasil, estas pesquisas em genética de população de espécies de Euglossini, estão concentradas a maior parte na porção sul e sudeste do país (SOFIA *et al.*, 2005; FREIRIA *et al.*, 2012; PENHA *et al.*, 2015; FRANTINE-SILVA *et al.*, 2017; MIRANDA *et al.*, 2019), nos grandes centros urbanos. Aspecto que muitas vezes limita os estudos a ambientes próximos a estes centros, devido a logística e uso de recursos financeiros, com poucos estudos realizados em áreas mais amplas do continente Sul-Americano (DICK *et al.*, 2004; LÓPEZ-URIBE *et al.*, 2014). A má distribuição da amostragem no espaço limita o poder de inferência de possíveis eventos geográficos e geológicos sobre as populações.

2.8 O MARANHÃO E SUAS ÁREAS DE TRANSIÇÃO

As áreas de transição são importantes para a manutenção da biodiversidade, por reunir organismos de diferentes formações vegetais em um mesmo espaço geográfico, contudo existe a dificuldade na definição destas áreas. Para Milan e Moro (2016), ecótono é a zona de transição entre formações vegetais adjacentes, que podem ser definidas de acordo com a escala geográfica e temporal. Estes autores traçam as modificações no termo ao longo do tempo, destacando elementos norteadores dele como a escala espacial, presença de uma zona de tensão entre dois ecossistemas e existência de valores intermediários de parâmetros utilizados para caracterizar os conjuntos de organismos destas áreas (densidade, cobertura e volume).

O conceito de ecótono pode variar desde micro-habitats até grandes escalas como o que se encontra no estado Maranhão. Neste sentido o Maranhão enquadra-se como um grande ecótono formado a partir da zona da tensão oriundas do contato entre grandes formações vegetais da Caatinga, Cerrado e Floresta Amazônica (REBÊLO *et al.*, 1999), fato que garante a confluência de elementos faunísticos destes três biomas, o que contribui para a elevada

diversidade genética e ecológica de abelhas registradas para a região (MARTINS *et al*. 2018; FERREIRA *et al.* 2019).

Em nível local, o estado apresenta um mosaico de ambientes que podem ser subdivididos em oito fitorregiões: (i) Restingas e Dunas; (ii) Cerrados meridionais; (iii) Campo aluvial flúvio-marinho (Baixada Maranhense); (iv) Zona mista de matas, Cocais e Cerrados; (v) Zona mista de matas e cocais; (vi) Amazônia maranhense; (vii) Zona de cerrado e caatinga; e (viii) Ilha de São Luís (ver REBÊLO *et al.*, 1999; MARTINS *et al.*, 2021). Embora esta subdivisão seja bem clara na literatura atual, existem áreas do estado ainda inexploradas quanto a sua fauna de abelhas.

Neste sentido, embora as abelhas das orquídeas sejam excelentes modelos e contam com elevado número de publicações disponíveis, dados primários de ocorrência de espécies ainda são escassos para muitas regiões do Brasil. Com destaque para as áreas dentro da Bacia Amazônica, centro de origem do grupo, domínio de Cerrado, Caatinga e ainda áreas de transição (RAMÍREZ *et al.*, 2010; SYDNEY *et al.*, 2010; PEREIRA; GONÇALVES; RAMOS, 2021). Entre estes ambientes transicionais, o Maranhão segue esta linha, com a maior parte das informações sobre o grupo limitada a porção norte do estado como enfatizado por Rebêlo e Silva (1999) em uma revisão do grupo. Estudos recentes destacam que a heterogeneidade ambiental, assim como a que ocorre no Maranhão, tem influência positiva na diversidade de abelhas Euglossini.

Opedal e colaboradores (2020) afirmam que a grande diversidade e abundância de abelhas das orquídeas em áreas heterogêneas, como encontradas em escala mais ampla em áreas de transição, deve-se a afinidade de plantas que servem de recursos florais a diversos habitats que podem variar desde o interior de grandes áreas florestais, borda de floresta, e ainda em ambientes perturbados. Corroborando a premissa destes autores, estudos realizados no Maranhão têm obtido elevados valores de diversidade de espécies (MARTINS *et al*., 2018; FERREIRA *et al.*, 2019), o que pode estar diretamente relacionado a esta heterogeneidade de ambiente e recursos florais desta região.

López-Uribe *et al*. (2014) estudando a tolerância fisiológica de três espécies de Eulaema, destaca a existência de uma área de refúgio pleistocênico na faixa litorânea do Maranhão (Figura 9). Estas áreas de refúgios são destacadas por permitir o estabelecimento de uma elevada diversidade genética e ecológica de espécies (FRANTINE-SILVA *et al.*, 2017). Aspecto que aliado a heterogeneidade da região pode contribuir para os elevados valores de diversidade genética encontrados para *Eg. cordata* amostrada em diferentes formações florestais do Maranhão (MARTINS *et al.*, 2021). Além disto, estudos ecológicos encontraram elevados valores de diversidade de espécies para áreas de Cerrado e Amazonia Maranhense

(MARTINS *et al.,* 2018; FERREIRA *et al.*, 2019). Tornando estas áreas interessantes para a realização de estudos sobre a variabilidade genética e diversidade de espécies de abelhas em geral.

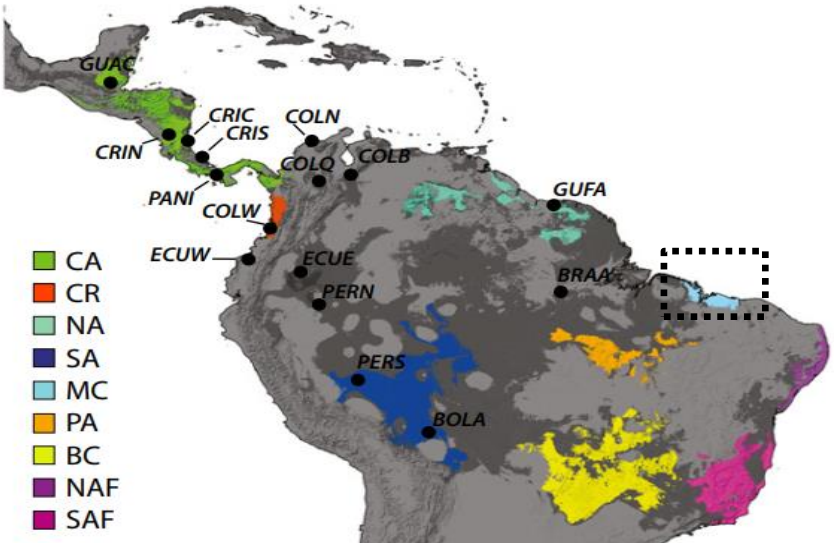

Figura 9 - Áreas de estabilidade e distribuição de amostras de *Eulaema cingulata* Fabricius, 1804 na América Central (CA), Região do Choco (CR), Norte da Amazônia (NA), Sul da Amazônia (SA), **Costa do Maranhão (MC)**, Amazonia Paraense (PA), Cerrado Brasileiro (BC), Norte da Mata Atlântica (NAF), Sul da Mata Atlântica (SAF). Adaptado de López-Uribe *et al.* (2014).

2.9 MUDANÇAS CLIMÁTICAS: GLACIAÇÕES DO PLEISTOCENO (2,58 M.A)

As florestas úmidas da América do Sul, que eram contínuas no Terciário, foram intercaladas por áreas de vegetação aberta e seca devido à dinâmica do clima neste período (OLIVEIRA-FILHO; RATTER, 1995). A principal hipótese biogeográfica para explicar o efeito da descontinuidade das florestas úmidas sobre os organismos nestes períodos foi a teoria dos refúgios (HAFFER, 1969; PENNINGTON *et al.*, 2004), que afirma que os refúgios seriam ilhas de florestas densas úmidas isoladas por vegetação aberta. No Pleistoceno, os eventos ocorridos durante as máximas glaciais causaram a retração das florestas, as quais se expandiram nos períodos mais quentes, efeito que foi contrário a vegetação aberta.

Existem várias críticas que questionam o modelo de refúgios entre elas: (i) A conectividade das Florestas Amazônica e Mata Atlântica através das matas de galeria podem ter aumentando o fluxo gênico e diminuído a diferenciação (BATALHA-FILHO *et al.*, 2013); (ii) Evidências geológicas apenas sugerem que houve mudanças climáticas e de paisagem na Amazônia, e não que essas mudanças realmente causassem especiação (COLINVAUX; DE OLIVEIRA; BUSH, 2000); (iii) Apenas as espécies ecologicamente bastante restritas foram afetadas, enquanto que as populações de espécies ecologicamente mais flexíveis podem não ter

sido efetivamente isoladas nos refúgios; (iv) padrões paleopalinológicos sugerem uma mata contínua na região amazônica (BUSH; OLIVEIRA, 2006).

Embora a Teoria de Refúgios seja a mais amplamente abordada para explicar a elevada biodiversidade das florestas tropicais, Leite e colaboradores (2016) estudaram a expansão da fauna de pequenos mamíferos florestais durante as glaciações do Quaternário através da combinação de modelos de distribuição e análise de coalescência em sequências de DNA de pequenos mamíferos na tentativa de buscar congruências entre os cenários: presente, período interglacial (~120.000 m.a) e última máxima glacial (~21.000 m.a). Com base em seus dados, estes autores postularam um modelo nomeado de "Floresta Atlântida", no qual mostra que houve uma expansão da fauna destes animais para abrigos no interior do continente durante o último período glacial. Os autores destacam ainda que no ambiente de Mata Atlântica é provável que a elevação do nível do mar e o relevo irregular da região tiveram maior contribuição na biogeografia histórica da Mata Atlântica, enquanto os refúgios florestais tiveram apenas papel secundário, mesmo para estes animais com alta afinidade florestal.

Ainda dentro da Teoria de refúgios, ao longo do Pleistoceno (últimos 2,58 milhões de anos) os movimentos de expansão e retração das florestas úmidas e de vegetação aberta foram influenciados pelos Máximos Glaciais (períodos mais secos) e períodos interglaciares. Durante este período a dinâmica da fauna foi diretamente influenciada por estes eventos, as espécies dependentes de florestas úmidas tiveram sua distribuição limitada por estes ciclos de retração e expansão das florestas, populações associadas a diferentes refúgios se diversificaram de maneiras distintas, causando o isolamento de algumas e a expansão de outras (BATALHA FILHO; MIYAKI, 2011). As matas ripárias presentes ao longo da Diagonal da Seca podem representar regiões complementares às florestas úmidas adjacentes a elas (PENNINGTON *et al.*, 2004; BATALHA-FILHO *et al.*, 2013) tornando-se refúgios a espécies com maior afinidade por florestas.

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4 CAPÍTULO I –

Genetic diversity and population structure of two Euglossini bee species in a host-parasite relationship

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Genetic diversity and population structure of two Euglossini bee species in a host-parasite relationship

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Abstract

In the current study, two euglossine species, *Exaerete smaragdina* and *Eulaema nigrita*, a cleptoparasite bee and its host, respectively, were used as models to: (i) access the genetic diversity and population structure of both species, sampled along a wide latitudinal range of Atlantic Forest, where the distribution of *El. nigrita* and *Ex. smaragdina* co-occurs; (ii) investigate the evolutionary history of these species through the Atlantic Forest, and in a wider scenario, to examine the evolutionary history of these species across others forest domains. Analyses involved males of *El. nigrita* and *Ex. smaragdina* sampled through Brazilian territory, including 19 sites in the Atlantic Forest. Bayesian Skyline Plot (BSP) was used to infer possible climate oscillations on population of both species over time. The BSP revealed expansion in effective population size for both species over the last 500 years. The BSP results aligned to the starlike configuration in the haplotype network, neutrality test, and population diversity patterns indicated population expansion of the two species during the late Pleistocene. Our findings suggest areas of potential refugia to the climatic oscillations of the Pleistocene in the Atlantic Forest in the Brazilian states of Espírito Santo for *El. nigrita* and Pernambuco for *Ex. smaragdina*.

Key words: ecological interaction, evolutionary history, orchid bees, parasitism, Pleistocene

INTRODUCTION

Orchid bees (Hymenoptera: Apidae: Euglossini) are a group of Neotropical pollinators known for their affinity with humid tropical forests (Dressler 1982), which have their origin attributed to Amazon basin (Ramírez *et al.* 2010). The tribe encompasses five living genera: *Eufriesea* Cockerell, *Euglossa* Latreille, *Eulaema* Lepeletier, *Aglae* Lepeletier & Serville and *Exaerete* Hoffmannsegg, (Dressler 1982, Roubik & Hanson 2004). While the first three show free living habit, *Aglae* and *Exaerete* are cleptoparasites of other euglossine genera. Specifically, *Aglae*, a monotypic genus, is an exclusive nest cleptoparasite of *Eulaema*, and *Exaerete* species are cleptoparasites of both *Eulaema* and *Eufriesea* (Dressler 1982, Roubik & Hanson 2004).

In the cleptoparasitic relationship, the female invades the nest of other species and lays her eggs in the cells of host bees (Garófalo & Rozen 2001, Danforth *et al.* 2019). Among orchid bees, one of the best known cleptoparasitic relationships is between the species *Ex. smaragdina* (Guérin, 1844) and *El. nigrita* Lepeletier, 19841. In the first review on orchid bee nesting biology, Zucchi *et al.* (1969) highlighted that 50% of the cells in a nest of *El. nigrita* were parasitized by *Ex. smaragdina,* revealing a ability of females of this latter species to lay their eggs in the cells of the host species. Subsequently, the cleptoparasitic behavior of *Ex. smaragdina* in the nest of *El. nigrita* was described in detail by Garófalo & Rozen (2001).

Eulaema nigrita and *Ex. smaragdina* have a broad geographic range, occurring from Mexico to southern Brazil (Nemésio 2009). Across the Brazilian territory, both species are also widely distributed and commonly found together in inventories conducted in different vegetation formations (Storck-Tonon *et al.* 2013, 2009, Silveira *et al.* 2015, Martins *et al.* 2018).

It has been suggested that favorable rates of parasitism are usually density-dependent and, consequently, will be enhanced where host populations are large (Wcislo 1987). Despite this, studies revealed that abundances of *El. nigrita* and *Ex. smaragdina* are not necessarily positively associated along their distribution. For instance, along the Atlantic Forest domain, it was

demonstrated that while the frequency in number of males of *El. nigrita*, surveyed in inventories, is high towards to the South whereas no correlation was found between frequencies of *Ex. smaragdina* and latitude (Nemésio & Silveira 2006). In fact, in several studies carried out through Atlantic Forest, the relative frequency of *Ex. smaragdina* varied consistently, in most studies independently from the frequencies of its host (Rebêlo & Garófalo 1997, Aguiar & Gaglianone 2012, Cordeiro *et al.* 2013). Despite this, a significant positive association between host–parasite ratio, involving both species, and latitude was found by Nemésio & Silveira (2006), who demonstrated that the relative frequencies of the parasite to its host are higher near the equator. They also suggested that there is some trend in frequencies of *Ex. smaragdina* decrease going further south across the Atlantic Forest (Nemésio & Silveira 2006). While it has been suggested that variations in abundance of some species of orchid bees, probably, reflects variables on smaller spatial scales (Lopes *et al.* 2022), it is still necessary further investigations on this theme before any generalization.

In this context, studies on the genetic diversity and population structure of these species and their evolutionary history could be helpful to better understand the current abundance and distribution of *Ex. smaragdina* and *El. nigrita* across the Atlantic Forest. Furthermore, considering that the relationship between cleptoparasitic bees and their hosts are usually specialized, and the parasitism successful is dependent on both presence and abundance of the hosts, population genetic studies involving parasites and their hosts can be valuable for supporting future conservation and management measures.

Many studies have shown the effects of Pleistocene, a period of climatic changes and geomorphological alterations in the Neotropical region, on the population structure and demography of different organisms in the Atlantic Forest, including different groups of bees (Batalha-Filho *et al.* 2010, Frantine-Silva *et al.* 2017). The current literature indicates that paleoclimatic instability, occurring during the Quaternary Period, impacted the Atlantic Forest,

shaping the genetic structure of some orchid bee species (López-Uribe *et al.* 2014, Frantine-Silva *et al.* 2017). Moreover, it was suggested that species showing narrower physiological tolerance probably experienced less suitable habitats during the Quaternary climatic oscillations (López-Uribe *et al.* 2014). Thus, orchid bees are excellent models to investigate the effect of climatic oscillations of the Plio-Pleistocene (between 5.3 million years ago–mya and 11,600 years ago) (López-Uribe *et al.* 2014).

Taking the above into consideration, the aims of the present study were: (i) to investigate genetic diversity and population structure of *El. nigrita* and *Ex. smaragdina* populations, sampled along a wide latitudinal range of Atlantic Forest, where the distribution of both species is co-occur; (ii) to make inferences on the evolutionary history of both species across the Atlantic Forest, and (iii) lastly, considering the wide distribution of both species through the Brazilian territory, we also investigated the evolutionary history of *El. nigrita* and *Ex. smaragdina* in a wider scenario, which included samples from others Brazilian forest domains.

MATERIALS AND METHODS

Study area and Samplings

Bee samplings were carried out between 2012 and 2019, in 19 localities within the Atlantic Forest (Figure 1-S). Of these 19 localities, males of *Ex. smaragdina* and *El. nigrita* were collected in 12, not necessarily coincident (Table I-S). As mentioned above, aiming to compare the set of haplotypes surveyed in areas of Atlantic Forest with other regions were both species are found, we included localities in the Amazon Forest (AM), and the Brazilian *Caatinga* and *Cerrado*. One area of the Andean Montane Forest located in the municipality of Santa Fé de Antioquia in Colombia was included (Table I-S). Two methods of collection were used, active collection (*e.g.*, Nemésio 2010) and passive collection (PET bottle traps) (*e.g.*, Santos Júnior *et al.* 2014, Martins *et al.* 2018), both using aromatic compounds (1,8-cineole, eugenol, methyl benzoate, methyl trans-cinnamate,

methyl salicylate, skatole, p-tolyl and vanillin) for attraction. The specimens collected were stored in alcohol in the freezer at -20°C, or directly in the freezer. Finally, to complete the sampling, 15 pinned (dry material) males of *Ex. smaragdina*, deposited in the collection of insects from the *"Centro de Coleções Taxonômicas da Universidade Federal de Minas Gerais"* – CCT-UFMG, were included in the analyses (Table II-S).

The DNA was extracted from the thoracic musculature or the hind leg of the bees, using the phenol-chloroform method (Sambrook & Russel 2001). In total, 118 specimens of *El. nigrita* and 81 of *Ex. smaragdina* were surveyed in our study. However, of these 97 of *El. nigrita* and 74 of *Ex. smaragdina*, since not all these specimens produced high-quality sequences (see Tables V-S and VI-S). The DNA pellet was re-suspended in 40 µl of LOW TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and 1 µl was used for spectrophotometer quantification (NanoDrop Thermo Scientific 2000). After this, the material was diluted in a solution at 20 ng/µl concentration.

DNA processing

Two mitochondrial markers, *COI* and *16S* genes, were chosen for our analysis, since they have shown some variation in other studies of orchid bees, *e.g., COI* (Dick *et al.* 2004, Nemésio *et al.* 2013) and *16S* (Frantine-Silva *et al.* 2017). In addition, we also analyzed a segment of the *Opsin* nuclear gene (Michel-Salzat *et al.* 2004).

Amplifications of all genes were performed in a final volume of 15 µl PCR reaction mix, including 0.1 µl Taq Polymerase Platinum – 5u/µl (Platinum, Invitrogen, Brazil), 0.6 µl $MgCl_2$ – 50mM, 0.6 µl primers – 25 µM, 1.5 µl reaction buffer – 10X, 1.2 µl dNTPs – 2.5mM, and 1 µl DNA – ~20 ng /µl. The process was conducted in the thermocycler using different annealing temperatures (48–57ºC see below). The amplification cycles were: 1 cycle of denaturation at 95ºC for 5 min; 37 cycles of denaturation at 95ºC for 45 s, primer annealing temperature for 30 s and extension at 72ºC for 90 s; and the last pass of final extension at 72ºC for 10 minutes. For

mitochondrial DNA, the *COI* (Hebert *et al.* 2004) and *16S* genes (Cameron *et al.* 1992) were used. In the case of nuclear DNA, the *Opsin* gene was chosen (Mardulyn & Cameron 1999) (Table II-S). All PCR products were visualized in 0.8% agarose gel.

The positive samples were purified through the polyethylene glycol 20% (PEG) method according to Santos Júnior *et al.* (2015). For the sequencing reactions, the same primers were applied as in the PCR reactions. After purification, the PCR products were sequenced through the ABI 3130x1 sequencer following the manufacturer's guidelines (Applied Biosystems). All the fragments were sequenced in forward and reverse directions. Sequenced fragments of the nuclear and mitochondrial genes were checked in Seqscape software 2.6 v. (Applied Biosystems, Darmstadt).

Genetic data analysis

The alignment of DNA sequences was made with the aid of the online tool MAFFT v. 7.475 (Katoh *et al.* 2017). For the alignment of the *16S* sequence, the option Q-INS-i (the secondary structure of RNA is considered) was selected. After this, the Gblocks program (Dereeper *et al.* 2008) was used to remove regions of ambiguous alignment of this mitochondrial gene. Other fragments were performed in the MAFFT default.

Genetic diversity and population structure

For both species, we estimated the genetic diversity parameters, including the number of haplotypes (*h*), haplotype diversity (Hd), and nucleotide diversity (π), using the set of samples from each locality with DnaSP software (Librado & Rozas 2009). Firstly, we estimated these parameters based on sequences of the three gene-segments amplified for the set of samples from all localities. In this latter analysis, genetic diversity measures were obtained for different clusters of each species, which were defined by spatial delimitation analyses performed in R software, using the

126 package Geneland, version 4.0.8 run (Guillot *et al.* 2005). These same genetic diversity parameters
127 were calculated separately for *El. nigrita* and *Ex. smaragdina* surveyed only in the Atlantic Forest
128 areas.

129 Due to sample limitations, the uncorrelated haplotype frequency model (UHF) was chosen
130 for some localities. The UHF model uses the Metropolis-Hasting algorithm to start from arbitrary
131 values for all unknown parameters and to modify them in such a way that after many iterations,
132 these values are close to the true values (Guillot *et al.* 2005). To identify genetic spatial
133 discontinuities among populations of both species, the Geneland was used in three independent
134 rounds. In the first, the number of possible populations ranges from 1 to 10 was defined in the
135 software. After this, based on the results, the software (*i.e.*, $K = 2$) was executed again two times,
136 using the number of clusters obtained for the program, to confirm the clustering. In all rounds, 10
137 million iterations were used of the Markov chain Monte Carlo (MCMC) and thinning by 100 to
138 estimate the number of populations and geographic limits of the individuals.

139 The Analysis of Molecular Variance (AMOVA), calculated through 10,000 iterations, was
140 carried out based only in sequences of *COI* gene, since amplified of this gene showed the highest
141 genetic diversity for both orchid bee species. Based on the result from Geneland analysis, a
142 hierarchical AMOVA was run, using the Tamura and Nei model, to consider variable base
143 frequencies with equal transversion rates and variable transition rates, calculated using Arlequin
144 for the results of each species (Excoffier & Lischer 2010).

145 To view the spatial distribution of the haplotypes that constructed the network, DnaSP was
146 used to create the haplotype data file, removing the invariable sites to run in Network v. 5 based
147 on the Median-joining algorithm (Bandelt *et al.* 1999). This network was used to view the spatial
148 distribution of haplotypes, *e.g.,* the clusters found by Geneland.

149 The population structure from the species was examined based on the Φ_{ST} statistic, calculated in
150 Arlequin. To test the existence of the correlation between genetic and geographical distances of

observations across a landscape, the Mantel test in Alleles in Space software was used, with 10,000 permutations (Miller 2005).

Population Demography and Migration

The neutrality tests of Tajima's D and Fu and Li's D were carried out in Arlequin v. 3.5.2.2 (Excoffier & Lischer 2010). In both cases, only the *COI* sequences used were used in the analyses of both species.

The PartitionFinder 2 program was used to find the best partitioning scheme for each data set (Lanfear *et al.* 2016). From the PartitionFinder 2 results, Beast v. 2.6.0 software (Bouckaert *et al.* 2014) was run, using the Bayesian Skyline Plot method (BSP) to infer possible oscillations in the effective population size (Ne) over time between both species. Three independent runs were performed in Beast, one for each type of posterior distribution of data (normal, exponential, and strict). Each run was performed according to the following parameters for the dataset of each species: 100 million steps of MCMC, incrementing 100 steps, UPGMA initial tree, and F81+I (*El. nigrita)* and F81 (*Ex. smaragdina*) substitution model. To calibrate the molecular clock, the date of the most recent common ancestor of *El. nigrita* and *Ex. smaragdina* (9 mya; sd = 1 mya) was used, as shown only by Ramírez *et al.* (2010).

Tracer software was used to plot the BSP results of the posterior distribution (only when the effective sample size was ESS > 200) (Rambaut *et al.* 2013). The determination of the best models of the posterior distribution of both species was based on the corrected Akaike Information Criterion (AICc).

To estimate the migration rate (M) between the clusters found in the Geneland results for each species, migration analysis was run in Migrate software, version 4.4.3 (Beerli 2016). This software uses the Metropolis-Hastings algorithm to calculate effective population sizes (Θ) and migration rates based on the coalescence theory. The following parameters were used for MCMC:

176 inheritance scalers of 0.25, recording 50,000 steps, incrementing 1,000 steps, number of concurrent
177 chains three replicates, visiting parameter values 150,000,000, and the first 10,000 genealogies
178 were discarded as burn-in. The UPGMA tree was used as the starting genealogy and static heating
179 with 4 automatic changes (temperatures were 1.0; 1.5; 3.0; 10^{11}) for each species.

180 Five models were evaluated to understand the migration dynamics of the groups found in
181 Geneland (see Figures 1 and 3): (1) a model with one population (panmictic population); (2) – a
182 full model with two population sizes and two migration rates, from cluster 1 to cluster 2, and from
183 cluster 2 to cluster 1 (*El. nigrita*: AF+AM ↔ TN; *Ex. smaragdina* AF ↔ AM); (3) a model with
184 two population sizes and one migration rate from cluster 2 to cluster 1 (*El. nigrita*: AF+AM ←
185 TN; *Ex. smaragdina* (AF) ← (AM); (4) a model with two population sizes and one migration rate
186 from cluster 1 to cluster 2 (*El. nigrita*: AF+AM → TN; *Ex. smaragdina* AF → AM; (5) a model
187 with two separate groups of populations or independent evolutionary lineages. To find the best
188 migration model indicated by Migrate for these species, the value of model probability from the
189 Bezier approximation score was calculated for the different models (ΔBAS), as proposed by Beerli
190 & Palczewski (2001).

191

192 **RESULTS**

193 **Genetic Diversity and Population Structure**

194 In total, 97 males of *El. nigrita* were analyzed for *COI* (586 bp), 77 for *16S* (~576 bp), and 58 for
195 *Opsin* (585 bp) genes (Tables I). The *COI* was conspicuously the most variable region for the
196 number of haplotypes (h = 29) and haplotype diversity (Hd = 0.732). The highest value of
197 nucleotide diversity was found for the *16S* segment (π = 0.037). The neutrality test of the *COI* gene
198 showed no significant values for *El. nigrita* (Table I).

199

Table I Genetic diversity measures obtained for *Eulaema nigrita* and *Exaerete smaragdina* along the Atlantic Forest (above), and for samples of different 37 localities distributed in Brazilian territory and one site from Colombia (below). Diversity measures shown by samples of both species surveyed in the Atlantic Forest neutrality tests of Tajima's D and Fu and Li's D only for the most variable gene (*COI*). Sample sizes (N), number of haplotypes (*h*), haplotype diversity (Hd), nucleotide diversity (π), and standard deviation (sd). *p < 0.05; **p<0.01. Values for the clusters were generated in Geneland software.

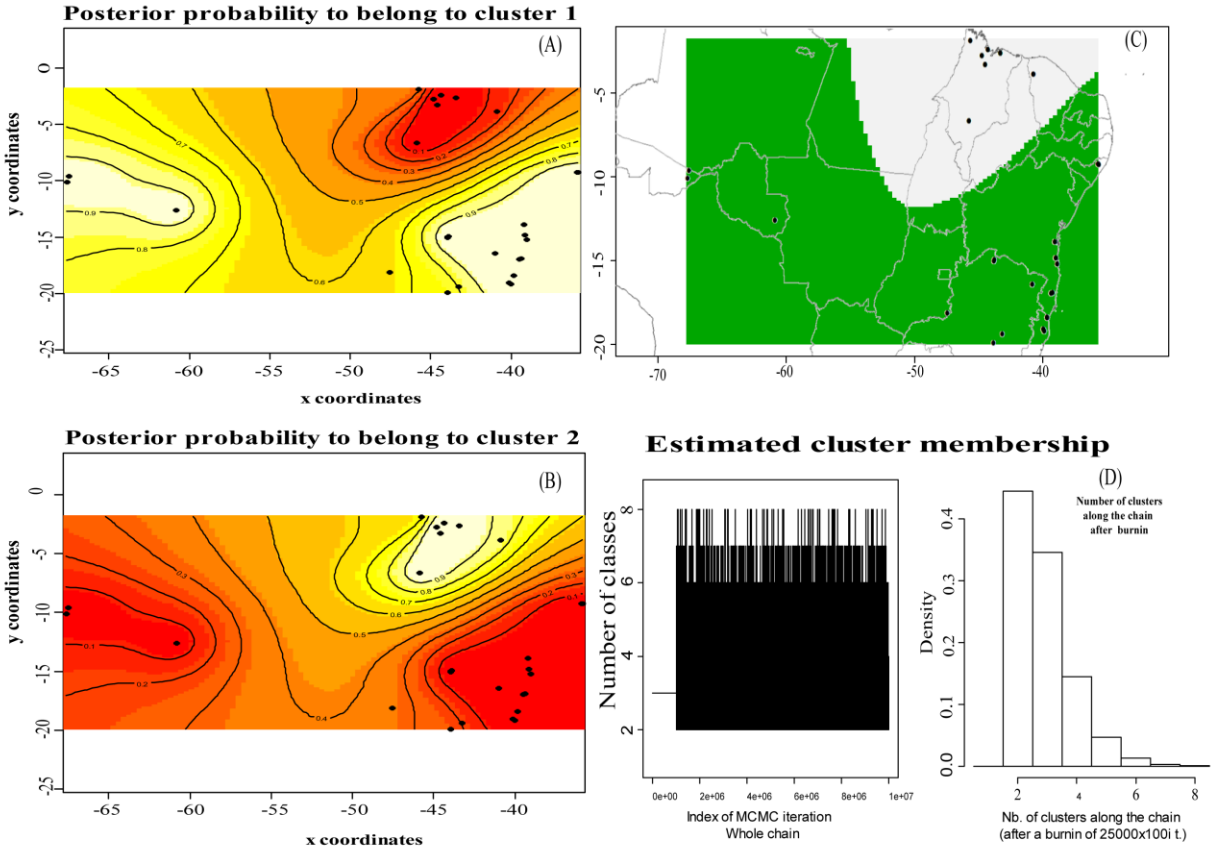
Species	*COI* gene	Genetic measures	AF only
Eulaema nigrita	***COI* (586 bp)**	**N**	56
		h	12
		Hd	0.438
		(± sd)	(± 0.007)
		π	0.002
		Tajima's D	-2.600***
		Fu and Li's D	-4.642**
Exaerete smaragdina	***COI* (660 bp)**	**N**	36
		h	8
		Hd	0.556
		(± sd)	(± 0.008)
		π	0.001
		Tajima's D	-2.029*
		Fu and Li's D	-3.089*

Species	Three genes	Genetic measures	Cluster 1	Cluster 2	Total
			AF + AM	TN	
Eulaema nigrita	***COI* (586 bp)**	**N**	64	33	97
		h	14	16	29
		Hd	0.484	0.873	0.732
		(± sd)	(± 0.006)	(± 0.002)	(± 0.002)
		π	0.007	0.012	0.017
		Tajima's D	-1.411	0.179	0.840
		Fu and Li's D	0.391	0.467	-0.763
	***16S* (~576 bp)**	**N**	45	32	77
		h	3	2	3
		Hd	0.279	0.125	0.234
		(± sd)	(± 0.006)	(± 0.005)	(± 0.003)
		π	0.039	0.024	0.037
	***Opsin* (585 bp)**	**N**	30	28	58
		h	8	5	9
		Hd	0.595	0.381	0.495
		(± sd)	(± 0.01)	(± 0.010)	(± 0.006)
		π	0.001	0.001	0.001

	Genes	Genetic measures	Cluster 1	Cluster 2	Total
			AF	AM	
		N	36	38	74
		h	9	7	14

Exaerete smaragdina	***COI* (660 bp)**	**Hd (± sd)**	0.551 (± 0.009)	0.485 (± 0.009)	0.677 (± 0.001)
		π	0.001	0.007	0.002
		Tajima's D	-2.100*	-1.840*	-2.183*
		Fu and Li's D	-3.311**	-1.541	-3.844**
	***16S* (~511 bp)**	**N**	9	30	39
		h	2	6	8
		Hd	0.222	0.310	0.567
		(± sd)	(± 0.027)	(± 0.011)	(± 0.006)
		π	0.0004	0.001	0.001
	***Opsin* (668 bp)**	**N**	3	16	19
		h	1	2	3
		Hd	0.000	0.400	0.433
		(± sd)	(± 0.000)	(± 0.012)	(± 0.013)
		π	0.001	0.001	0.001

The spatial delimitation of the clusters based on the Geneland UHF model (only for *COI* gene) pointed to the existence of a range from $K = 2$ clusters for *El. nigrita*, with the haplogroup AF+AM encompassing all localities in the Amazon and Atlantic forests (AC2, AC4, AL, BA1, BA2, BA3, BA4, BA5, ES1, ES2, ES3, GO, MG1, MG2, MG3, MG4, and RO1) and only the Transitional areas (TN) in the northeast Brazil haplogroup in the states of Ceará and Maranhão (CE, MA1, MA2, MA3, MA4, MA5, and MA6) (Figure 1). The AF+AM cluster showed Hd = 0.484 and $\pi = 0.0071$. Analyzing separately the set of 56 males of *El. nigrita* sampled only along the AF, the number of haplotypes ($h = 12$) and estimates of Hd (0.438 ± 0.007) and π (0.002) were very close to those found for AF+AM. Distinctively from the results found for AF+AM, the neutrality values of Tajima's ($D = -2.029$; $p < 0.05$) and Fu and Li's tests ($D = -3.089$; $p < 0.05$), obtained for *El. nigrita* samples from AF, were statistically significant. Concerning to TN cluster, the value of Hd (0.873) found for TN was noticeably higher when compared to AF+AM cluster. Respectively, 13 and 10 private haplotypes were found to TN and AF+AM.



224 **Figure 1** - Population Clusters (K=2) identified using the UHF model implemented in Geneland
225 Software for the COI gene of *Eulaema nigrita*. Cluster 1 includes the Amazon and Atlantic forests
226 together, Cluster 2 showed areas of the Maranhão and Ceará in the area called, in this paper,
227 Transitional area in northeastern Brazil (A), (B), and (C). The Lighter Colors Indicate Higher
228 Probability Values >0.09. Diagrams and graph of the cluster formations (D).
229

230 Among the total of 29 haplotypes found for the *COI* gene of *El. nigrita*, H1 (50.51%) was
231 the most common among the localities sampled, being predominant in populations that inhabit the
232 Amazon and Atlantic forests (Figure 2). On the other hand, 23 private haplotypes were identified
233 for this species (Figure 2A). The Median-joining network haplotypes showed a starlike
234 configuration close to H1, with several departing private haplotypes, evolving from one to two
235 mutational steps, following a complex network with two medium vectors and several mutational
236 steps (Figure 2B). The pattern in the frequency of H1 in these analyzed populations showed a
237 breakup in the frequency of this haplotype between the humid tropical forests

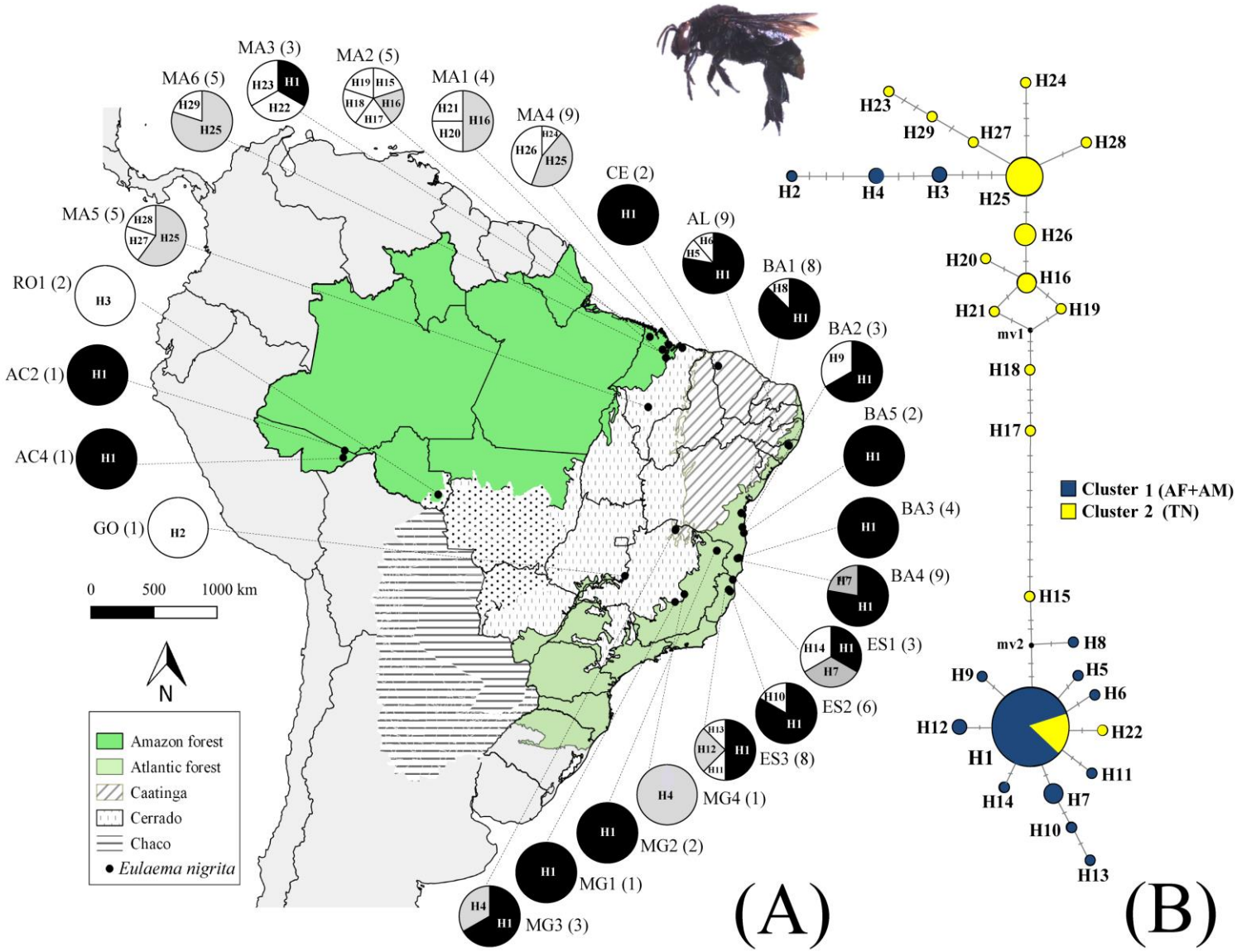


Figure 2 - Twelve sites and frequency of haplotypes present in a fragment of the *COI* gene of *Eulaema nigrita* from Atlantic forest and other different forest domains in the South America. (A) Pie charts indicate the frequency and distribution of each haplotype. Common haplotypes (black), haplotypes shared by at least 2 sites (gray), and private haplotypes (white). (B) Median-joining haplotype network for 29 haplotypes (H1-H29) for two haplogroups (Cluster 1 and 2), the blue color represents cluster 1 that includes the Amazon and Atlantic forests, while the yellow color is cluster 2 which represents the localities of northeast Brazil (CE, MA1, MA2, MA3, MA4, MA5, and MA6), and medium vectors (mv). Site codes are the same as in Table 1, where AC, AL, BA, CE, ES, GO, MA, MG, and RO correspond to the usual abbreviations of the following Brazilian states Acre, Alagoas, Bahia, Ceará, Espírito Santo, Goiás. Maranhão, Minas Gerais, and Rondônia

For *Ex. smaragdina,* 74 males were analyzed for the *COI* gene (660 bp), 39 for *16S* (~511 bp), and 19 for *Opsin* (668 bp). In this species, the number of haplotypes and Hd were more variable for *COI* (*h* =14 haplotypes; Hd = 0.677), followed by *16S* (*h* = 9; Hd = 0.567), and *Opsin* (*h* = 3; Hd = 0.433); the highest measure of nucleotide diversity (π = 0.002) was also found for the *COI* region (Table I). The demographic scenario analysis, performed through the neutrality tests of Tajima's D and Fu and Li's D, indicated population expansion only for *Ex. smaragdina* (*COI*: Tajima's D= -2.1840, $p < 0.01$ and Fu and Li's D = -3.8445, $p < 0.01$). In this species, the UHF identified two haplogroups (*K* = 2), which separate the Amazon and Atlantic Forest regions (Figure 3).

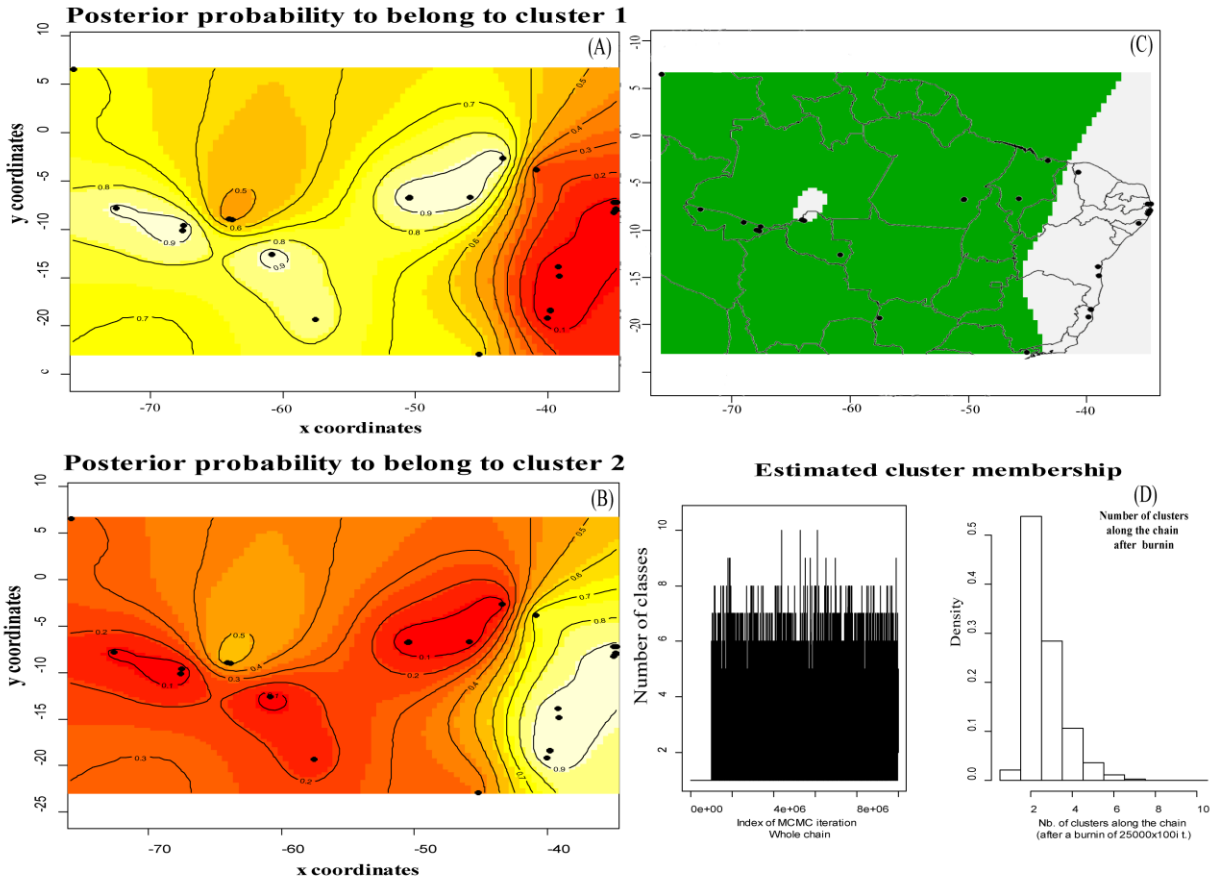


Figure 3 - Population Clusters (K=2) identified using the UHF model implemented in Geneland Software for the COI gene of *Exaerete smaragdina*. Cluster 1 includes the localities in the Amazon biome, while Cluster 2 showed localities in the Atlantic Forest (A), (B), and (C). The Lighter Colors Indicate Higher Probability Values >0.09. Diagrams and graphs of the cluster formations (D).

252 The AF haplogroup includes the localities CE, PB1, PB2, PE1, PE2, PE3, PE4, BA1, BA2,
253 ES1, and ES2 in the Atlantic Forest. The AM haplogroup includes localities in the Amazon Forest
254 (AC1, AC2, AC4, AC5, CO, MA4, MA5, MS, PA, RO1, RO2, and SP). AM showed h = 7, Hd =
255 0.485, and π = 0.0079 while the AF haplogroup showed h= 9, Hd =0.551, and π = 0.0013. The
256 private haplotypes for the two clusters were, respectively, seven and five haplotypes.
257 Analyzing the haplotype network for *Ex. smaragdina*, it is possible to notice that the H2
258 (41.89%) and H3 (39.18%) haplotypes were the most frequent (Figure 4A). The network
259 haplotypes showed a double starlike configuration, with the H2 haplotype predominant in localities
260 in the Amazon Forest and some sites in the Atlantic Forest (Figure 4B), while the H3 haplotype
261 was present mainly in the population from the east and northeast of Brazil in the Atlantic Forest.
262 This haplotype was also present in a single locality in west Brazil (RO2). Twelve private
263 haplotypes from one to five mutational steps from H2 and H3 were randomly present along the
264 sampling areas.
265 For both species, the highest value of genetic structuring was found among populations
266 within clusters (*El. nigrita* - Φ_{ST} = 0.840; $p < 0.001$ and *Ex. smaragdina* - Φ_{SC} = 0.374; $p < 0.001$)
267 (Tables III-S and VI-S). Our data point to different patterns of population structure in population
268 levels for both species. In the case of the *El. nigrita*, the highest difference was found for the TN
269 haplogroup (Table III-S), whereas for the *Ex. smaragdina* the highest structuring was revealed for
270 localities in the Amazon Forest compared to other points in the Atlantic Forest (Table IV-S).
271 *Eulaema nigrita* did not show a significant correlation between genetic and geographic distances
272 ($r = 0.031$; $p > 0.05$). *Exaerete smaragdina* exhibited a positive correlation between these variables
273 (Mantel test: $r = 0.452$; $p < 0.05$) (Figure 2S), indicating the existence of greater gene flow among
274 geographically closer populations.

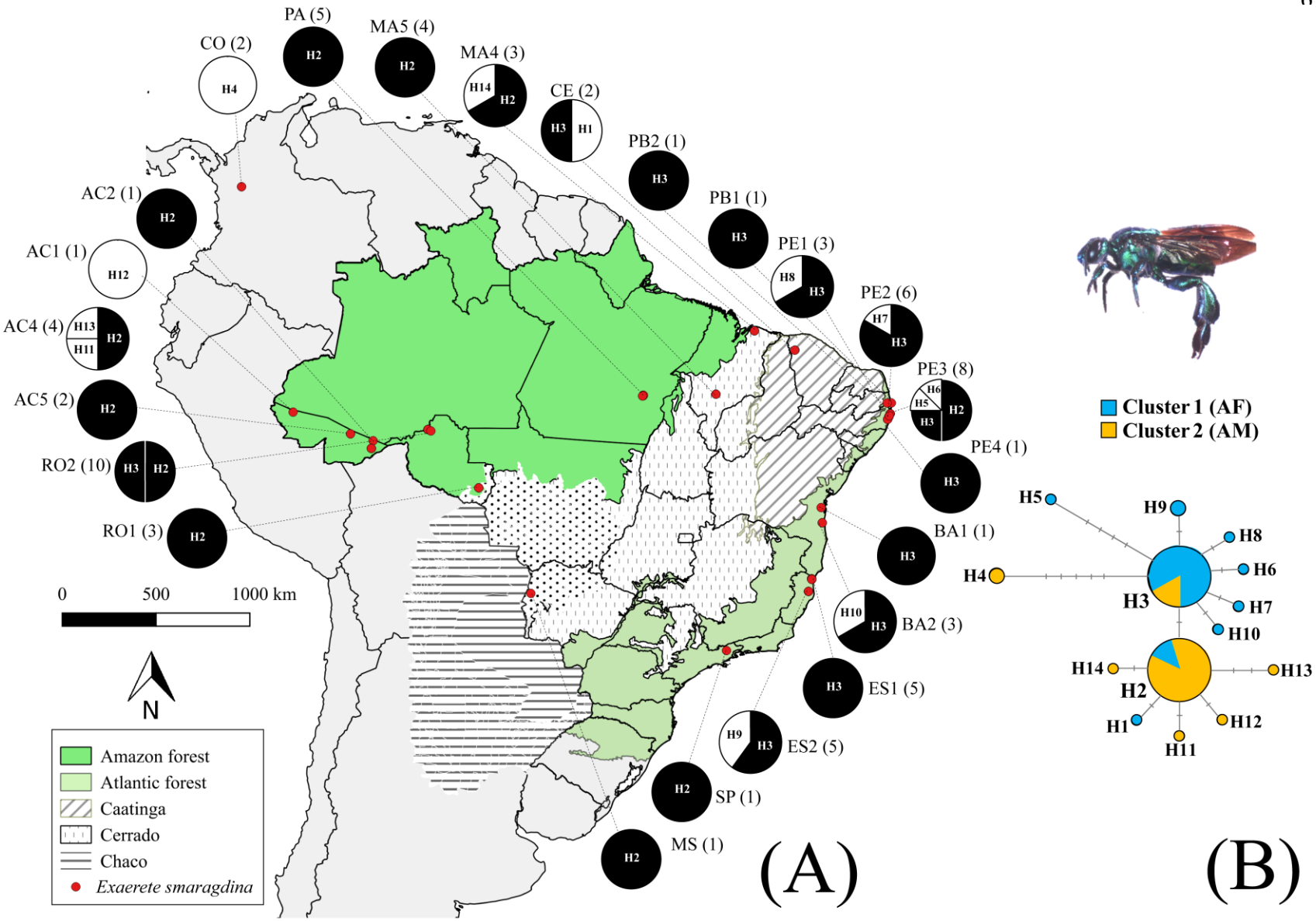


Figure 4 - Eleven sites and frequency of haplotypes present in a fragment of the COI gene of *Exaerete smaragdina* from Atlantic forest and other different forest domains in the South America. (A) Pie charts indicate the frequency and distribution of each haplotype. Haplotypes common (black), haplotypes shared by at least 2 sites (gray) and private haplotypes (white). (B) Median-joining network haplotype for 14 haplotypes (H1-H14) for two haplogroups (Cluster 1 and 2), light blue color represents the cluster 1 which is part the Atlantic forests with the localities CE, PB1, PB2, PE1, PE2, PE3, PE4, BA1, BA2, ES1, and ES2. Orange color in the network is represented by the other west of Brazil in the Amazon domain. Site codes are the same as in the Table 1 AC, BA, CE, ES, GO, MA, MS, PA, PB, PE, RO and SP correspond to usual abbreviations of the following Brazilian states, Acre, Bahia, Ceará, Espirito Santo, Maranhão, Mato Grosso do Sul, Pará, Paraiba, Pernambuco and Rondônia. The CO site correspond a localition of Santa Fe de Antioquia in the Colombia country

Population Demography and Migration

The BSP revealed stability in effective population size (Ne) for both species in most of the Plio-Pleistocene period, with demographic expansion of about 0.5 mya (Figure 5). The full (AF+AM ↔ TN) model was selected by Migrate as the best model for *El. nigrita*, indicating that migration between the AF+AM and TN groups of populations was the more likely explanation for our data set. In the case of *Ex. smaragdina*, the best model shows that migration from the AM to AF (AF ← AM) groups of populations is more explanatory than the other hypotheses (Table II).

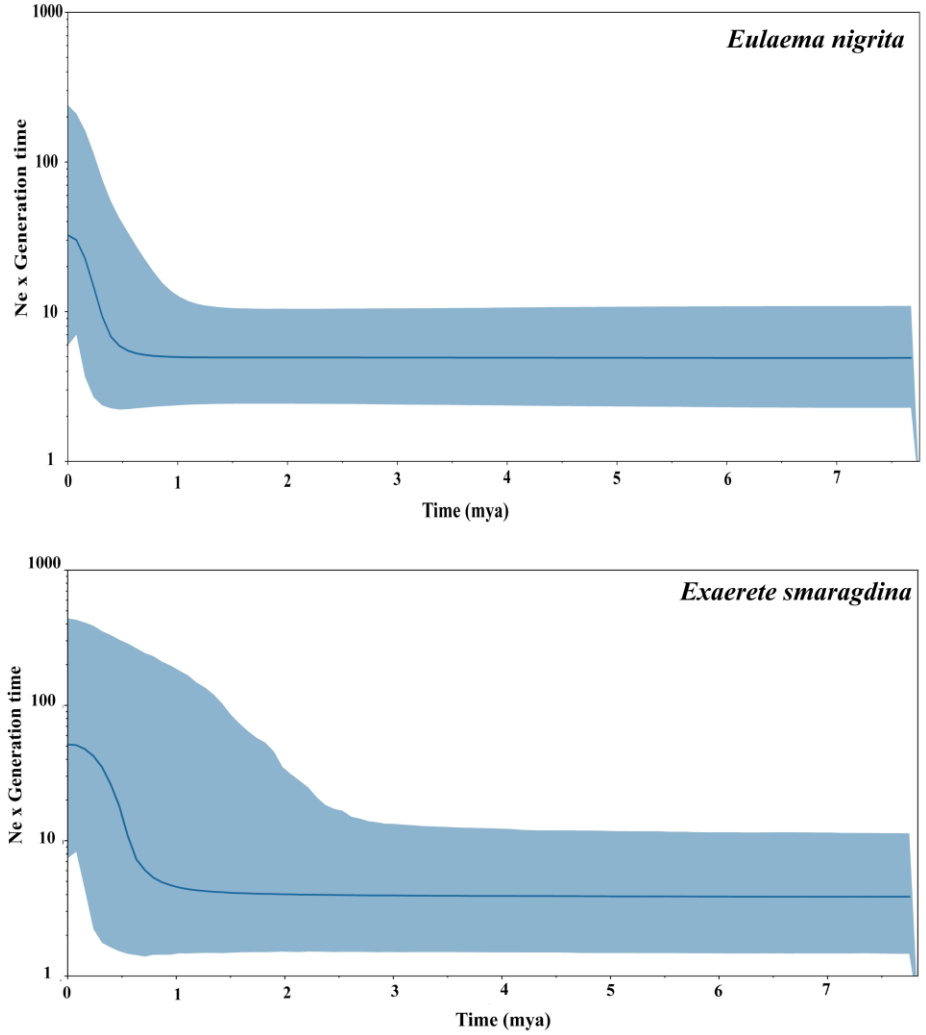


Figure 5 - Bayesian Skyline Plot (BSP) based on *COI* gene exhibiting changes in effective population size of *Eulaema nigrita* (above) and *Exaerete smaragdina* (down). The strict clock was used to infer the demographic history of populations. The dark blue horizontal line shows median BSP estimate and the blue area shows upper and lower 95% limits of the posterior density.

Tabela II Comparison of migration models generated in Migrate 4.4.3 from the *COI* gene of two species of orchid bees (*El. nigrita* and *Ex. smaragdina*) in a cleptoparasite and host relationship. The arrows indicate the direction of migration between the groups, and * the best model.

***Eulaema nigrita* (AM+AF/ TN)**			
Models (n)	**Bezier approximation score**	**ΔBAS**	**Model probability**
1 Panmictic population	-1531,668.691	358,22627	0.000
2 (AF+AM) ↔ (TN)	-1352,555.556	0	0.994*
3 (AF+AM) ← (TN)	-1373,405.241	41,699.37	0.000
4 (AF+AM) → (TN)	-1357,654.873	10,198.634	0.006
***Exaerete smaragdina* (AM/ AF)**			
Models (n)	**Bezier approximation score**	**ΔBAS**	**Model probability**
1 Panmictic population	-1106,657.717	145,91671	0.000
2 (AF) ↔ (AM)	-1035,972.831	4,546.938	0.081
3 (AF) ← (AM)	-1033,699.362	0	0.786*
4 (AF) → (AM)	-1035,475.257	3,551.79	0.133

*$p < 0.05$

DISCUSSION

Pleistocene climate oscillations may have contributed to the current genetic structuring of the species studied here, as reported for other orchid bees (López-Uribe *et al.* 2014, Frantine-Silva *et al.* 2017), as well as other bee groups (Carvalho & Del Lama 2015, Miranda *et al.* 2016, 2017). The network configuration patterns, spatial delimitation reported by Geneland, migration models, and BSP, indicated that the distribution of genetic diversity in populations of *El. nigrita* and *Ex. smaragdina* throughout the Atlantic Forest would have a distinct evolutionary history relative to the TN and Amazon Forest, respectively. In both cases, possibly influenced by isolations and subsequent population expansions from refugia areas, enabling demographic expansion of both species during the Pleistocene, and corroborating with data reported for bees (Santos Júnior *et al.* 2015, Miranda *et al.* 2016, 2017), and other organisms (Haffer 1969; Carnaval *et al.* 2009).

Genetic Structure and Genetic Diversity

Although the present study focused on information from the mitochondrial *COI* region, which is less variable than non-genic regions (introns and microsatellites), the haplotypic diversity values

304 were found for the TN cluster of *El. nigrita* were higher when compared to what has been reported
305 for the mitochondrial DNA of other orchid bee species (López-Uribe *et al.* 2014, Frantine-Silva *et*
306 *al.* 2017). At the same time, data obtained here for both species also suggest clues to recent
307 population expansions throughout the Atlantic Forest, markedly influencing the distribution of
308 genetic diversity among populations of *El. nigrita* and *Ex. smaragdina*.
309
310 **Population Demography and Migration**
311 Data from the present study place the population expansions of *Ex. smaragdina* and *El. nigrita*
312 during the Pleistocene, a period that included sequences of glaciation events that changed forest
313 patterns around the world. The cycles of glaciations promoted expansion and retraction of the arid
314 environments of South America (Haffer 1969; Werneck 2011), providing opportunities for species
315 from more forested environments (*e.g.*, *Ex. smaragdina*) and adapted to open areas (*e.g.*, *El.*
316 *nigrita*) to establish (Carvalho & Almeida 2011).
317 Dick *et al.* (2004), studying the possible effect of the Andean cordilleras on the same
318 species, did not find a phylogeographical structure in Amazon euglossines. This fact may be
319 explained by the recent population expansion of the bees through different environments in South
320 America. Another possible explanation for this expansion may be the permanence of large areas of
321 Amazon formation that remained unchanged by the events of climatic oscillations in the
322 Pleistocene (Colinvaux *et al.* 2000). Following this line, the BSP showed stability between 2 and
323 7 mya for the two species (see Figure 7), which may have represented a period of great climatic
324 instability on the demographic pattern of populations during the Plio-Pleistocene. There was
325 expansion in the effective size of both species only in the late Pleistocene, when the climatic
326 changes seem to have similarly affected the historical demography patterns of both *El. nigrita* and
327 *Ex. smaragdina*, as reported for other bee species (Miranda *et al.* 2016, 2017, Frantine-Silva *et al.*
328 2017). When there was climate stability, a higher Ne (Ne=~80) was observed for the *Ex.*

329 *smaragdina* compared to *El. nigrita*. Concerning this fact, it has been suggested that *El. nigrita* is
330 not the only host of *Ex. smaragdina* throughout the distribution of both species (Nemésio & Silveira
331 2006). These latter authors highlight the high abundance of *Ex. smaragdina* in several areas where
332 *El. nigrita* is absent in the Amazon Basin, pointing out *El. cingulata* (Fabricius) or *El. meriana*
333 (Olivier) as potential hosts of *Ex. smaragdina*, a supposition still to be proved (Nemésio & Silveira
334 2006).

335 Overall, clearer evidence of population expansion was obtained for *Ex. smaragdina*, which
336 revealed significant values for Tajima's D and Fu and Li's D, as well as the starlike configuration
337 in the haplotype network. In addition, the combination $Hd > 0.5$ together with $\pi < 0.5\%$, according
338 to Grant & Bowen (1998), may suggest a population bottleneck followed by rapid population
339 growth and accumulation of mutations. In the case of *El. nigrita*, the population expansion was
340 also indicated by both Tajima's D and Fu and Li's D estimates and the starlike configuration, is
341 readily apparent among the Atlantic Forest in the haplotype network, including most haplotypes
342 separated by only one mutational step. On the other hand, the haplotypic ($Hd < 0.5$) and nucleotide
343 ($\pi > 0.5\%$) diversities of the AF+AM cluster suggest divergence among geographically subdivided
344 populations (Grant & Bowen 1998), which seems plausible since the AF+AM analysis included
345 haplotypes H3 (RO1) and H2 (GO) that belong to the TN cluster haplogroup, and this possibly
346 influenced the genetic diversity estimates and neutrality tests. In any case, BSP results agreed that
347 both species possibly had expansion in the effective size in the late Pleistocene, starting about
348 500,000 years ago, although the times of onset of expansion and the routes taken may present
349 important differences between the species.

350 In the case of *El. nigrita*, comparisons between AF+AM and TN brought information within
351 the evolutionary history of the species. The TN group, besides showing haplotypic diversity twice
352 as high as AF+AM, and with 13 of its 16 haplotypes being private, also showed no signs of recent
353 evolutionarily population expansions. In fact, the $Hd > 0.5$ and $\pi > 0.5\%$ values obtained for TN

suggest a large and stable population with a long evolutionary history or secondary contact between different lineages (Grant & Bowen 1998). At the same time, TN showed no significant values in neutrality tests and its haplotypes are less related than those in AF+AM, including several mutational steps, indicating distinct and longer-established populations than those in the Atlantic Forest. Due to failures during the sequencing process of the *Ex. smaragdina* samples, the sampling of the TN region for this species was very limited, which affects comparisons for this area.

Indeed, expansions from populations that remained in refuge areas during the Pleistocene climatic oscillations seem plausible for both species in the present study. This scenario is not new and has already been reported for stingless bees (Carvalho & Del Lama 2015, Miranda *et al.* 2016, 2017), as well as in other taxa, such as mammals (Vivo 1997, Leite *et al.* 2016), lizards (Werneck 2011, Pellegrino *et al.* 2011), birds (Haffer 1969, Batalha-Filho *et al.* 2013), and plants (Pennington *et al.* 2004, Martini *et al.* 2007), reinforcing the role of these areas as a shelter for high genetic diversity.

The Pleistocene models of identification of stable (refuge areas) versus unstable areas are one of the hypotheses to explain the high diversity of species in the Neotropical region (Haffer 1969, Carnaval & Moritz 2008, Carnaval *et al.* 2009). These areas have an important role in the conservation of bees, sheltering high genetic diversity (Carvalho & Del Lama 2015). In this sense, some studies on orchid bees in different scales such as transcontinental (López-Uribe *et al.* 2014) and Atlantic Forest areas found these areas to be important refuges for orchid bee species (Garraffoni *et al.* 2017, Miranda *et al.* 2019). Among the other several theories trying to explain the biodiversity of South America, the Forest refuge hypothesis is the most accepted (Haffer 1969). In the Pleistocene, refuges corresponded to stable environments during the climatic change of this epoch (Carnaval & Moritz 2008, Carnaval *et al.* 2009). López-Uribe *et al.* (2014) tested the effect of climatic instability of the Pleistocene in three Eulaema species, using mitochondrial (*COI* and *Cyt* b) and nuclear (microsatellites) markers allied to niche modeling. The authors suggested three

predictions for the orchid bee species with respect to historical climatic instability; (1) there was an uneven reduction in the number of forest refuge areas during the dry period of the Pleistocene for all orchid bee species, (2) orchid bees species with low physiological tolerance may show a stronger spatial phylogeographical structure following the refuge hypothesis, and (3) the spatial patterns of genetic diversity of these bees are the consequence of isolation and colonization events during several cycles of forest contraction and expansion. Both species studied here seem to fit these hypotheses.

Although the genetic data from the present study do not clearly show the refuge area from which the population of *El. nigrita* expanded in the Atlantic Forest, it seems quite plausible that TN is within or near one of these areas, maintaining ancestral genetic diversity patterns and possibly influencing other areas in the Amazon region, as suggested by haplotype H3 in RO1 and H2 in GO. In fact, López-Uribe *et al.* (2014) found a suitable area during the Last Glacial Maxima (LGM, 21kya) on the Maranhão coast (TN cluster) for *El. cingulata*. Currently, *El. nigrita* and *El. cingulata* cohabit in several ecosystems from rainforest environments to open formations of *Cerrado* and *Caatinga* in South America (Silveira *et al.* 2015, Martins *et al.* 2018), which may suggest possible cohabitation of these species in the suitable area of the Maranhão coast during the LGM, in the same area as the TN cluster (López-Uribe *et al.* 2014). Studying refuge areas for stingless bees (Meliponini), Camargo & Pedro (2003) also pointed out areas in Maranhão and Pará states as regions of climate stability during the Pleistocene. In addition, Martins *et al.* (2021) found high levels of genetic structuring for *Euglossa cordata* (Linnaeus, 1758), reinforcing the importance of the TN refuge in this region for bee conservation.

Starting from the idea that the Amazon Basin is the original center of the Euglossini (Dressler 1982, Ramírez *et al.* 2010), it is possible that *El. nigrita* was isolated in the TN due to unfavorable environmental conditions during the glaciation events in the Pleistocene. After these climate oscillations, the species studied found conditions to establish themselves and exhibited

404 high demographic expansion due to the high dispersion capacity of the males, in different moments
405 during the Pleistocene. Concurrently, it seems clear that populations of *El. nigrita* from the Atlantic
406 Forest expanded from a refuge area different from TN, including an evolutionary history that
407 involves the previous dispersion of the species to the east coast of South America, as demonstrated
408 by Miranda *et al.* (2019) who identified priority areas for the conservation of ten orchid bee species,
409 among these the two studied here, through ecological niche modeling.

410 In the case of *Ex. smaragdina*, genetic data suggest expansion from at least two refuges,
411 one influencing the Amazon populations more markedly and the other more evident among the
412 Atlantic Forest populations. Although more conclusive discussions require further studies, one of
413 the areas of highest genetic diversity for *Ex. smaragdina* was in one of the Pernambuco sites (PE3),
414 precisely within one of the suggested refuge areas for the Atlantic Forest suggested for both species
415 by Miranda *et al.* (2019). In this context, it seems plausible that the current distribution of genetic
416 diversity of mitochondrial DNA of *Ex. smaragdina* included reflections of an expansion from
417 Pernambuco, dispersing over generations towards the south of the Atlantic Forest formation.
418 Concurrently, populations still dispersing in the north-south direction could be facing more
419 difficulty establishing in the current scenario, and this may be contributing to the lower frequency
420 in smaller latitudes.

421 In some Euglossini genera, such as *Eulaema* and *Exaerete,* the dispersion capacity is
422 directly related to body size (Janzen 1971, Dick *et al.* 2004, Pokorny *et al.* 2015). In this case,
423 larger bees have higher advantages such as thermal tolerance, high energy to fly long distances,
424 and the capacity to colonize new areas (founder effect) (Dick *et al.* 2004). Thus, the best migration
425 model was full (AF+AM ↔ TN) for *El. nigrita*, indicating that migration between clusters occurs
426 in both directions. For *Ex. smaragdina,* the best model showed dispersion in the direction (AF ←
427 AM), and this pattern appears to reflect the historical pattern that the whole group follows (Nemésio
428 2009), which seems plausible since the Amazon is reported as the original center of the Euglossini

429 (Dressler 1982, Ramírez *et al*. 2010). *Eulaema nigrita* did not show this pattern because AM and
430 AF were grouped, and TN showed different haplotypes.

431 In fact, AM and AF clusters examined for *Ex. smaragdina* showed similar values of genetic
432 diversity and very close haplogroups separated by only one mutation. This also indicates common
433 founder populations for AM and AF, and these data suggest that the populations of this species are
434 well established in these forest formations (Nemésio & Silveira 2006). However, the lack of higher
435 genetic diversity values is probably the consequence of the non-sampling of the original center of
436 *Ex. smaragdina*, due to limitations in our sampling and failures during the sample sequencing
437 process.

438 According to López-Uribe *et al.* (2014), the higher structuration observed in mtDNA and
439 correlation of geographical and genetic distance is the result of a different dispersion capacity of
440 Euglossini males (up to 40 km in a few days) (Dressler 1982, Pokorny *et al.* 2015) and females (up
441 to 23 km in the same day) (Janzen 1971). Moreover, the genetic transmission of the material of the
442 mtDNA that is exclusively maternal (Avise 2009), associated with the higher intraspecific mutation
443 rate, is the possible cause of the structuration reported for some studies (López-Uribe *et al.* 2014,
444 Penha *et al.* 2015).

445 Interestingly, the samples of *Ex. smaragdina* in the present study showed a direct
446 relationship between genetic and geographical variables (Mantel test: $r = 0.452$; $p<0.05$),
447 corroborating isolation by distance, which is possibly influenced by the dispersal pattern after
448 population expansion. A clear pattern of genetic divergence was observed for populations separated
449 by more than 418 km, highlighting samples from Espírito Santo with the highest isolation levels.
450 In the case of *El. nigrita*, the absence of this correlation was already expected, since TN and
451 AF+AM populations include different haplogroups.

452

453

CONCLUSION

Despite the closely related ecological relationship between *El. nigrita* and *Ex. smaragdina*, our data reveal different patterns of structure for these orchid bee species, suggesting different evolutionary histories for both species throughout their distribution. On the other hand, the concomitant patterns of population expansion for the studied species, such as the starlike configuration, the presence of few mutations between haplotypes, and significant values for neutrality tests suggest similar response patterns to climate oscillations during the Pleistocene for both species. The highest values of genetic diversity detected for *El. nigrita* within the Atlantic Forest (ES1 and ESP3) and TN region, outside the AF, point out these areas as potential refuges for this during the Pleistocene. For *Ex. smaragdina,* Pernambuco (PE3) may have provided suitable conditions for the survival and maintenance of the high genetic diversity of orchid bees, as highlighted for other orchid bee species. Thus, the preservation of these environments becomes vital for the maintenance of the populations of these important elements of the Neotropical fauna

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Author contributions

DCM, JESJR and PMCA, participated in the study sample design. DCM and JESJR carried out the field activities and molecular genetic analysis. DCM and JESJR carried out all the computational

479 analysis of molecular data. SHS and DGF contributed with some genetic data re-interpretation. All

480 authors participated in the writing of the final document.

481

482 **Disclosure statement**

483 All authors declare no potential conflict of interest.

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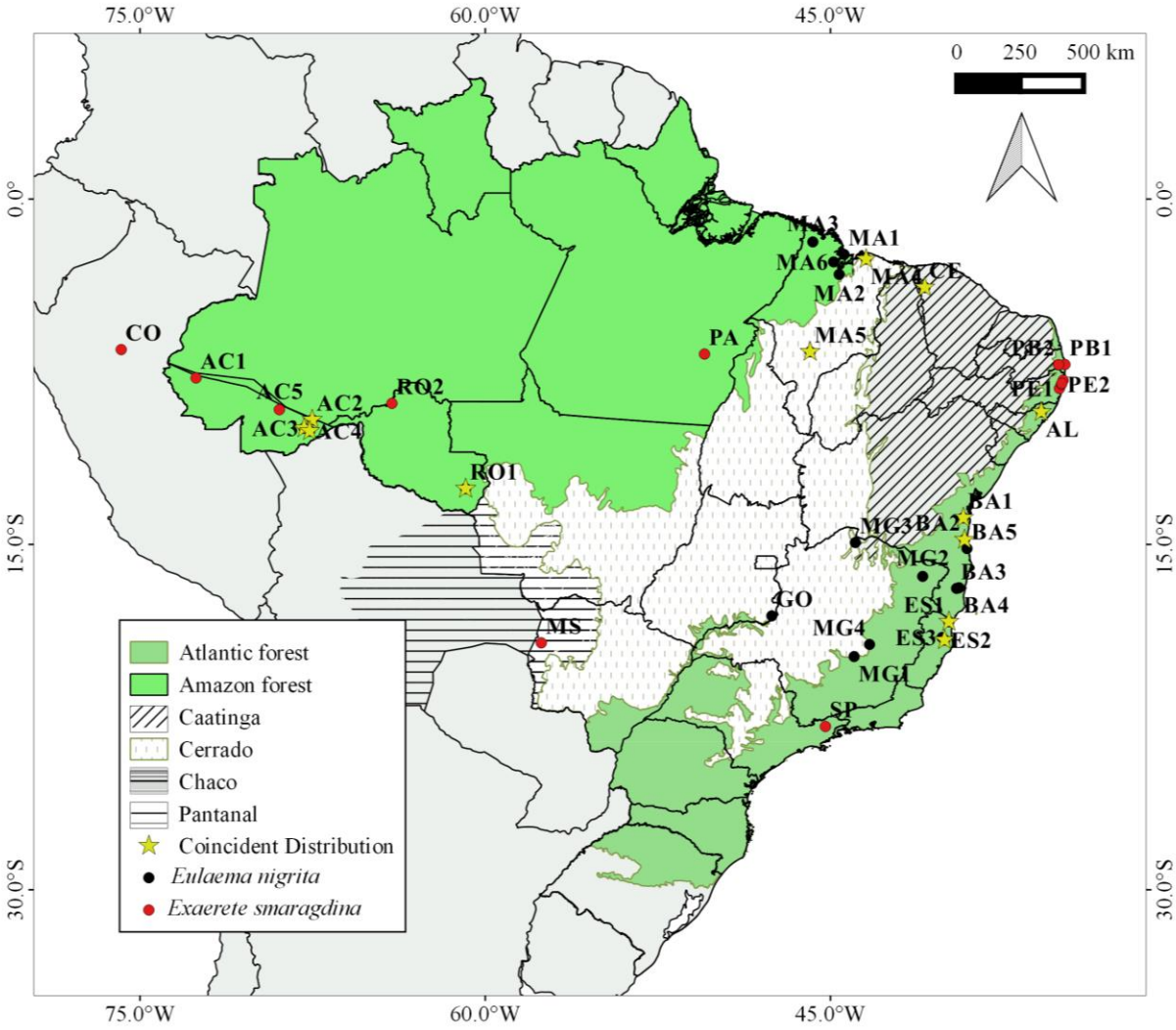
Supplementary Material: Figures and Tables



484 **Figure 1S.** Sampling sites at 19 locations belonging to the Atlantic Forest and other Brazilian
485 vegetation formations for populations of *Eulaema nigrita* and *Exaerete smaragdina*.

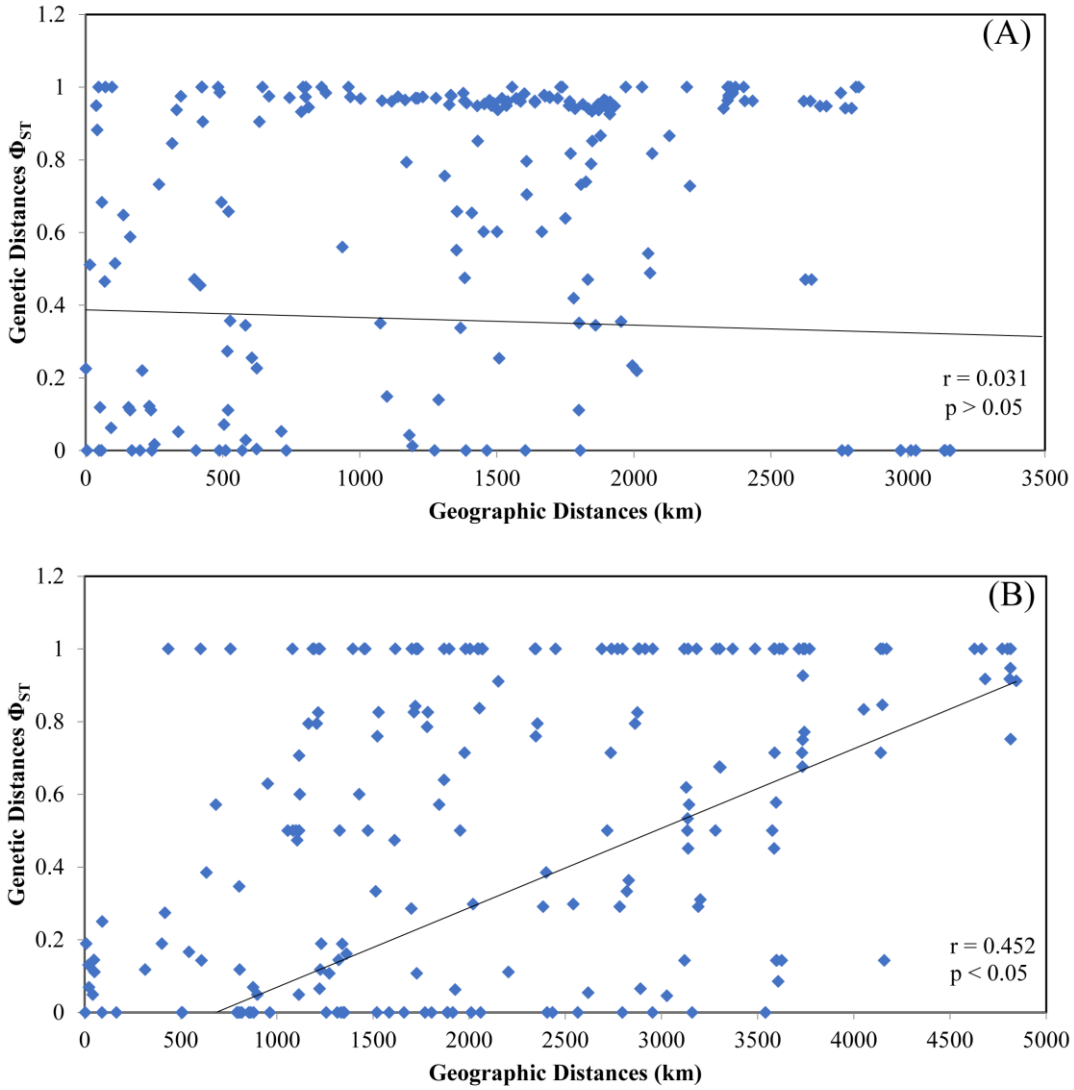


Figure 2S. Relationship between the genetic (Φ_{ST}) and geographical distances (km) from the COI segment of *Eulaema nigrita* (Mantel test: r = 0.031; p>0.05) (A) and *Exaerete smaragdina* (Mantel test: r = 0.452; p<0.05) (B) populations.

Table I-S Localities, code, and geographic coordinates for samples of *Eulaema nigrita* and *Exaerete smaragdina* captured in the Amazon Forest, Atlantic Forest, *Caatinga*, and *Cerrado* in Brazil, and in the locality of Santa Fé de Antioquia in Colombia. Localities within Atlantic Forest are shown in bold.

Localities	Code	El	Ex	Latitude	Longitude
Cruzeiro do Sul, Acre	AC1		1	-7.771389	-72.565833
Humaitá Forest State Park, Porto Acre, Acre	AC2	1	1	-9.589139	-67.516472
Zoobotanical Park of UFAC, Rio Branco, Acre	AC3	8	5	-9.951389	-67.873083
Catuaba Experimental Farm of the UFAC, Senador Guiomard, Acre	AC4	1	4	-10.075722	-67.627361
Cazumba-Iracema Experimental Farm, Senador Madureira, Acre	AC5		2	-9.150556	-68.944722
Murici Ecological Station, Murici, Alagoas	AL	9	1	-9.247222	-35.838889
Michelin Ecological Reserve, Igrapiúna, Bahia	BA1	9	1	-13.845556	-39.226389
UESC Campus, Ilhéus, Bahia	BA2	3	3	-14.795278	-39.172778
Itamaraju, Bahia	BA3	4		-16.914167	-39.521389
Monte Pascoal National Park, Porto Seguro, Bahia	BA4	9		-16.885278	-39.413056
Biological Reserve of Una, Una, Bahia	BA5	3		-15.183333	-39.066944
Ubajara National Park, Ubajara, Ceará	CE	2	2	-3.838833	-40.900528
Santa Fe de Antioquia, Colômbia	CO		2	-6.540156	-75.816786
National Forest of Rio Preto, Conceição da Barra, Espírito Santo	ES1	5	5	-18.357778	-39.855000
Reverse Natural of Vale, Linhares, Espírito Santo	ES2	7	5	-19.156389	-40.042222
Biological Reserve of Sooretama, Sooretama, Espírito Santo	ES3	11		-19.042500	-40.148056
Davinópolis, Goiás	GO	1		-18.105486	-47.548891
Alcântara, Maranhão	MA1	5		-2.406198	-44.408382
Anajatuba, Maranhão	MA2	5		-3.275683	-44.619375
Candido Mendes, Maranhão	MA3	3		-1.863333	-45.767222
Humberto de Campos, Maranhão	MA4	9	4	-2.610505	-43.451591
Mirador State Park, Formosa da Serra Negra, Maranhão	MA5	5	4	-6.632303	-45.884514
São Bento, Maranhão	MA6	5		-2.732145	-44.863949
UFMG Ecological Station, Belo Horizonte, Minas Gerais	MG1	1		-19.873964	-43.973523
Biological Reserve of the Mata Escura, Jequitinhonha, Minas Gerais	MG2	6		-16.390556	-40.992778
Lagoa do Cajueiro State Park, Matias Cardoso, Minas Gerais	MG3	3		-14.920556	-43.919167
Morro do Pilar, Minas Gerais	MG4	1		-19.348083	-43.303361
Corumbá, Mato Grosso do Sul	MS		1	-19.279217	-57.563953
Água Azul do Norte, Pará	PA		5	-6.733833	-50.472667
State Park Trilha dos Cinco Rios, João Pessoa, Paraíba	PB1		1	-7.190109	-34.811405
Cruz do Espírito Santo, Paraíba	PB2		1	-7.195281	-35.088570
Cabo de Santo Agostinho, Pernambuco	PE1		3	-8.229583	-35.050333
Camaragibe, Pernambuco	PE2		6	-8.047773	-34.965639
Igarassu, Pernambuco	PE3		8	-7.869885	-34.886786
State Park Dois Irmãos, Recife, Pernambuco	PE4		1	-8.002670	-34.945407
Chupinguaia, Rondônia	RO1	2	4	-12.579058	-60.851300
Porto Velho, Rondônia	RO2		10	-8.874917	-64.053028
Aparecida, São Paulo	SP		1	-22.910611	-45.217917

Table II-S Specimens sequenced for the genetic analyses, based on segments of genes *16S, Opsin,* and *COI* (base pair size = bp), with their geographic origins, which were used in phylogeographic and biogeographic analyses. Brazil: AC – Acre; AL – Alagoas; BA – Bahia; CE – Ceará; ES – Espírito Santo; MA – Maranhão; MG – Minas Gerais; MS – Mato Grosso do Sul; PA – Pará; PB – Paraíba; PE – Pernambuco; RO – Rondônia; SP – São Paulo; UFAC – Universidade Federal do Acre (AC); UESC – Universidade Estadual de Santa Cruz (BA). The blank spaces in the table are samples which were not amplified or sequenced.

Species	**Collection locality**	**Voucher**	**Latitude**	**Longitude**	***16S* (bp)**	***Opsin* (bp)**	***COI* (bp)**
Eulaema nigrita Lepeletier, 1841	Ubajara National Park, Ubajara, CE	1207204	-3.838833	-40.900528	576		586
Eulaema nigrita Lepeletier, 1841	Ubajara National Park, Ubajara, CE	1207569	-3.847500	-40.888361	576		586
Eulaema nigrita Lepeletier, 1841	Davinópolis, GO	1208611	-18.105486	-47.548891	576		586
Eulaema nigrita Lepeletier, 1841	Chupinguaia, RO	1221064	-12.579058	-60.851300	576		586
Eulaema nigrita Lepeletier, 1841	Chupinguaia, RO	1221110	-12.579058	-60.851300	576		586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	43509	-9.217500	-35.877778		585	586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	43510	-9.217500	-35.877778			586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	43515	-9.217500	-35.877778		585	586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	43551	-9.217500	-35.877778	576		586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	43573	-9.223056	-35.879167		585	586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	43905	-9.207778	-35.908889	576	585	586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	44071	-9.247222	-35.838889			586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	44078	-9.247222	-35.838889			586
Eulaema nigrita Lepeletier, 1841	Murici Ecological Station, Murici, AL	44096	-9.247222	-35.838889			586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46433	-16.885278	-39.413056			586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46479	-16.885278	-39.413056			586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46545	-16.885278	-39.413056		585	586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46564	-16.885278	-39.413056	576	585	586

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Eulaema nigrita Lepeletier, 1841	Itamaraju, BA	46719	-16.914167	-39.521389		585	586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46753	-16.878056	-39.415556	576		586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46754	-16.878056	-39.415556	576		586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46755	-16.878056	-39.415556	576		586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46819	-16.878056	-39.415556	576		586
Eulaema nigrita Lepeletier, 1841	Monte Pascoal National Park, Porto Seguro, BA	46855	-16.878056	-39.415556			586
Eulaema nigrita Lepeletier, 1841	Itamaraju, BA	46892	-16.914167	-39.521389			586
Eulaema nigrita Lepeletier, 1841	Itamaraju, BA	46893	-16.914167	-39.521389			586
Eulaema nigrita Lepeletier, 1841	Itamaraju, BA	46902	-16.914167	-39.521389		585	586
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47444	-13.845556	-39.226389		585	586
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47606	-13.833611	-39.248889		585	586
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47623	-13.833611	-39.248889	576		
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47682	-13.833611	-39.248889	576		586
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47707	-13.833611	-39.248889	576		586
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47727	-13.834167	-39.248889		585	586
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47795	-14.795278	-39.172778		585	586

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47798	-14.795278	-39.172778		585	586
Eulaema nigrita Lepeletier, 1841	Michelin Ecological Reserve, Igrapiúna, BA	47881	-14.795278	-39.172778		585	586
Eulaema nigrita Lepeletier, 1841	UESC Campus, Ilhéus, BA	47885	-14.795278	-39.172778	576		586
Eulaema nigrita Lepeletier, 1841	UESC Campus, Ilhéus, BA	47894	-14.795278	-39.172778	576		586
Eulaema nigrita Lepeletier, 1841	UESC Campus, Ilhéus, BA	50778	-14.795278	-39.172778		585	586
Eulaema nigrita Lepeletier, 1841	Natural Vale Reserve, Linhares, ES	48149	-19.156389	-40.042222	576		586
Eulaema nigrita Lepeletier, 1841	Natural Vale Reserve, Linhares, ES	48204	-19.156389	-40.042222			586
Eulaema nigrita Lepeletier, 1841	Natural Vale Reserve, Linhares, ES	48256	-19.156389	-40.042222		585	586
Eulaema nigrita Lepeletier, 1841	Natural Vale Reserve, Linhares, ES	49041	-19156389	-40042222			586
Eulaema nigrita Lepeletier, 1841	Natural Vale Reserve, Linhares, ES	49049	-19.156389	-40.042222		585	
Eulaema nigrita Lepeletier, 1841	Natural Vale Reserve, Linhares, ES	49114	-19156389	-40042222		585	586
Eulaema nigrita Lepeletier, 1841	Natural Vale Reserve, Linhares, ES	49269	-19.156389	-40.042222		585	586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	48672	-19.042500	-40.148056	576		
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	48682	-19.042500	-40.148056	576		586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	48715	-19.042500	-40.148056	576		586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	48754	-19.042500	-40.148056	576		586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	48787	-19.042500	-40.148056		585	
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	48804	-19.055639	-40.147222		585	
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	49012	-19.042500	-40.148056	576		586

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	49018	-19.042500	-40.148056	576		586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	49021	-19.042500	-40.148056		585	586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	49024	-19.042500	-40.148056	576		586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Sooretama, Sooretama, ES	49039	-19.042500	-40.148056	576		586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of the Mata Escura, Jequitinhonha, MG	49447	-16.390556	-40.992778			586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of the Mata Escura, Jequitinhonha, MG	49521	-16.390556	-40.992778	576		586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of the Mata Escura, Jequitinhonha, MG	49527	-16.390556	-40.992778	576		
Eulaema nigrita Lepeletier, 1841	Biological Reserve of the Mata Escura, Jequitinhonha, MG	49579	-16.350000	-41.091944	576	585	
Eulaema nigrita Lepeletier, 1841	Biological Reserve of the Mata Escura, Jequitinhonha, MG	49582	-16.350000	-41.091944	576		
Eulaema nigrita Lepeletier, 1841	Biological Reserve of the Mata Escura, Jequitinhonha, MG	49585	-16.350000	-41.091944	576		
Eulaema nigrita Lepeletier, 1841	Lagoa do Cajueiro State Park, Matias Cardoso, MG	1504843	-14.920556	-43.919167	576		586
Eulaema nigrita Lepeletier, 1841	Lagoa do Cajueiro State Park, Matias Cardoso, MG	1504913	-14.993056	-43.951667	576		586
Eulaema nigrita Lepeletier, 1841	Lagoa do Cajueiro State Park, Matias Cardoso, MG	1505042	-14.989167	-43.949722	576		586
Eulaema nigrita Lepeletier, 1841	Morro do Pilar, MG	1501312	-19.348083	-43.303361	576		586

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Eulaema nigrita Lepeletier, 1841	UFMG Ecological Station, Belo Horizonte, MG	1208638	-19.873964	-43.973523	576		586
Eulaema nigrita Lepeletier, 1841	National Forest of Rio Preto, Conceição da Barra, ES	49665	-18.357778	-39.855000	576	585	586
Eulaema nigrita Lepeletier, 1841	National Forest of Rio Preto, Conceição da Barra, ES	50024	-18.366389	-39.847778		585	586
Eulaema nigrita Lepeletier, 1841	National Forest of Rio Preto, Conceição da Barra, ES	50096	-18.357778	-39.855000		585	586
Eulaema nigrita Lepeletier, 1841	National Forest of Rio Preto, Conceição da Barra, ES	50218	-18.387778	-39.852500		585	
Eulaema nigrita Lepeletier, 1841	National Forest of Rio Preto, Conceição da Barra, ES	50282	-18.387778	-39.852500		585	
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Una, Una, BA	50383	-15.183333	-39.066944			586
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Una, Una, BA	50508	-15.168611	-39.065000	576		
Eulaema nigrita Lepeletier, 1841	Biological Reserve of Una, Una, BA	50674	-15.168611	-39.065000		585	586
Eulaema nigrita Lepeletier, 1841	Catuaba Experimental Farm of the UFAC, Senador Guiomard, AC	AC147	-10.075722	-67.627361			586
Eulaema nigrita Lepeletier, 1841	Humaitá Florest State Park, Porto, AC	AC242	-9.589139	-67.516472	576	585	586
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC423	-9.955472	-67.873889	576		
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC424	-9.955472	-67.873889	576		
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC425	-9.955472	-67.873889	576		
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC426	-9.955472	-67.873889	576		
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC428	-9.955472	-67.873889	576		

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC429	-9.955472	-67.873889	576		
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC431	-9.955472	-67.873889	576		
Eulaema nigrita Lepeletier, 1841	Zoobotanical Park of UFAC, Rio Branco, AC	AC432	-9.955472	-67.873889	576		
Eulaema nigrita Lepeletier, 1841	Anajatuba, MA	AJ122	-3.275683	-44.619375	576	585	586
Eulaema nigrita Lepeletier, 1841	Anajatuba, MA	AJ123	-3.275683	-44.619375	576	585	586
Eulaema nigrita Lepeletier, 1841	Anajatuba, MA	AJ124	-3.275683	-44.619375	576	585	586
Eulaema nigrita Lepeletier, 1841	Anajatuba, MA	AJ1387	-3.275683	-44.619375	576	585	586
Eulaema nigrita Lepeletier, 1841	Anajatuba, MA	AJ388	-3.275683	-44.619375	576	585	586
Eulaema nigrita Lepeletier, 1841	Alcântara, MA	AL100	-2.406198	-44.408382		585	586
Eulaema nigrita Lepeletier, 1841	Alcântara, MA	AL101	-2.406198	-44.408382	576	585	586
Eulaema nigrita Lepeletier, 1841	Alcântara, MA	AL102	-2.406198	-44.408382	576	585	586
Eulaema nigrita Lepeletier, 1841	Alcântara, MA	AL103	-2.406198	-44.408382	576	585	586
Eulaema nigrita Lepeletier, 1841	Alcântara, MA	AL104	-2.406198	-44.408382	576		
Eulaema nigrita Lepeletier, 1841	Candido Mendes, MA	CM267	-1.863333	-45.767222	576		586
Eulaema nigrita Lepeletier, 1841	Candido Mendes, MA	CM268	-1.863333	-45.767222			586
Eulaema nigrita Lepeletier, 1841	Candido Mendes, MA	CM67	-1.863333	-45.767222	576		586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB56	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB57	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB58	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB59	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB60	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB61	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB62	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB98	-2.610505	-43.451591	576	585	586
Eulaema nigrita Lepeletier, 1841	Humberto de Campos, MA	HB99	-2.610505	-43.451591	576	585	586

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Eulaema nigrita Lepeletier, 1841	Mirador State Park, Formosa da Serra Negra, MA	MD01	-6.632303	-45.884514	576	585	586
Eulaema nigrita Lepeletier, 1841	Mirador State Park, Formosa da Serra Negra, MA	MD02	-6.632303	-45.884514	576	585	586
Eulaema nigrita Lepeletier, 1841	Mirador State Park, Formosa da Serra Negra, MA	MD03	-6.632303	-45.884514	576	585	586
Eulaema nigrita Lepeletier, 1841	Mirador State Park, Formosa da Serra Negra, MA	MD04	-6.632303	-45.884514	576	585	586
Eulaema nigrita Lepeletier, 1841	Mirador State Park, Formosa da Serra Negra, MA	MD05	-6.632303	-45.884514	576	585	586
Eulaema nigrita Lepeletier, 1841	São Bento, MA	SB74	-2.732145	-44.863949	576	585	586
Eulaema nigrita Lepeletier, 1841	São Bento, MA	SB75	-2.732145	-44.863949	576	585	586
Eulaema nigrita Lepeletier, 1841	São Bento, MA	SB76	-2.732145	-44.863949	576	585	586
Eulaema nigrita Lepeletier, 1841	São Bento, MA	SB77	-2.732145	-44.863949	576	585	586
Eulaema nigrita Lepeletier, 1841	São Bento, MA	SB78	-2.732145	-44.863949	576	585	586
Exaerete smaragdina (Guérin, 1844)	Ubajara National Park, Ubajara, CE	1207649	-3.831500	-40.900667			660
Exaerete smaragdina (Guérin, 1844)	Ubajara National Parck, Ubajara, CE	1316725	-3.837861	-40.910250			660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, Rondônia, RO	1218414	-8.969000	-63.872444	511		660
Exaerete smaragdina (Guérin, 1844)	Santa Fe de Antioquia, Colombia	1403287	-6.540156	-75.816786		668	660
Exaerete smaragdina (Guérin, 1844)	Santa Fe de Antioquia, Colombia	1403288	-6.540156	-75.816786	511		660
Exaerete smaragdina (Guérin, 1844)	National Forest of Rio Preto, Conceição da Barra, ES	1403827	-18.348333	-39.851917	511		660
Exaerete smaragdina (Guérin, 1844)	National Forest of Rio Preto, Conceição da Barra, ES	1404049	-18.391139	-39.835889	511		660
Exaerete smaragdina (Guérin, 1844)	National Forest of Rio Preto, Conceição da Barra, ES	1404429	-18.351447	-39.835425			660
Exaerete smaragdina (Guérin, 1844)	National Forest of Rio Preto, Conceição da Barra, ES	1404482	-18.391139	-39.835889			660

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413406	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413407	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413408	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413409	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413411	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413413	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413414	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Igarassu, PE	1413415	-7.869885	-34.886786			660
Exaerete smaragdina (Guérin, 1844)	Camaragibe, PE	1413428	-8,047773	-34.965639			660
Exaerete smaragdina (Guérin, 1844)	Camaragibe, PE	1413429	-8,047773	-34.965639			660
Exaerete smaragdina (Guérin, 1844)	Camaragibe, PE	1413430	-8,047773	-34.965639			660
Exaerete smaragdina (Guérin, 1844)	Camaragibe, PE	1413431	-8,047773	-34.965639			660
Exaerete smaragdina (Guérin, 1844)	Camaragibe, PE	1413432	-8,047773	-34.965639			660
Exaerete smaragdina (Guérin, 1844)	Camaragibe, PE	1413433	-8,047773	-34.965639			660
Exaerete smaragdina (Guérin, 1844)	Cabo de Santo Agostinho, PE	1413434	-8.229583	-35.050333			660
Exaerete smaragdina (Guérin, 1844)	Cabo de Santo Agostinho, PE	1413436	-8.229583	-35.050333			660
Exaerete smaragdina (Guérin, 1844)	Cabo de Santo Agostinho, PE	1413437	-8.229583	-35.050333			660
Exaerete smaragdina (Guérin, 1844)	State Park Dois Irmãos, Recife, PE	1413439	-8.002670	-34.945407			660
Exaerete smaragdina (Guérin, 1844)	State Park Trilhha dos Cinco Rios, João Pessoa, PB	1413440	-7.190109	-34.811405			660
Exaerete smaragdina (Guérin, 1844)	Cruz do Espírito Santo, PB	1413441	-7.195281	-35.088570			660
Exaerete smaragdina (Guérin, 1844)	Aparecida, SP	1414858	-22.910611	-45.217917	511		660
Exaerete smaragdina (Guérin, 1844)	Mirador State Park, Formosa da Serra Negra, MA	1415266	-6.632303	-45.884514	511		660
Exaerete smaragdina (Guérin, 1844)	Mirador State Park, Formosa da Serra Negra, MA	1415267	-6.632303	-45.884514	511		660
Exaerete smaragdina (Guérin, 1844)	Mirador State Park, Formosa da Serra Negra, MA	1415268	-6.632303	-45.884514	511		660

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Exaerete smaragdina (Guérin, 1844)	Mirador State Park, Formosa da Serra Negra, MA	1415269	-6.632303	-45.884514	511		660
Exaerete smaragdina (Guérin, 1844)	Água Azul do Norte, PA	1524257	-6.733833	-50.472667	511	668	660
Exaerete smaragdina (Guérin, 1844)	Água Azul do Norte, PA	1524323	-6.696694	-50.466333	511		660
Exaerete smaragdina (Guérin, 1844)	Água Azul do Norte, PA	1524341	-6.696694	-50.466333	511	668	660
Exaerete smaragdina (Guérin, 1844)	Água Azul do Norte, PA	1524342	-6.696694	-50.466333	511		660
Exaerete smaragdina (Guérin, 1844)	Água Azul do Norte, PA	1524398	-6.746861	-50.514444	511		660
Exaerete smaragdina (Guérin, 1844)	Murici Ecological Station, Murici, AL	44118	-9.247222	-35.838889	511	668	
Exaerete smaragdina (Guérin, 1844)	UESC Campus, Ilhéus, BA	46957	-14.795278	-39.172778			660
Exaerete smaragdina (Guérin, 1844)	Michelin Ecological Reserve, Igrapiúna, BA	47861	-13.833611	-39.248889	511	668	660
Exaerete smaragdina (Guérin, 1844)	Natural Vale Reserve, Linhares, ES	48219	-19.156389	-40.042222	511		660
Exaerete smaragdina (Guérin, 1844)	Natural Vale Reserve, Linhares, ES	48336	-19.156389	-40.042222	511	668	660
Exaerete smaragdina (Guérin, 1844)	Natural Vale Reserve, Linhares, ES	48349	-19.156389	-40.042222	511		660
Exaerete smaragdina (Guérin, 1844)	Natural Vale Reserve, Linhares, ES	49042	-19.156389	-40.042222	511		660
Exaerete smaragdina (Guérin, 1844)	Natural Vale Reserve, Linhares, ES	49433	-19.156389	-40.042222	511		660
Exaerete smaragdina (Guérin, 1844)	National Forest of Rio Preto, Conceição da Barra, ES	49975	-18.357778	-39.855000			660
Exaerete smaragdina (Guérin, 1844)	Chupinguaia, RO	53104	-12.579058	-60.851300	511		660
Exaerete smaragdina (Guérin, 1844)	Chupinguaia, RO	53240	-12.579058	-60.851300	511		660
Exaerete smaragdina (Guérin, 1844)	Chupinguaia, RO	53245	-12.579058	-60.851300			660
Exaerete smaragdina (Guérin, 1844)	Chupinguaia, RO	53246	-12.579058	-60.851300	511		660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76013	-8.874917	-64.053028			660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76188	-8.874917	-64.053028	511		660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76190	-8.874917	-64.053028			660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76227	-8.874917	-64.053028	511		660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76228	-8.874917	-64.053028			660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76513	-8.874917	-64.053028			660

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76533	-8.874917	-64.053028			660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76540	-8.874917	-64.053028	511		660
Exaerete smaragdina (Guérin, 1844)	Porto Velho, RO	76554	-8.874917	-64.053028	511		660
Exaerete smaragdina (Guérin, 1844)	UESC Campus, Ilhéus, BA	77253	-14.795278	-39.172778			660
Exaerete smaragdina (Guérin, 1844)	UESC Campus, Ilhéus, BA	77435	-14.795278	-39.172778			660
Exaerete smaragdina (Guérin, 1844)	Cazumba-Iracema Experimental Farm, Senador Madureira, AC	AC01	-9.150556	-68.944722	511	668	660
Exaerete smaragdina (Guérin, 1844)	Catuaba Experimental Farm of the UFAC, Senador Guiomard, AC	AC145	-10.075722	-67.627361	511		660
Exaerete smaragdina (Guérin, 1844)	Catuaba Experimental Farm of the UFAC, Senador Guiomard, AC	AC146	-10.075722	-67.627361	511		660
Exaerete smaragdina (Guérin, 1844)	Cruzeiro do Sul, Acre	AC15	-7.771389	-72.565833		668	660
Exaerete smaragdina (Guérin, 1844)	Catuaba Experimental Farm of the UFAC, Senador Guiomard, AC	AC155	-10.075722	-67.627361	511	668	660
Exaerete smaragdina (Guérin, 1844)	Catuaba Experimental Farm of the UFAC, Senador Guiomard, AC	AC156	-10.075723	-67.627369		668	
Exaerete smaragdina (Guérin, 1844)	Catuaba Experimental Farm of the UFAC, Senador Guiomard, AC	AC157	-10.075722	-67.627361	511	668	660
Exaerete smaragdina (Guérin, 1844)	Sena Madureira, Acre	AC30	-9.150556	-68.944722	511	668	660
Exaerete smaragdina (Guérin, 1844)	Humaitá Florest State Park, Porto, AC	AC330	-9.589139	-67.516472	511		660
Exaerete smaragdina (Guérin, 1844)	Zoobotanical Park of the UFAC, Rio Branco, AC	AC366	-9.951389	-67.873083	511	668	
Exaerete smaragdina (Guérin, 1844)	Zoobotanical Park of the UFAC, Rio Branco, AC	AC392	-9.951389	-67.873083	511	668	
Exaerete smaragdina (Guérin, 1844)	Zoobotanical Park of the UFAC, Rio Branco, AC	AC405	-9.955472	-67.873889		668	
Exaerete smaragdina (Guérin, 1844)	Zoobotanical Park of the UFAC, Rio Branco, AC	AC417	-9.955472	-67.873889	511	668	

Species	Collection locality	Voucher	Latitude	Longitude	*16S* (bp)	*Opsin* (bp)	*COI* (bp)
Exaerete smaragdina (Guérin, 1844)	Zoobotanical Park of the UFAC, Rio Branco, AC	AC421	-9.955472	-67.873889	511	668	
Exaerete smaragdina (Guérin, 1844)	Humberto de Campos, MA	HB100	-2.610505	-43.451591		668	
Exaerete smaragdina (Guérin, 1844)	Humberto de Campos, MA	HB101	-2.610505	-43.451591		668	660
Exaerete smaragdina (Guérin, 1844)	Humberto de Campos, MA	HB102	-2.610505	-43.451591		668	660
Exaerete smaragdina (Guérin, 1844)	Humberto de Campos, MA	HB103	-2.610505	-43.451591		668	
Exaerete smaragdina (Guérin, 1844)	Humberto de Campos, MA	HB104	-2.610505	-43.451591		668	660
Exaerete smaragdina (Guérin, 1844)	Corumbá, MS	72455	-19.279217	-57.563953			660

Table III-S Pairwise Φ_{ST} values for 586 bp of DNA fragments of the mitochondrial *COI* gene from 97 sequences of *Eulaema nigrita* sampled in 24 localities in different vegetable formations in the Brazilian territory. The Φ_{ST} values are in the lower left of the matrix and the distance in kilometers between localities in the upper right. Significant Φ_{ST} values and the geographic distance between the respective pairs of samples are exhibited in bold. Negative and not significant values = 0.000.

		AC2	AC4	AL	BA1	BA2	BA3	BA4	BA5	ES1	ES2	ES3	GO	MG1	MG2	MG3	MG4	RO1	CE	MA1	MA2	MA3	MA4	MA5	MA6
	AC2	---	57.47	3,481	3,119	3,135	3,136	3,146	3,152	3,138	3,143	3,127	2,352	2,781	2,974	2,633	2,820	803	3,010	2,679	2,626	2,554	2,771	2,405	2,620
	AC4	0.000	---	3,488	3,120	3,138	3,132	3,150	3,155	3,129	3,136	3,120	2,342	2,759	2,972	2,629	2,810	793	3,028	2,702	2,648	2,583	2,794	2,433	2,644
	AL	0.000	0.000	---	623	714	935	930	746	1,099	1,192	**1,181**	1,600	1,459	966	1,075	1,378	**2,755**	816	**1,212**	**1,170**	1,367	**1,116**	**1,139**	**1,230**
	BA1	0.000	0.000	0.000	---	105	341	338	150	505	595	584	1,003	836	340	517	744	**2,346**	1,127	**1,390**	**1,310**	1,508	**1,326**	**1,081**	**1,381**
	BA2	0.000	0.000	0.000	0.000	---	238	232	43	402	490	482	966	759	262	511	669	2,359	1,226	**1,489**	**1,409**	1,604	**1,428**	**1,165**	**1,473**
AF+AM	**BA3**	0.000	0.000	0.000	0.000	0.000	---	12	198	162	257	244	861	571	168	519	483	2,345	1,464	**1,692**	**1,609**	1,799	**1,639**	**1,332**	**1,673**
	BA4	0.000	0.000	0.000	0.000	0.000	0.000	---	195	170	261	251	876	582	176	527	489	**2,360**	1,455	**1,693**	**1,608**	1,800	**1,638**	**1,334**	**1,676**
	BA5	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	357	449	441	959	732	241	523	645	2,370	1,273	1,539	1,452	1,648	**1,480**	1,206	1,519
	ES1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	92	81	813	465	249	577	380	2,342	1,612	**1,834**	**1,750**	1,936	**1,785**	**1,454**	**1,814**
	ES2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	16	802	423	323	624	347	**2,346**	1,699	**1,913**	**1,825**	2,011	**1,870**	**1,522**	1,891
	ES3	0.000	0.000	**0.042**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	787	410	304	606	332	**2,327**	1,686	**1,912**	**1,807**	1,994	**1,847**	**1,503**	**1,871**
	GO	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	425	720	519	470	1,556	1,739	1,766	1,664	1,800	1,763	1,278	1,722
	MG1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	487	543	96	1.970	1,804	1,930	1,832	2,000	1,903	1,476	1,899
	MG2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	362	423	2,193	1,387	1,587	**1,501**	1,691	**1,535**	**1,203**	**1,570**
	MG3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	500	1,860	1,271	1,383	1,287	1,459	**1,354**	**936**	**1,353**
	MG4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	2,030	1,732	1,878	1,780	1,953	1,844	1,430	1,848
	RO1	0.000	0.000	**0.984**	**0.972**	0.000	0.000	**0.986**	0.000	0.000	**0.977**	**0.941**	0.000	0.000	0.000	0.000	0.000	---	2,400	2,129	2,059	2,052	**2,204**	**1,769**	**2,067**
	CE	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	420	**418**	583	**315**	**634**	427
	MA1	0.000	0.000	**0.969**	**0.956**	**0.955**	**0.974**	**0.971**	0.000	**0.940**	**0.959**	**0.925**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	100	**162**	**107**	**495**	**59**
TN	**MA2**	0.000	0.000	**0.793**	**0.755**	**0.654**	**0.704**	**0.796**	0.000	**0.639**	**0.739**	**0.732**	0.000	0.000	**0.602**	0.000	0.000	0.000	**0.602**	0.000	---	206	**150**	**396**	**69**
	MA3	0.000	0.000	0.000	0.000	0.000	0.000	0.351	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.587**	0.000	---	**267**	**521**	**137**
	MA4	0.000	0.000	**0.961**	**0.951**	**0.948**	**0.958**	**0.962**	**0.949**	**0.940**	**0.953**	**0.933**	0.000	0.000	**0.949**	**0.658**	0.000	**0.728**	**0.949**	**0.515**	**0.511**	**0.733**	---	520	156
	MA5	0.000	0.000	**0.973**	**0.963**	**0.965**	**0.978**	**0.975**	0.000	**0.953**	**0.966**	**0.937**	0.000	0.000	**0.970**	**0.560**	0.000	**0.817**	**0.970**	**0.683**	**0.471**	**0.658**	0.000	---	442
	MA6	0.000	0.000	**0.973**	**0.962**	**0.964**	**0.978**	**0.975**	0.000	**0.952**	**0.965**	**0.936**	0.000	0.000	**0.969**	**0.551**	0.000	**0.817**	0.000	**0.683**	**0.465**	**0.648**	0.000	0.000	---

Table IV-S Pairwise Φ_{ST} values for the *COI* gene from 74 sequences of *Exaerete smaragdina* sampled in 23 localities in different vegetable formations in the Brazilian territory, and a sample from Colombia. The Φ_{ST} values are in the lower left of the matrix and the distance in kilometers between localities in the upper right. Significant Φ_{ST} values and the geographic distance between the respective pairs of samples are exhibited in bold. Negative not significant values = 0.000.

		BA1	BA2	CE	ES1	ES2	PB1	PB2	PE1	PE2	PE3	PE4	SP	AC1	AC2	AC4	AC5	CO	MA4	MA5	MS	PA	RO1	RO2
	BA1	---	105	1,127	505	595	879	864	770	796	812	796	1,187	3,714	3,119	3,120	3,285	4,628	1,326	1,081	2,044	1,460	2,346	2,763
	BA2	0.000	---	1,226	402	490	967	951	854	876	898	881	1,099	3,730	3,135	**3,138**	3,301	4,683	1,428	**1,165**	1,473	**1,529**	**2,355**	2,783
	CE	0.000	0.000	---	1,612	1,699	769	743	807	804	801	803	2,160	3,541	3,010	3,028	3,159	4,052	315	634	2,485	**1,106**	2,400	2,619
	ES1	0.000	0.000	0.000	---	92	1,351	1,334	1,231	1,252	1,273	1,256	759	3,736	3,138	**3,129**	**3,303**	4,815	**1,785**	**1,454**	1,869	**1,728**	**2,342**	2,819
	ES2	0.000	0.000	0.000	0.000	---	1,439	1,424	1,321	1,340	**1,362**	1,346	683	3,734	3,143	**3,136**	3,306	4,845	**1,870**	**1,522**	1,844	**1,781**	**2,346**	**2,829**
AF	**PB1**	0.000	0.000	0.000	0.000	0.000	---	30	118	96	76	91	2,065	4,170	3,614	3,625	3,770	4,799	1,084	1,224	2,798	1,733	2,915	3,228
	PB2	0.000	0.000	0.000	0.000	0.000	0.000	---	115	96	78	91	2,048	4,139	3,584	3,597	3,739	4,771	1,057	1,194	2,772	1,702	2,885	3,195
	PE1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	23	43	27	1,953	4,140	3,576	**3,586**	3,733	4,811	1,119	**1,208**	2,718	**1,712**	**2,863**	3,191
	PE2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	21	5	1,976	4,148	3,588	**3,596**	**3,743**	**4,814**	**1,116**	**1,215**	2,736	**1,720**	**2,875**	3,202
	PE3	0.000	0.000	0.000	0.000	**0.161**	0.000	0.000	0.000	0.000	---	16	1,996	4,158	3,597	3,607	3,754	**4,816**	1,114	1,222	2,753	1,727	2,891	3,213
	PE4	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	1,981	4,151	3,589	3,599	3,746	4,814	1,116	1,216	2,740	1,722	2,880	3,206
	SP	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	3,370	2,796	2,776	2,953	4,664	2,256	1,805	1,346	1,888	2,011	2,541
	AC1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	603	608	435	1,616	3,281	2,954	2,070	2,449	1,395	953
	AC2	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	57.47	166	2,004	2,771	2,405	1,520	1,915	806	**394**
	AC4	0.000	**0.451**	0.000	**0.619**	**0.533**	0.000	0.000	**0.451**	**0.578**	0.000	0.000	0.000	0.000	0.000	---	174	**2,053**	2,794	2,433	1,486	1,927	792	**418**
AM	**AC5**	0.000	0.000	0.000	**1.000**	0.000	0.000	0.000	0.000	**0.771**	0.000	0.000	0.000	0.000	0.000	0.000	---	1,897	2,916	2,565	1,661	2,060	963	543
	CO	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.947**	**0.752**	0.000	0.000	0.000	0.000	**0.837**	0.000	---	3,735	3,630	3,487	3,182	2,690	**2,151**
	MA4	0.000	0.000	0.000	**0.826**	**0.640**	0.000	0.000	0.000	**0.707**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	520	2,399	900	2,204	2,384
	MA5	0.000	**0.795**	0.000	**1.000**	**0.760**	0.000	0.000	**0.795**	**0.825**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	1,886	509	1,770	2,020
	MS	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	1,584	817	1,343
	PA	0.000	**0.826**	**0.474**	**1.000**	**0.786**	0.000	0.000	**0.826**	**0.843**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	1,314	1,514
	RO1	0.000	**0.795**	0.000	**1.000**	**0.760**	0.000	0.000	**0.795**	**0.825**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	---	539
	RO2	0.000	0.000	0.000	0.000	**0.363**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	**0.274**	0.000	**0.911**	0.000	0.000	0.000	0.000	0.000	---

506 **Table V-S** Genetic measures from mtDNA genes (*16S, COI)* and one nuDNA (*Opsin*) of *Eulaema nigrita* sampled in 25 localities in the Brazilian
507 territory and also a sample from Colombia and identification of the cluster to which the sample belongs according to the Geneland results (*K*),
508 sample sizes (N), number of haplotypes (*h*), haplotype diversity (Hd), nucleotide diversity (π). Samples without computed data failed in the
509 sequence. Group 1 = AF+AM; Group 2 = TN.

			mtDNA								nuDNA			
Localities	**Sites**	**Group**	***16S* (576 bp)**				***COI* (586 bp)**				***Opsin* (585 bp)**			
		K	**N**	***h***	**Hd (sd)**	**π**	**N**	***H***	**Hd (sd)**	**Π**	**N**	***h***	**Hd (sd)**	**π**
Porto Acre	AC2	1	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000
Rio Branco	AC3	1	8	1	0.000 ± 0.000	0.000								
Senador Guiomard	AC4	1					1	1	0.000 ± 0.000	0.000				
Murici	AL	1	2	2	1.000 ± 0.500	0.195	9	3	0.417 ± 0.191	0.001	4	2	0.667 ± 0.204	0.001
Igrapiúna	BA1	1	3	1	0.000 ± 0.000	0.000	8	2	0.250 ± 0.180	0.001	6	2	0.600 ± 0.129	0.001
Ilhéus	BA2	1	2	1	0.000 ± 0.000	0.000	3	2	0.667 ± 0.314	0.001	1	1	0.000 ± 0.000	0.000
Itamaraju	BA3	1					4	1	0.000 ± 0.000	0.000	2	1	0.000 ± 0.00	0.000
Porto Seguro	BA4	1	5	1	0.000 ± 0.000	0.000	9	2	0.389 ± 0.164	0.001	2	2	1.000 ± 0.500	0.002
Una	BA5	1	1	1	0.000 ± 0.000	0.000	2	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000
Conceição da Barra	ES1	1	1	1	0.000 ± 0.000	0.000	3	3	1.000 ± 0.272	0.002	5	2	0.400 ± 0.237	0.001
Linhares	ES2	1	1	1	0.000 ± 0.000	0.000	6	2	0.333 ± 0.215	0.001	4	3	0.833 ± 0.222	0.002
Sooretama	ES3	1	8	3	0.607 ± 0.164	0.049	8	4	0.750 ± 0.139	0.003	3	3	1.000 ± 0.272	0.002
Davinópolis	GO	1	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000				
Belo Horizonte	MG1	1	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000				
Jequitinhonha	MG2	1	5	2	0.400 ± 0.237	0.078	2	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000
Matias Cardoso	MG3	1	3	2	0.667 ± 0.314	0.130	3	2	0.667 ± 0.314	0.030				
Morro Pilar	MG4	1	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000				
Chupinguaia	RO1	1	2	1	0.000 ± 0.000	0.000	2	1	0.000 ± 0.000	0.000				
Ubajara	CE	2	2	1	0.000 ± 0.000	0.000	2	1	0.000 ± 0.000	0.000				
Alcântara	MA1	2	4	1	0.000 ± 0.000	0.000	4	3	0.833 ± 0.222	0.002	4	2	0.500 ± 0.265	0.001
Anajatuba	MA2	2	5	1	0.000 ± 0.000	0.000	5	5	1.000 ± 0.126	0.012	5	2	0.400 ± 0.237	0.001

Candido Mendes	MA3	2	2	2	1.000 ± 0.500	0.195	3	3	1.000 ± 0.272	0.023			
Humberto de Campos	MA4	2	9	1	0.000 ± 0.000	0.000	9	3	0.667 ± 0.105	0.002	9	1	0.000 ± 0.000	0.000
Formosa da Serra Negra	MA5	2	5	1	0.000 ± 0.000	0.000	5	3	0.700 ± 0.218	0.001	5	1	0.000 ± 0.000	0.000
São Bento	MA6	2	5	1	0.000 ± 0.000	0.000	5	2	0.400 ± 0.237	0.001	5	3	0.700 ± 0.218	0.002
Total	25	2	77	3	0.234 ± 0.003	0.037	97	29	0.732 ± 0.002	0.017	5	9	0.495 ± 0.006	0.001

510

Table VI -S Genetic measures from mtDNA genes (*16S, COI*) and one nuDNA (*Opsin*) of *Exaerete smaragdina* sampled in 25 localities in the Brazilian territory and also a sample from Colombia and identification of the cluster to which the sample belongs according to the Geneland results (*K*), sample sizes (N), number of haplotypes (*h*), haplotype diversity (Hd), nucleotide diversity (π). Samples without computed data failed in the sequence. Group 1 = AF; Group 2 = AM.

			mtDNA								nuDNA			
Localities	**Sites**	**Grou**	***16S* (511 pb)**				***COI* (660 pb)**				***Opsin* (668**			
		K	**N**	***h***	**Hd (sd)**	**π**	**N**	***h***	**Hd (sd)**	**π**	**N**	***h***	**Hd (sd)**	**π**
Murici	AL	1	1	1	0.000 ± 0.000	0.000					1	1	0.000 ± 0.000	0.000
Igrapiúna	BA1	1	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000
Ilhéus	BA2	1					3	2	0.667 ± 0.314	0.001				
Conceição da Barra	ES1	1	2	2	1.000 ± 0.500	0.002	5	1	0.000 ± 0.000	0.000				
Linhares	ES2	1	5	1	0.000 ± 0.000	0.000	5	2	0.600 ± 0.175	0.001	1	1	0.000 ± 0.000	0.000
Joao Pessoa	PB1	1					1	1	0.000 ± 0.000	0.000				
Cruz do Espírito Santo	PB2	1					1	1	0.000 ± 0.000	0.000				
Cabo de Santo Augostinho	PE1	1					3	2	0.667 ± 0.314	0.001				
Camaragibe	PE2	1					6	2	0.333 ± 0.215	0.001				
Igarassu	PE3	1					8	4	0.750 ± 0.139	0.003				
Recife	PE4	1					1	1	0.000 ± 0.000	0.000				
Chupinguaia	RO1	2	3	1	0.000 ± 0.000	0.000	3	1	0.000 ± 0.000	0.000				
Porto Velho	RO2	2	5	1	0.000 ± 0.000	0.000	10	2	0.556 ± 0.075	0.001				
Aparecida	SP	1	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000				
Cruzeiro do Sul	AC1	2					1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000
Porto Acre	AC2	2	1	1	0.000 ± 0.000	0.000	1	1	0.000 ± 0.000	0.000				
Rio Branco	AC3	2	4	1	0.000 ± 0.000	0.000					5	1	0.000 ± 0.000	0.000
Senador Guiomard	AC4	2	4	1	0.000 ± 0.000	0.000	4	3	0.833 ± 0.222	0.002	2	1	0.000 ± 0.000	0.000
Senador Madureira	AC5	2	2	1	0.000 ± 0.000	0.000	2	1	0.000 ± 0.000	0.000	2	2	1000 ± 0.500	0.002
Ubajara	CE	2					2	2	1.000 ± 0.500	0.003				
Santa Fé de Antioqui	CO	2	1	1	0.000 ± 0.000	0.000	2	1	0.000 ± 0.000	0.000				
Humberto de Campos	MA4	2					3	2	0.667 ± 0.314	0.001	4	1	0.000 ± 0.000	0.000
Formosa da Serra Negra	MA5	2	4	4	1000 ± 0.177	0.003	4	1	0.000 ± 0.000	0.000				
Corumbá	MS	2					1	1	0.000 ± 0.000	0.000				
Água Azul do Norte	PA	2	5	1	0.000 ± 0.000	0.000	5	1	0.000 ± 0.000	0.000	2	1	0.000 ± 0.000	0.000
Total	25	2	39	8	0.557 ± 0.006	0.001	73	14	0.677 ± 0.001	0.002	19	3	0.433 ± 0.013	0.001

515 **Table VII - S** Analysis of Molecular Variance (AMOVA) using the Geneland results for the UHF model (K = 2) for COI of *Eulaema nigrita*.
516 *p < 0.001

Source of Variation	df	Sum of squares	Percentage of variation	Φ
Among groups	1	240.649	65.69	$\Phi_{CT} = 0.656$*
Among populations Within groups	22	173.927	22.32	$\Phi_{SC} = 0.650$*
Within populations	73	70.156	11.98	$\Phi_{ST} = 0.840$*
Total	96	484.732		

517 **Table VIII-S** Analysis of Molecular Variance (AMOVA) using the Geneland results for the UHF model (K = 2) for COI of *Exaerete*
518 *smaragdina*. *p<0.001

Source of Variation	df	Sum of squares	Percentage of variation	Φ
Among groups	1	8.988	29.21	$\Phi_{CT} = 0.292$*
Among populations Within groups	21	18.786	26.50	$\Phi_{SC} = 0.374$*
Within populations	51	16.158	44.29	$\Phi_{ST} = 0.557$*
Total	73	43.932		

5 CAPÍTULO II –

Orchid bees (Apidae: Euglossini) in Cerrado remnants in northeast Brazil

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Orchid bees (Apidae: Euglossini) in Cerrado remnants in northeast Brazil

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Abstract

There is a general lack of information concerning the diversity of bees belonging to the Euglossini tribe in Cerrado areas closest to the Brazilian Amazon. The state of Maranhão is situated in the northeast Brazilian Cerrado and has become the agricultural frontier of the country due to the advancement of monoculture and cattle farming. These activities have suppressed animal and plant populations in large areas of the Cerrado remnants, for which we have not yet obtained adequate scientific knowledge of local species. The objective of this study was to conduct a survey of Euglossini fauna in the northeast Cerrado. We evaluated the variation in species richness, composition and abundance between two distinct vegetation types: Cerrado sensu stricto (s.s.) and gallery forest. Male bees were captured from 7:00 a.m. to 5:00 p.m. over two consecutive days. Captures were carried out once per month from July 2012 to December 2013, using a combination of passive and active collection techniques, including baited traps, as well as a collector with an entomological net to collect bees near traps. We collected a total of 766 Euglossini males belonging to 24 species and five genera. The most abundant species were *Eulaema bombiformis*, *Eulaema nigrita* and *Eulaema cingulata* for the gallery forest site, whereas *Eulaema nigrita*, *Euglossa melanotricha* and *Euglossa cordata* were more abundant in the Cerrado *s.s*. The gallery forest yielded a higher number of male Euglossini (n = 503, 21 species) compared with the Cerrado *s.s*. (n = 263, 16 species). The presence of seven exclusive species in the gallery forest and three in the Cerrado *s.s*. indicated that both environments are important for the maintenance of Euglossini species in this region and highlight the increasing need for conservation programmes for the protection of Cerrado environments.

Keywords Baited traps; cerrado sensu strict; euglossine bees; Euglossini; gallery forest; savannah

Introduction

The Brazilian Cerrado is one of the top 25 global biodiversity hotspots due to the high number of endemic species, many of which live under the constant threat of decline (Myers *et al.* 2000). This vast and heterogeneous environment has been negatively affected by increasing anthropogenic activity (Ratter *et al.* 1997; Ratter 2002; Durigan *et al.* 2007), which has resulted in disturbances such as habitat fragmentation, invasion by exotic species, soil erosion, water pollution and ecosystem degradation, among others (Klink and Machado 2005). These processes increasingly restrict Cerrado remnants to areas further away from large, urban centres and conserva- tion units.

The Cerrado biome in the state of Maranhão locally called the 'Cerrado Maranhense' occupies an area of 10 million ha and corresponds to 30% of the state territory and 5% of the total Brazilian Cerrado (Conceição and Castro 2009). The Cerrado Maranhense has become the agricultural frontier of Brazil, with a heavy investment in the expansion of agribusiness in recent years (Sicsú and Lima 2000; Conceição and Castro 2009). This has drastically altered the landscape, transforming the natural landscapes into extensive pastures and monoculture systems.

The expansion of soybean, eucalyptus and sugarcane plantations has been reported to negatively affect biological communities in these areas. For example, Barreto *et al.* (2012) evaluated the effects of soybean expansion on bird and mammal communities through modelling in the city of Balsas in southern Maranhão; they estimated declines of 61% and 42%, respectively, due to the destruction of natural areas from agricultural and livestock activities. This is a worrying scenario, and the biological knowledge of several taxa in the area is limited, including beetles (Luçardo *et al.* 2014), birds (Rocha *et al.* 2015), bats (Sousa *et al.* 2013) and bees, the last of which play an important role in the maintenance of native plant species (Silberbauer- Gottsberger and Gottsberger 1988).

Of the Cerrado bee fauna, species in the tribe Euglossini are noteworthy owing to their role as potential bioindicators of environmental quality (Parra-H and Nates-Parra 2007; Ramalho *et al.* 2009; Mateus *et al.* 2015). Some studies in these environments have revealed different ecological aspects regarding the Euglossini community, such as the negative effects on the richness and composition of the orchid flora in agricultural landscapes (Mendes *et al.* 2008; Giehl *et al.* 2013; Nascimento *et al.* 2015), preference of certain species for open-forest habitats (Nemésio and Faria 2004; Anjos-Silva *et al.* 2006; Alvarenga *et al.* 2007; Faria and Silveira 2011; Silva 2012; Silveira *et al.* 2015; Martins *et al.* 2016; Tosta *et al.* 2017), and the importance of environmental hetero- geneity in the maintenance of the Euglossini community (Antonini *et al.* 2016; Moreira *et al.* 2017).

However, most of these studies were conducted in the Cerrado in southeast Brazil, with few samples of the Euglossini fauna of the Cerrado areas closest to the Amazon Forest region, considered as the centre of origin of orchid bees (Rebêlo 2001). According to Storck-Tonon *et al.* (2009), advancing the research on the Euglossini fauna will require sampling effort, the temporal separation of collections, and studies encompassing a greater variety of environments. Taking such steps will increase the probability of finding more species, including rare species, for better estimates of species richness of a given area. For Euglossini bees specifically, community level studies can be facilitated by using a high quantity of aromatic baits as lures to attract males. This methodology has been efficient in various entomological surveys and ecological studies in different ecosystems, including in areas of the Cerrado (Nemésio 2016).

We conducted a survey of Euglossini fauna in the Cerrado Maranhense, evaluating the variation in species richness, composition and abundance among distinct vegetation types of Cerrado *sensu stricto* and gallery forest. We also verified sampling adequacy for the two environments and analysed male preferences for attractive baits.

Materials and methods

Study areas

The study was conducted in Formosa da Serra Negra (6.2547°S, 46.1031°W), one of the municipalities that comprises the Mirador State Park (MSP). The park occupies an area of approximately 766,781 ha and is located in the southern-central region of Maranhão (SEMA 2011), which also includes the following municipalities: Colinas, Fernando Falcão, Fortaleza dos Nogueiras, Sambaíba, Loreto, São Félix do Balsas, São Domingos do Azeitão, Pastos Bons, Sucupira do Norte, Mirador and Tuntum (Figure 1). The MSP area contains distinct environments typical of the Cerrado, including Cerrado sensu stricto, cerradão (arboreal Cerrado) and gallery forest, and is delimited by the Alpercatas and Itapecuru Rivers (Santos and Conceição 2010). The purpose of establishment of the MSP in 1980 was to protect the sources of the Alpercatas and Itapecuru Rivers, the latter being the primary water supply for the capital city of São Luís, located 579 km from the site.

The climate is dry and sub-humid with an average annual rainfall of approximately 1200 mm. There is a distinct rainy season from October to April and a dry season from May to September. The mean maximum temperature ranges from 31.4°C to 33°C, and the minimum temperature ranges from 19.5°C to 21°C (Conceição *et al*. 2011).

Sampling

Two vegetation types common to the MSP were selected: one gallery forest (GF) bordering the

Alpercatas River (6.3756°S, 45.534°W), and one Cerrado *sensu stricto* (Css) (6.3832°S, 45.5358°W). There was a distance of approximately 1 km between sites. The collections were carried out in each environment from 7:00 a.m. to 5:00 p.m. over two consecutive days, once per month from July 2012 to December 2013 (total sampling effort = 360 hours).

We used a combination of passive and active collection techniques to sample male Euglossini, including baited traps and active search by a collector using an entomological net (Silva *et al*. 2009; Silveira *et al*. 2015). Collected specimens were placed in a lethal chamber containing ethyl acetate and packed in plastic bags labelled with the date and hour of collection, and the bait substance used in the traps, when applicable. Specimens were then taken to the laboratory for analysis.

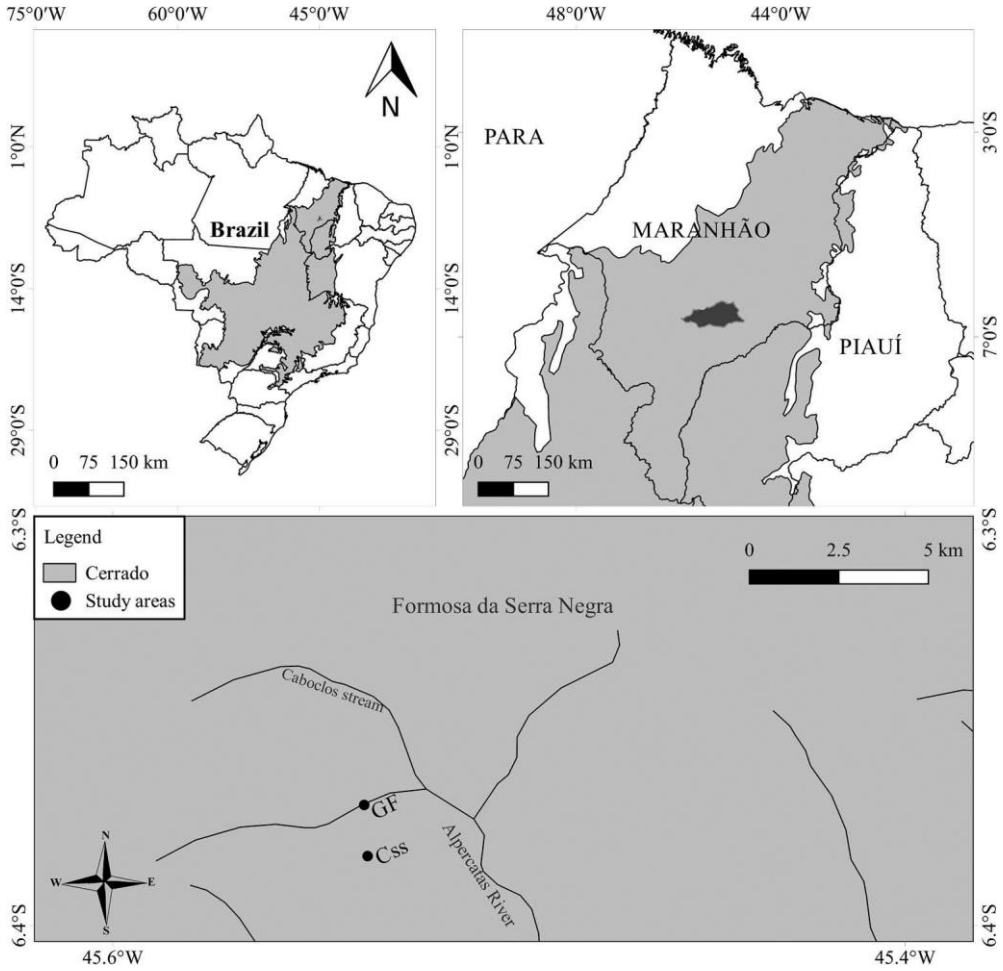


Figure 1 Distribution of the Cerrado biome in Brazil and Maranhão (grey area). Dark area represents the geographic location of Mirador State Park, MA, and the points correspond to study areas in gallery forest (GF) and Cerrado sensu stricto (Css) (QGIS Software 2.18, Quantum GIS Development Team 2017).

Odoriferous traps were constructed using plastic PET bottles with three lateral holes in which three inverted bottlenecks were inserted for the entrance of Euglossini males (see Ramalho *et al.* 2009 for additional details). Traps were fastened onto tree branches and

suspended at a height of 1.5 m above the ground, with 8 m between traps. Five odoriferous substances were used to attract bees: methyl cinnamate, eucalyptol, eugenol, methyl salicylate and vanillin. We used three replicates for each of the five sub- stances (i.e. 15 traps in total); baits consisted of cotton swabs soaked with the respective aromatic substances and were fixed inside the traps. The captured specimens were identified at the species level and deposited into the Laboratory Bee Collection (LEACOL) at the Federal University of Maranhão.

Data analysis

We used the Shannon–Wiener index to evaluate species diversity, using the following formula: $H' = -\Sigma$ pi ln(pi), where pi is the proportion of each species in the sample and ln is the Naperian logarithm of this proportion (Pielou 1975). To test the monthly differences in Shannon diversity values between the two areas, a Student's *t*-test ($p < 0.05$) was used. We used Pielou's equity index (1966) to verify species distributions in the communities, with the following formula: $J' = H'/H'_{max}$, where H' is the number derived from the Shannon–Wiener diversity index and H'_{max} is the Naperian log (ln) total number of species in the sample.

The degree of dominance for the two areas was measured using the Berger–Parker index (*d*), which is determined by the following expression: $d = N_{max}/N$, where *d* is the degree of dominance, N_{max} is the number of individuals of the most abundant species, and *N* is the total number of individuals sampled. This index expresses the proportional importance of the most abundant species in a given sample (Magurran 1988). These statistical tests were carried out using PAST version 3.0 (Hammer *et al.* 2001).

We calculated percentage similarity between the two habitats using the Renkonen index. The Renkonen coefficient is defined as $PS\Sigma_{min}$ (p1i, p2i) and is the sum of the minimum frequencies of taxa found at the two study sites (Wolda 1981). Differences in the proportion of males caught between the rainy and dry periods were analysed using a chi-squared test. To evaluate the influence of abiotic factors on the abundance and richness of Euglossini species for both study sites, we used a Pearson's correlation (*r*) to compare the mean values for temperature, relative humidity and rainfall to the numbers of individuals (gallery forest, GF_A and Cerrado *sensu stricto*, Css_{Ab}) and species richness (GF_R and Css_R) throughout the year. These analyses were performed in R (R Development Core Team 2015). Sampling efficiency was evaluated using species accu- mulation curves and Bootstrap, Chao 2, Jackknife 1 and Jackknife 2 ecological indices using the Estimate 9 programme (Colwell 2013).

Results

Community structure

We collected a total of 766 Euglossini males, belonging to 24 species and five genera. The genus with the highest representation was *Euglossa* (13 species), followed by *Eulaema* (4), *Exaerete* (2) and *Aglae* (1). The most abundant species were *Eulaema bombiformis*, *Eulaema nigrita* and *Eulaema cingulata*, which together represented 65.2% of the total sample for the gallery forest, while *Eul. nigrita*, *Euglossa melanotricha*, and Euglossa cordata represented 62.7% of the total sample in the Cerrado. All other species in both environments had relative abundances of < 10%. According to the Berger–Parker index (d), *Eul. bombiformis* was the dominant species (d = 0.39) in the gallery forest physiognomy, while *Eul. nigrita* (d = 0.26) was the dominant species in the Cerrado.

The gallery forest yielded a higher number of Euglossini males (n = 503, 21 species) than the Cerrado *s.s.* (n = 263, 16 species). Both environments presented similar diversity values, but the t-test showed differences between samples from the two sites ($t = 3.15$, $df = 34$, $p < 0.05$). The Cerrado *s.s.* presented slightly higher diversity and evenness index values ($H' = 2.101$; $J' = 0.7578$) than the gallery forest environment ($H' = 2.014$; $J' = 0.6724$) (Table 1).

The Renkonen coefficient of similarity showed 46.8% similarity in species composition between sites. Some species had greater abundance in the gallery forest environment, such as *Eul. bombiformis* (40% GF and 3.4% Css), *Eul. cingulata* (9.1% GF and 6.4% Css) and *Eulama meriana* (9.7% GF and 1.9% Css). Meanwhile, *Eul. nigrita* (26.6% Css and 16.1% GF), *Eug. melanotricha* (24.3% Css and 8% GF), *Eug. cordata* (11.8% Css and 3.4% GF), *Euglossa securigera* (9.5% Css and 2.4% GF) and *Euglossa fimbriata* (7.2% Css and 1.4% GF) showed higher activity in the Cerrado s.s. The remaining species were found in only one of the two habitats.

Table 1 Abundance (= number of individuals) of Euglossini bees (n) captured in the Mirador State Park in a gallery forest (GF) and Cerrado *sensu stricto* (Css) from July 2012 to December 2013.

Species	GF (%)	Css (%)	Total (%)
Aglae caerulea Lepeletier & Serville	2 (0.4)		2 (0.3)
Eufriesea auriceps (Friese)		1 (0.4)	1 (0.1)
Eufriesea surinamensis (Linnaeus)	1(0.2)		1 (0.1)
Eufriesea vidua (Moure)	1 (0.2)		1 (0.1)
Euglossa (Euglossa) amazonica Dressler	1 (0.2)		1 (0.1)
Euglossa (Euglossa) bidentata Dressler		1 (0.4)	1 (0.1)
Euglossa (Euglossa) cordata (Linnaeus)	17 (3.4)	31 (11.8)	48 (6.3)

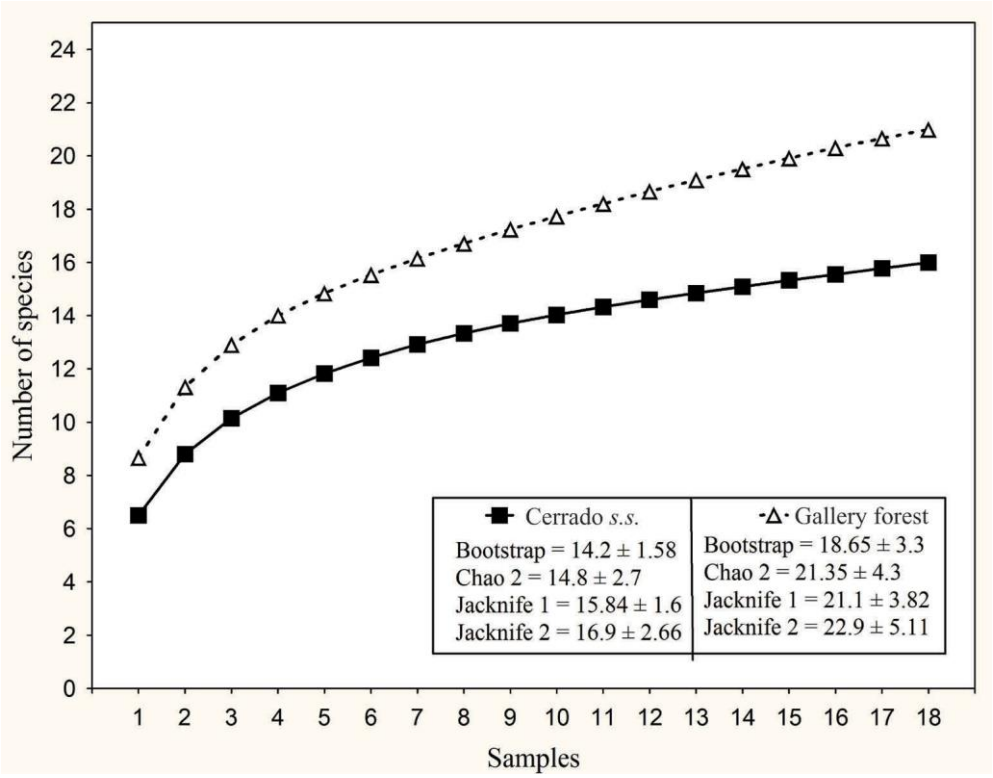
Euglossa (Euglossa) despecta Moure	2 (0.4)		2 (0.3)
Euglossa (Euglossa) fimbriata Rebêlo & Moure	7 (1.4)	19 (7.2)	26 (3.4)
Euglossa (Euglossa) hemichlora Cockerell		3 (1.1)	3 (0.4)
Euglossa (Euglossa) melanotricha Moure	34 (6.8)	64 (24.3)	98 (12.8)
Euglossa (Euglossa) modestior Dressler	2 (0.4)	1 (0.4)	3 (0.4)
Euglossa (Euglossa) platymera Dressler	1 (0.2)		1 (0.1)
Euglossa (Euglossa) pleosticta Dressler	10 (2.0)	5 (1.9)	15 (2)
Euglossa (Euglossa) securigera Dressler	12 (2.4)	25 (9.5)	37 (4.8)
Euglossa (Euglossa) townsendi Cockerell	1 (0.2)	4 (1.5)	5 (0.7)
Euglossa (Glossura) ignita Smith	1 (0.2)	1 (0.4)	2 (0.3)
Eulaema (Eulaema) bombiformis (Packard)	201 (40)	9 (3.4)	210 (27.4)
Eulaema (Apeulaema) cingulata (Fabricius)	46 (9.1)	17 (6.5)	63 (8.2)
Eulaema (Apeulaema) pseudocingulata Oliveira	11 (2.2)	7 (2.7)	18 (2.3)
Eulaema (Eulaema) meriana (Olivier)	49 (9.7)	5 (1.9)	54 (7)
Eulaema (Apeulaema) nigrita Lepeletier	81 (16.1)	70 (26.6)	151 (19.7)
Exaerete frontalis (Guérin)	14 (2.8)		14 (1.8)
Exaerete smaragdina (Guérin)	9 (1.9)		9 (1.2)
Abundance	503	263	766
Richness	21	16	
Equity Index (J')	0.6724	0.7578	
Berger–Parker index (d)	0.39	0.26	
Renkonen index	46.8		

The species accumulation curve (Figure 2), had a greater tendency to stabilize for the Cerrado compared with the gallery forest, indicating that greater sampling effort is probably needed for the latter. In general, Euglossini males remained active throughout the study period (Figure 3), with the highest peaks in activity during drought ($\chi^2 = 156.33$, gl = 8, $p > 0.05$). We note that the most abundant species in the gallery forest, *Eul. bombiformis*, yielded the most specimens in the intermediate period between the rainy and dry seasons (March–August 2013).

The highest activity for *Eul. nigrita* in the gallery forest occurred in the months of November 2012, April 2013 and October 2013, whereas *Eul. cingulata* showed peaks in activity in September and December 2012. For the Cerrado s.s., *Eul. nigrita* had the highest abundance between March and May 2013. *Euglossa melanotricha* males were more active in the months of May and November 2013. We found *Eug. cordata* in every month of the study period.

For the gallery forest, there was a positive correlation between abundance and

193 temperature (GF_{Ab}: $r = 0.491$, $p < 0.05$) and between the number of individuals and species
194 (GF_R: $r = 0.674$, $p < 0.05$). There was a negative correlation between temperature and the number
195 of individuals in Cerrado s.s. (Css_{Ab}: $r = -0.480$, $p < 0.05$) and number of species (Css_R: $r =$
196 -0.544, $p < 0.05$). The number of individuals and species was positively correlated (Css_R: $r =$
197 0.674, $p < 0.05$), and there was no relationship between rainfall and richness or abundance (GF_{Ab}:
198 $r = -0.965$, $p = 0.348$; GF_R: $r = -0.0663$, $p = 0.516$; Css_{Ab}: $r = 0.569$, $p = 0.577$; Css_R: $r = 0.813$,
199 $p = 0.427$) for either environment.



200 **Figure 2** Species accumulation curve generated from four richness estimators (Bootstrap, Chao 2,
201 Jackknife 1, and Jackknife 2) for Euglossini caught with bait traps at Mirador State Park, MA. The line
202 above with triangular markers shows the sampling adequacy for the gallery forest site, whereas the line
203 below with square markers shows the sampling adequacy for Cerrado *sensu stricto*.
204
205 *Bait attractiveness*
206 Of the five odoriferous substances used (Table 2), eucalyptol was most efficient in attracting
207 bees ($n = 276$; 15 sp.), followed by eugenol ($n = 142$; 12 sp.), methyl salicylate ($n = 194$; 9 sp.),
208 methyl cinnamate ($n = 80$; 9 sp.) and vanillin ($n = 74$; 10 sp.). The latter was only attractive for
209 *Eufriesea* species. *Eulaema bombiformis* was most attracted to methyl salicylate baits and
210 occasionally visited methyl cinnamate baits, whereas *El. nigrita* was most attracted to vanillin
211 and eucalyptol – primarily the latter. Some species, including *Euglossa townsendi*, *Euglossa*
212 *amazonica* and *Euglossa platymera*, were exclusively attracted to eucalyptol. *Aglae caerulea*
213 was only attracted to methyl cinnamate baits, and *Euglossa hemichlora* was exclusively

attracted to methyl salicylate, and Euglossa bidentata to vanillin baits. However, we cannot assume unique associations, because only a few individuals of these species were captured.

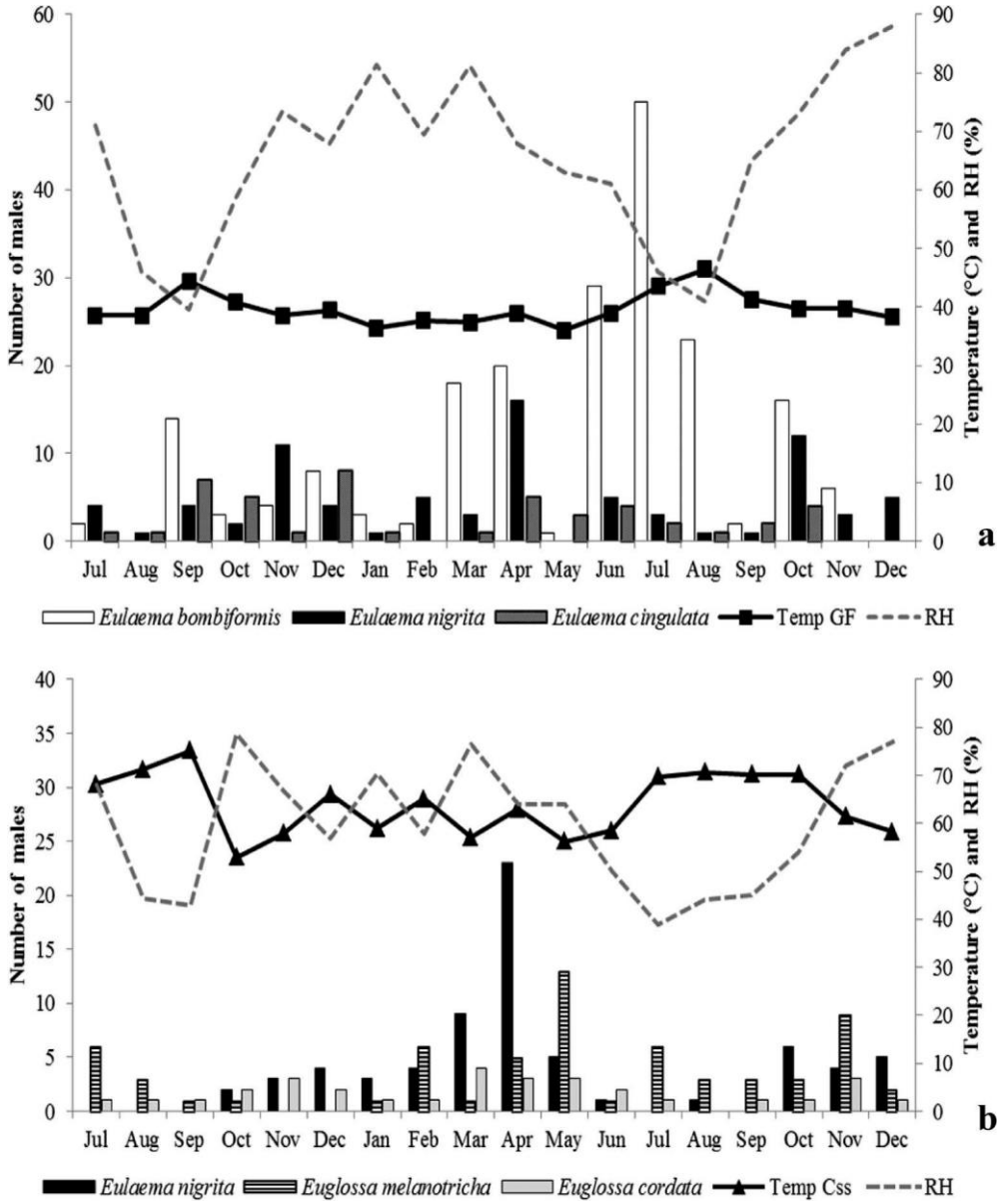


Figure 3 Distribution of the most abundant Euglossini species during 18 months of sampling, and monthly mean temperature and humidity in two areas of the Cerrado biome in Mirador State Park, MA: gallery forest (a) and Cerrado *sensu stricto* (b).

Daily activity for male Euglossini was similar between environments (Figure 4), with 70.3% of the visits to the baits occurring in the morning, with a peak in abundance between 10:00 a.m. and 11:00 a.m. for the gallery forest and between 11:00 a.m. and 12:00 p.m. for the Cerrado, then decreasing progressively from midday to late afternoon. Contrasting patterns of visitation schedule of baits of the most representative species were shown in both environments. *Eulaema bombiformis* visited the baits throughout the day, with its highest activity peak between 8:00 a.m. and 9:00 a.m., when the temperature ranged between 22.8°C and 24.1°C in

227 the gallery forest (Figure 4a). In the Cerrado *s.s.*, in which there is a decrease in the activity of *Eul.*
228 *nigrita* and *Eul*. c*ingulata* after the period between 11:00 a.m. and 12:00 p.m. and the highest
229 peaks of *Eug*. *melanotricha* and *Eug*. *cordata* at the same time, when the temperature was above
230 28.8°C (Figure 4b).
231
232 **Table 2** Numbers of Euglossini males attracted by five chemical compounds in the Mirador
233 State Park from July 2012 to December 2013.

	Gallery Forest					Cerrado *s.s.*				
Species	**EC**	**EG**	**MC**	**MS**	**VN**	**EC**	**EG**	**MC**	**MS**	**VN**
Aglae caerulea Lepeletier & Serville			2							
Eufriesea auriceps (Friese)										1
Eufriesea surinamensis (Linnaeus)					1					
Eufriesea vidua (Moure)					1					
Euglossa amazonica Dressler	1									
Euglossa bidentata Dressler										1
Euglossa cordata (Linnaeus)	17					28	3			
Euglossa despecta Moure	1	1								
Euglossa fimbriata Rebêlo & Moure	1	6				17	2			
Euglossa hemichlora Cockerell									3	
Euglossa melanotricha Moure	27	5		2		35	27	2		
Euglossa modestior Dressler	1		1				1			
Euglossa platymera Dressler	1									
Euglossa pleosticta Dressler	3	2	2		3	4				1
Euglossa securigera Dressler	6	4	1	1		6	19			
Euglossa townsendi Cockerell	1					4				
Euglossa ignita Smith				1		1				
Eulaema bombiformis (Packard)			58	143				2	7	
Eulaema cingulata (Fabricius)		35	1	1	9		14			3
Eulaema pseudocingulata Oliveira		8			3		3			4
Eulaema meriana (Olivier)	8	4	9	27	1	1			4	
Eulaema nigrita Lepeletier	62			1	18	40		1	2	27
Exaerete frontalis (Guérin)	5	6	1	2						
Exaerete smaragdina (Guérin)	6	2			1					
Abundance	140	73	75	178	37	136	69	5	16	37

Richness	14	10	8	7	9	9	7	3	4	6

Eucalyptol (EC), eugenol (EG), methyl cinnamate (MC), methyl salicylate (MS) e vanillin (VN).

234

235 **Discussion**

236 Species richness at the MSP in the present study was higher than that found in other studies
237 performed in areas of the Cerrado biome (Rebêlo and Cabral 1997; Nemésio and Faria 2004;
238 Alvarenga *et al.* 2007; Mendes *et al.* 2008; Justino and Augusto 2010; Faria and Silveira 2011;
239 Silva 2012; Giehl *et al.* 2013; Pires *et al.* 2013; Nascimento *et al.* 2015; Oliveira-Junior *et al.*
240 2015; Silveira *et al.* 2015; Moreira *et al.* 2017; Tosta *et al.* 2017). One possible explanation for
241 the higher observed richness is that the state of Maranhão lies in an ecotone area, between the
242 Cerrado of the Central Plateau, northeastern Caatingas and Amazonian forest (Rebêlo and Silva
243 1999). These bees have a high flight capacity and can cover broad areas of continuous forest
244 (Janzen 1971). The gallery forests in the MSP are thought to be transit zones for bees travelling
245 to the eastern-most areas of the Amazon basin. There is evidence to support this, including the
246 presence of *A. caerulea* in these environments (see Martins *et al.* 2016), formerly considered to
247 be strictly Amazonian.

248 Some studies describe the Cerrado Euglossini fauna as a subset of humid forest species,
249 with no endemic species (Anjo-Silva 2008; Faria and Silveira 2011). Our data corroborate this
250 assertion, as most of the species captured in the gallery forest are also common in the Amazon,
251 including *A. caerulea*, Eufriesea vidua, *Eug. amazonica*, *Eug. hemichlora* and *Eug. platymera*.
252 It is thus likely that the gallery forests of the Cerrado Maranhense constitute an important route
253 for the dispersion of Amazon species and potentially act as refuges from the high temperatures
254 and extreme conditions of the savanna ecosystem.

255 Other species common to the Amazon environment, such as *Eg. decorata* and *Eg.*
256 *augaspis*, were also captured using beta-ionone bait in the MSP, in a gallery forest 5 km away
257 from the study areas (Martins 2015, unpublished, personal communication). Moura and
258 Schlindwein (2009) captured Euglossini males on the banks of the São Francisco River, and
259 suggested that the presence of common bees in a forest environment in riparian forest
260 vegetation shows the importance of these sites for colonization by Euglossini species. Based
261 on this premise, and in view of our results, we assert that the gallery forests present in the MSP
262 may serve as a connection between areas of Cerrado and humid forest.

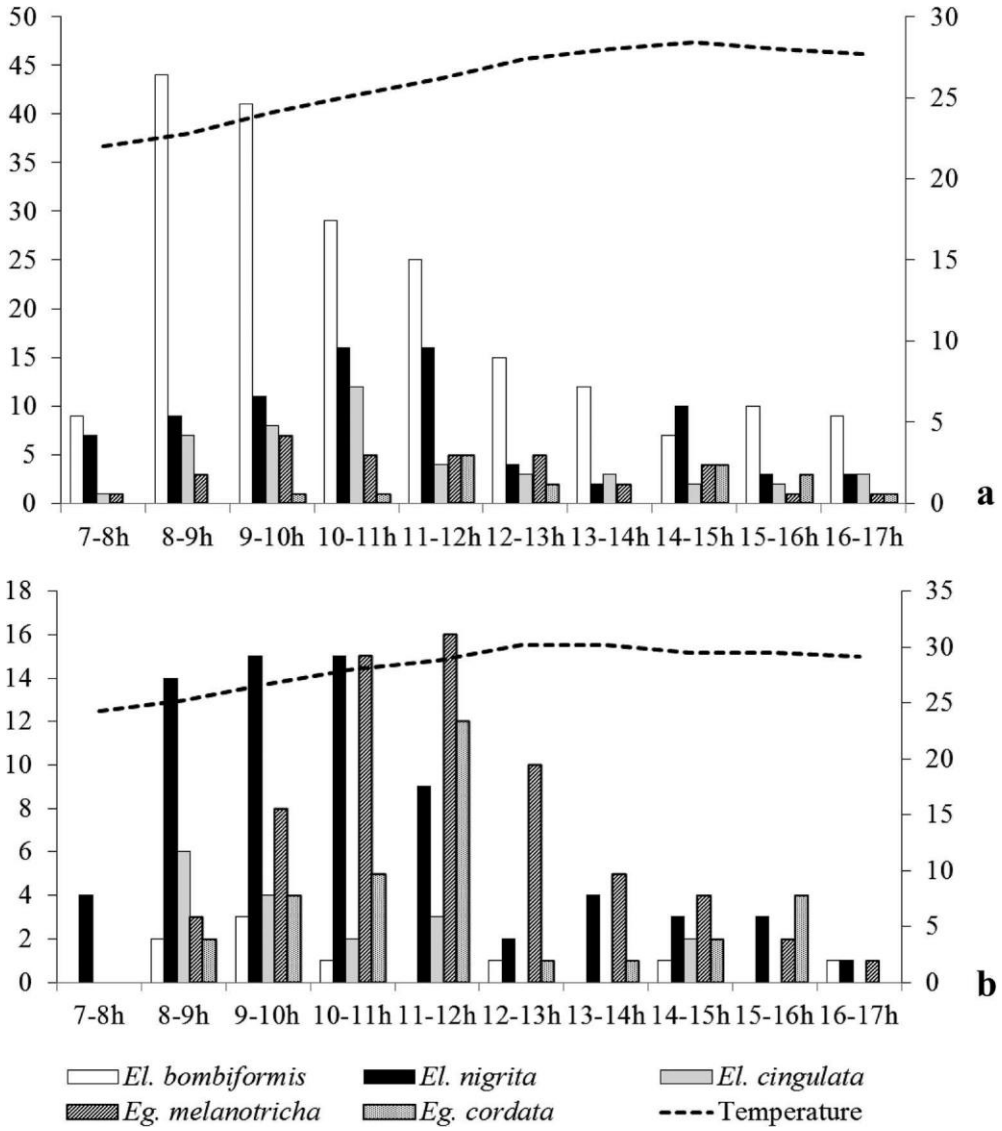


Figure 4 Influence of temperature on the patterns of daily activity of the five most abundant species in gallery forest (a) and Cerrado *sensu stricto* (b) of the Mirador State Park, MA.

The Cerrado is a dry corridor that runs between the two main regions of rainforest of tropical South America: the amazon forest in the northeast and the Atlantic forest in the east and southeast (Oliveira-Filho and Ratter 1995). In Brazil, the Cerrado is the second largest domain and connects these two separate regions by a network of vegetation types, including Gallery forests. Some forest-related euglossine bee species have been found in gallery forests, such as *Euf. vidua* and *Eug. platymera* (this study); *Euglossa pleosticta*, *Euglossa modestior* and *Euglossg avicula* (Silva 2012); *Eufriesea ornata* and *Euglossa gaianii* (Carvalho *et al*. 2006), and *A. caerulea* (Martins *et al*. 2016). These findings reinforce the hypothesis that the disjunction between the Amazon and Atlantic forests is interconnected by strips of gallery forests between the two a fore mentioned rainforest regions. Therefore, it is possible that such an interconnection network might help to sustain a high diversity of euglossine bees in the

277 Cerrado domain.

278 A study by Silveira *et al.* (2015) assessing the influence of forest formations on
279 Euglossini diversity in the Cerrado of Minas Gerais suggested that the presence of forest
280 environments, such as semi-deciduous seasonal forest, guarantees high diversity of Euglossini
281 males in the Cerrado domain, because these sites allow for the colonization of the associated
282 *Euglossa* species. In our study, eight species were captured only in gallery forest. Among these
283 species *Euf. vidua* and *Eug. platymera* – common species in the Amazon region (Dressler 1982;
284 Moure *et al.* 2012) – were captured for the first time in Brazilian Cerrado.

285 Analysis of the sampling sufficiency revealed a strong trend toward stability for species
286 in the Cerrado s.s. However, a greater sample effort was needed for the gallery forest to
287 stabilize the curve. Other studies in the Cerrado domain obtained similar results, showing only
288 a trend toward curve stability (Silveira *et al*. 2011, 2015). One possible cause for this is a lack
289 of affinity of rare species in this environment for the bait types used (Rebêlo and Garófalo
290 1997), and inefficiency of the sampling protocols adopted by researchers in studies of
291 Euglossini communities in Cerrado (Nemésio 2016). Another factor that can influence sample
292 efficiency is the availability of natural resources. According to Armbruster (1993), changes in
293 the structure of the Euglossini community between nearby sites may simply reflect the
294 temporal variation in the arrangement of natural sources of aromatics, because the males can
295 congregate in a determined area in response to the availability of these resources. In a field
296 observation, Tonhasca *et al*. (2003) found a high density of males of *Eul. nigrita* and *Eul.*
297 *cingulata* on sap-exuding trees, aroids and exposed soil in a forest fragment of the Atlantic
298 Forest in Rio de Janeiro. In this case, it is possible that the greater availability of aromas in the
299 gallery forest is related to the absence of stabilization of the collector curve.

300 The gallery forest exhibited higher abundance and lower diversity compared with
301 Cerrado s.s., and the differences were primarily attributed to the dominance of *Eul.*
302 *bombiformis* (d = 0.39), which was attracted predominantly by methyl salicylate baits and
303 represented 40% of the total sample from the gallery forest community. Other studies have also
304 reported a preference of this species for methyl salicylate (Morato *et al.* 1992; Brosi 2009;
305 Storck-Tonon *et al.* 2009). In the current study, *Eul. bombiformis* also visited other bait types,
306 but to a lesser extent.

307 The high flight capacity of the Euglossini bees (Janzen 1971) probably influenced our
308 results, as 13 species were common among the sites (Renkonen coefficient = 46.8%). According
309 to Neves and Viana (2003), although some species are found predominantly in the forest
310 environment, individuals might visit neighbouring ecosystems in search of floral resources. For
311 example, *Eug. hemichlora* is a common species in humid forests that is also captured in Cerrado.

Euglossa melanotricha, Eug. cordata, Eug. securigera, and *Eug. fimbriata* were more frequently captured in the Cerrado area, which demonstrated the ability of these species to seek resources in open areas. Faria and Silveira (2011) conducted a study in the Cerrado domain in southeastern Brazil, in Cerrado s.s., and riparian forest sites. The authors found three species restricted to the most open environment: *Eug.* fi*mbriata, Eug. townsendi* and *Euglossa* sp. They also found higher diversity for study sites in Cerrado s.s. and suggested that the riparian forests in the Cerrado apparently do not serve as routes of entry for forest-dependent euglossine species. However, it is possible that the short distance between the study areas and the surrounding agricultural matrix inhibits humid forest species from transiting to Cerrado, which are often not abundant and tend to be more difficult to sample.

The presence of cleptoparasitic species, such as *Exaerete frontalis*, *Exaerete smarag*dina and *A. caerulea* (Martins *et al.* 2016), may reflect the high degree of preservation of the area, because these three species are hosts of the abundant genus *Eulaema*. Silva *et al.* (2015) used ecological niche modelling to cross environmental variables and host species presence data for *Eul. nigrita* to predict the distribution of *A. caerulea* and found that *Eul. nigrita* had no influence on the occurrence of *A. caerulea*. Although the interactions between host and cleptoparasitic species within the Euglossini are not well understood (Silva *et al.* 2015), based on the data collected by Silva *et al.* (2015), we could infer that *A. caerulea* occupies a niche that is more closely associated with forest remnants and a more preserved habitat, such as that found in MSP. However, *Eul. nigrita* seems to be more associated with open areas (Peruquetti *et al.* 1999).

Regarding the applied methodology, the observed predominance of Eulaema species was expected due to the use of odoriferous traps during sampling. This tendency to capture male *Eulaema* more frequently than Euglossa has been well described in the literature (Nemésio and Morato 2004; Justino and Augusto 2010), and is often attributed to the aggression between males of these two genera, as well as to the ability of *Euglossa* to escape from traps (Justino and Augusto 2010). Even when sampling used an active collector with a catch net to reduce the negative effects of the trap escape on estimates of community richness, the results of the interaction between *Eulaema* and *Euglossa* species in traps did not change.

Several biotic factors can influence the daily activity of Euglossini males in the aromatic baits, such as the availability of natural sources of aromatic substances, supply of nectar and activity of females, as well as abiotic factors such as temperature, humidity and windspeed. Temperature is highlighted by several authors (Armbruster and Berg 1994; Oliveira 1999; Santos and Sofia 2002) as the main regulator of the activity in odoriferous baits.

Studies in the Central Amazon emphasize the variation in temperature between 24.5° C

and 27°C as the optimum point for a higher rate of visitation of the Euglossini in the baits (Oliveira 1999). In a study conducted in forest fragments in Parana, Santos and Sofia (2002) observed that bees were more present in the baits at temperatures between 22.2°C to 26.5°C and relative humidity of 75.6% and 71.8%. Our data for the gallery forest are consistent with these values; *Eul. bombiformis* and *Eul. nigrita* were more active in the gallery forest when the temperature ranged between 22.8°C and 26.2°C.

In contrast, the Cerrado *s.s* exhibited more contrasting results because *Eug. melanotricha* and *Eug. cordata* showed the highest peaks of activity in the half- day period when the temperature varied around 28.8°C. According to Santos and Sofia (2002), the bees of the genus *Euglossa* visited the baits more frequently when temperatures were higher, but within the limit that allows the regulation of body temperature. Differences in the vegetation cover and resource availability between vegetation types might have influenced visitation rates to baits. In the case of Cerrado *s.s*., higher temperatures may impede thermoregulation for some species at certain times of the day during sampling (Stone 1993), whereas in gallery forest, temperatures permit higher and prolonged bee activity near baits. Armbruster and Berg (1994) suggested that air temperature seems to be the main driver of daily patterns of Euglossini activity, lending support to the hypothesis that bees tend to avoid foraging in the afternoon when temperatures are higher, probably to avoid overheating.

Although both individual and species abundances were higher during the dry season (Rebêlo and Garófalo 1997; Neves and Viana 2003), species of the genus *Eufriesea* (*Eufriesea auriceps* and *Eufriesea surinamensis*) were captured only in the rainy season. These bees are extremely seasonal, and many of them are active only in the rainy season because of diapause in the pre-pupal stage; some species have only one generation per year (Kimsey 1987; Rebêlo 2001), which limits activity during some months of the year. *Eulaema nigrita, Eug. melanotricha, Eug. cordata, Eug. securigera* and *Eug. fimbriata* – which were well represented among the samples – are considered common in several ecosystems (Rebêlo *et al.* 2003), among them the Caatinga (Neves and Viana 2003) and Cerrado (Silveira *et al.* 2015).

Eulaema nigrita is sometimes associated with disturbed (Peruquetti *et al.* 1999) or more open environments, and has been reported to occur in the Cerrado biome (Silveira *et al.* 2015). In the MSP, *Eul. nigrita* was commonly sampled in both collection areas. The high abundance of *Eul. nigrita* and *Eug. melanotricha* in Cerrado *s.s.* has been reported in other studies of the Cerrado in Minas Gerais (Alvarenga *et al.* 2007; Justino and Augusto 2010; Faria and Silveira 2011; Silveira *et al.* 2015; Antonini *et al.* 2016). This demonstrates the flexibility of these species to exploit resources in areas with lower plant cover, in contrast to habits observed for the rest of the Euglossini.

382 The thermal amplitude found in both study areas may have provided different
383 responses regarding the attractiveness of certain types of baits to bees, because the baits used
384 have different degrees of volatility. In the gallery forest, the increase in temperature during the
385 morning was directly related to the rate of visitation of *Eul. bombiformis* males to the methyl
386 salicylate (Morato *et al.* 1992), because this bait has relative volatility. However, in the
387 afternoon, when temperatures are generally higher, this species tended to increase visitation
388 to the methyl cinnamate baits that have lower volatility compared with methyl salicylate
389 (Williams and Whitten 1983).
390 In the case of Cerrado s.s., the temperature demonstrates a negative effect on the
391 community, as highlighted by the drastic fall in activity of species of *Eulaema*. However,
392 between 11:00 a.m. to 12:00 p.m. when the temperature was high, we recorded the highest
393 peak activity of *Eug. melanotricha* and *Eug. cordata*, demonstrating an affinity for eugenol. This
394 bait is less volatile than the other baits used because of its high molecular weight (Armbruster and
395 McCormick 1990; Rebêlo 2001), which makes it more efficient in the hottest hours of the day.
396 Eucalyptol is recognized as the most efficient bait in studies with Euglossini bees due to its
397 low molecular weight and high volatility (Williams and Whitten 1983; Nemésio 2012).
398 However, the use of complementary scents or scents that are less attractive – such as methyl
399 cinnamate and vanillin – were useful for the sampling of infrequent species such as *A. caerulea*
400 (*see* Martins *et al.* 2016) and species of the genus *Eufriesea* (*Euf. surinamensis, Euf. auriceps,*
401 *Euf. vidua*). According to Nemésio (2016), the use of a greater number of aromatic compounds
402 in xeric areas (like the Brazilian Cerrado and northeast Caatinga) makes it possible to overcome
403 the issue of low abundance of Euglossini in these environ- ments. Additionally, this helps to
404 attract species that present greater specificity for a certain aroma – a fact evidenced by the
405 captured species mentioned above.
406 The Cerrado domain contains several ecosystems ranging from fields to forest for-
407 mations; this factor promotes high β diversity for the Euglossini. Furthermore, the greater
408 stability of abiotic factors in gallery forest makes the Cerrado an important habitat for species
409 from adjacent environments, primarily *Euglossa* from humid forest environments (Silveira *et*
410 *al.*2015). Although our samples were limited, with only one study site in each type of environ-
411 ment separated by 1 km, our data suggest that the MSP gallery forest may serve as a route of
412 dispersion for Amazon species in the Cerrado domain. This hypothesis is supported by the
413 presence of seven species unique to this environment (*i.e.* compared with only three in Cerrado
414 s.s.). However, these two habitats shared 13 species, demonstrating that the vegetation
415 formations together contribute to the maintenance of the group in this region and are important
416 areas for the implementation for Cerrado conservation programmes.

As the new agricultural frontier, Maranhão has attracted the attention of large land-owners and companies from the southern region of Brazil (Barreto *et al.* 2012). These groups have invested in planting soybeans and eucalyptus, causing extensive damage to the Cerrado Maranhense. Although this diverse formation has the capacity to recover from damages caused by natural events such as fires, it may not rebound as easily from anthropogenic disturbance.

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Orchid bees (Apidae: Euglossini) in the biodiversity hotspot of eastern Amazon

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Orchid bees (Apidae: Euglossini) in the biodiversity hotspot of eastern Amazon

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Short title: Orchid bees in eastern Amazon

Abstract

The Environmental Protection Area (APA) of *Reentrâncias Maranhenses* (RM), located at the eastern end of the Amazon Basin, is of great importance for biodiversity conservation because of its high species diversity. Through the diversity profile based on Hill numbers, this study intends to: (i) investigate the possible variations in richness, composition, and abundance between two collection methods (bait traps and insect nets); (ii) verify the effect of distribution fluctuation in the abundance of orchid bees during the year, and (iii) calculate the sample-size coverage through the Hill numbers. Thus, orchid bee males were captured using insect nets and bait traps in the municipalities of Cururupu (CP) and Mirinzal (MZ) on the western coast of the state of Maranhão, Brazil. A total of 3,019 Euglossini males were collected from 42 species of five genera. The most abundant species were *Eulaema cingulata* (26.37%), *Euglossa cordata* (22%), *Eg. piliventris* (9.18%), and *Eg. viridis* (6.06%). The analyses based on Hill number showed no difference between the sites sampled with the two methods, however, there were difference in the composition of species between the environments. Our data indicated that insect nets and bait traps can be used in a complementary manner to increase sampling efficiency and that both were important when attempting to effectively record euglossine species richness in the eastern Amazon.

Keywords: Maranhense Amazon / complementary methods / insect net / bait trap / aromatic compound / tropical forest

Introduction

Euglossini bees are important elements of tropical forest fauna because they are responsible for the pollination of around 60 families of native and cultivated plants (Dressler 1982, Ramírez *et al.* 2002; Roubik and Hanson 2004,). There are five genera of euglossine bees: *Euglossa* Latreille, 1802; *Eulaema* Lepeletier, 1841; *Exaerete* Hoffmannsegg, 1817; *Aglae* Lepeletier & Serville, 1825; and *Eufriesea* Cockerell, 1908 (Moure *et al.* 2022), distributed from northern Mexico to northern Argentine.

The coevolutionary interactions between Euglossini bees and Orchidaceae led the group to be popularly known as the orchid bees (Dressler 1968). While orchids provide the male bees with chemical compounds that are possibly used in the synthesis of sex pheromones (Dressler 1982), they, in turn, contribute to the cross-pollination of the plants, which maintains genetic diversity (Zayed 2009). Dodson *et al.* (1969) discovered that male euglossines can be artificially attracted by synthetic substances, which made it possible to research different points along the Neotropical region (Nemésio and Morato 2006; Storck-Tonon *et al.* 2009).

In the Amazonian domain, surveys of orchid bees are often irregularly distributed, generally made over a short period of time, and are conducted along the great rivers or near highways (Oliveira and Campos 1996*;* Silva and Rebêlo 1999; Santos Júnior *et al.* 2014; Antonini *et al.* 2017). The small number of studies undertaken in the Amazon region is a cause for concern because, as hypothesized by Ramírez *et al.* (2010), this region could be the center of origin and diversification for orchid bees. In addition, increasing deforestation over the last few decades in the region (Maués and Oliveira 2010) makes these areas of the Amazonian domain

a priority for studies on the diversity of euglossine bees because much information could potentially be lost. The studies on euglossine bees have concentrated on the central and western regions of Amazon (Morato 1992; Oliveira and Campos 1996; Abrahamczyk *et al.* 2011; Santos Júnior *et al.* 2014; Antonini *et al.* 2017, Botsh *et al* 2017). In the extreme eastern portion of this domain, where Maranhão state is located, research is even more limited.

The Maranhão is located in an ecotone area between the Caatinga, Cerrado, and Amazonian domains (Rebêlo and Silva 1999), these habitats are connected by forest formations such as riparian forests and "cocais forest" (palm forest) that allow the displacement of forest-dependent species

López-Uribe *et al.* (2014) investigating the effects of climate change in the Pleistocene on the phylogeography of three species of *Eulaema* Lepeletier using mitochondrial and nuclear markers, hypothesized that the Maranhão coast might have been a potential Pleistocene refugia in the past. Overlapping this area, in the western portion of this Brazilian state there is the Area of Environmental Protection (or APA from Portuguese) of *Reentrâncias Maranhenses* (RM) considered the east limit of the Amazon basin (Martins and Oliveira 2011, SEMA 2011), due to the presence of a remnant of the Amazon forest in this region is interesting for the knowledge of the euglossine fauna. Three studies have been conducted in the RM, with the highest richness values found in Carutapera (24 species), Alcântara (14sp) and Vitória do Mearim (11sp) (Albuquerque *et al.* 2001; Brito and Rêgo 2001; Ferrerira *et al.* 2019), but these studies used a limited number of aromatic compounds.

One of the major problems in the use of diversity indices are the criteria for their choice, besides the weight that is given to rare species,

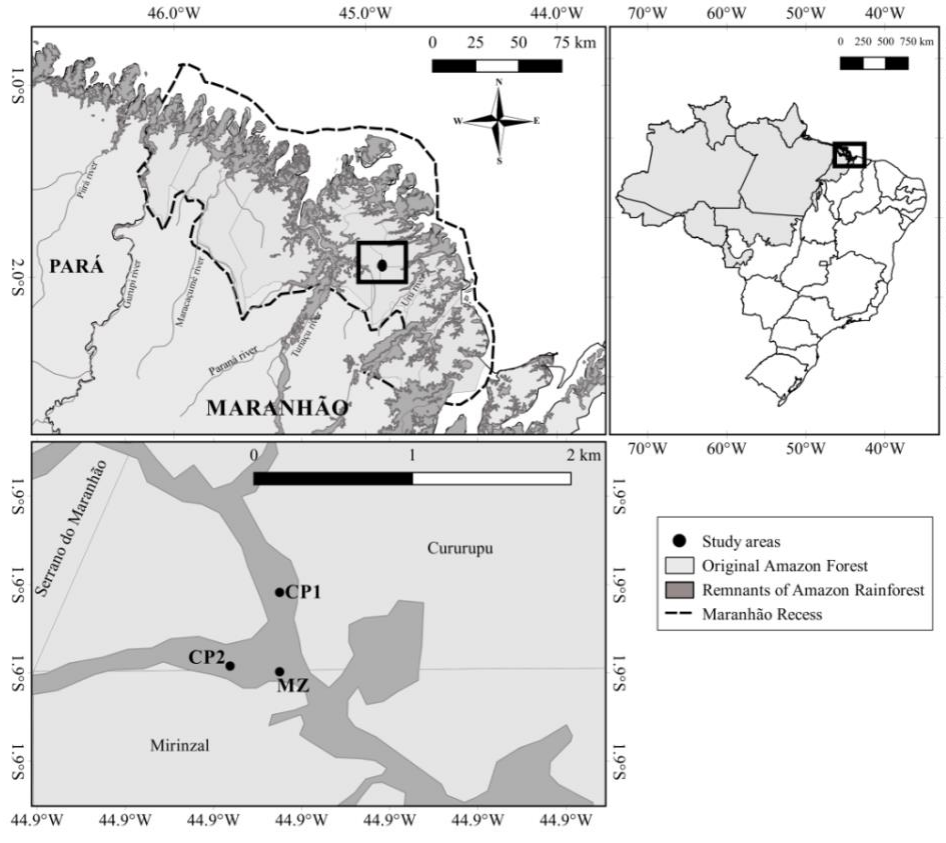
aspects that may be contoured through Hill numbers. With this analysis is possible to standardize the main indices (species richness - q = 0, Shannon diversity - q = 1 and Simpson diversity - q = 2), and sample-size coverage, data commonly used in community analysis (Hsieh *et al.* 2016), plotting intuitive curve graphs and easy to compare.

In this context, this study intended to: (i) investigate the possible variations in richness, composition, and abundance between two collection methods (bait traps and insect nets), based on the diversity profile of Hill numbers; (ii) verify the effect of distribution fluctuation in the abundance of orchid bees during the year, and (iii) calculate the sample-size coverage through the Hill numbers.

Materials and methods

Study areas

The study was carried out in remnants of Amazon forest along the boundaries between the municipalities of Cururupu and Mirinzal on the western coast of the state of Maranhão, northeast Brazil (Figure 1). These localities are part of the 14 municipalities that represents the RM and occupy an area of 2.680.911,2 ha of terrestrial and marine ecosystems (SEMA 2011). According to Koppen's classification, the study site climate is As, which is characterized by a tropical climate with a dry summer season (Alvares *et al.* 2013). The average annual rainfall is 1,486 mm and the rainy period is from January to June (SEMA 2011).



142 **Figure** 1 **-** Study areas located in the municipalities of Cururupu (CP1 and
143 CP2) and Mirinzal (MZ). The dotted area represents the Area of
144 Environmental Protection of *Reentrâncias Maranhenses* (RM) in the
145 eastern Amazon.
146

147 *Bee capture*

148 The method used to collect the bees followed the one developed by
149 Storck-Tonon *et al.* (2009) with the following modifications. The
150 distances between the sample points varied from 500 m to 1000 m; there
151 was monthly sampling; a larger amount of bait was used, and sand was
152 placed in the entrances of the traps. There were monthly collections
153 between August 2015 and July 2016, which took place on two consecutive
154 days between 8:00 am and 4:00 pm. There was no collection in March

2016 because of high rainfall, which made it impossible to carry out field activities.

Insect nets were used to collect the samples in Mirinzal (MZ: 44°54′42.3″W, 1°56′43.1″S, altitude 28 m), which are separated 1 km and 300 m, respectively for CP1 and CP2 described later in the text. A transect was drawn into the forest and two sets of cotton swabs containing the following aromatic compounds were distributed along the transects: benzyl acetate, methyl cinnamate, benzyl benzoate, beta-ionone, eucalyptol, eugenol, methyl salicylate, and vanillin (Sigma-Aldrich, Co., 3050 Spruce street, St. Louis MO 63103 USA 314-771-5765) (Rebêlo and Garófalo 1997).

Two areas were selected for passive collection by bait traps. These were both in the municipality of Cururupu (CP1: 44°54′51.9″W, 1°56′41.7″S, altitude 16 m; CP2: 44°54′42.2″W, 1°56′27.3″S, altitude 18 m), these sites are separated 600 m apart from each other. The traps were constructed according to Ramalho *et al.* (2009). A set of bait traps was placed in the sub-forest at each sampling point. Each set of bait traps consisted of eight PET (polyethylene terephthalate) bottles, contained a piece of cotton soaked with one of the aromatic compounds mentioned above.

The bait traps were installed at a minimum height of 1.5 m above the ground and separated from each other by a distance of 8 m. The aromatic compounds were replaced in the early morning of each day. After 24 h of sampling, the captured specimens were removed, sacrificed using deadly vials, and stored for future assembly and identification. After assembly and identification, the captured specimens were deposited in the reference collection of the Bee Studies Laboratory of the Federal University of Maranhão (LEA/UFMA).

Data analysis

The rarefaction and extrapolation models based on Hill numbers were used to compare the structures of the orchid bees communities in the different sites sampled according to Chao *et al* (2014). This diversity index family comes to solve the main problems of analysis of communities such as the influence of sampling effort and the non-incorporation of data of relative abundance of the species in the analysis of communities. The Hill numbers to bring them together in the single ordering graphics parameters such as species richness (q=0), Shannon-Wiener indexes (q=1), and Simpson index (q=2) facilitates the comparison between different communities in time and space (Chao *et al* 2014, Hsieh et 2016). This analysis to compare the orchid bees communities sampled was used the parameter q =0, of Hill number performed from 1,000 bootstrap replications and 95% confidence intervals (Chao *et al* 2014).

Circular statistic was applied for tests to the annual fluctuations of the most abundant species, in this case the species with abundance above 5% for the total community. From this, each month one of the 12 months of the year are converted into angles of 30º, beginning in January (30º) and ending in December (330º), generating metrics such as mean angle (r), mean vector, length of the mean vector (R) used to assess the concentration of bees. Rayleigh test of uniformity was applied based significance of mean angle (r) calculated through the software Oriana (Kovach Computing Services, 2012) to test the following hypotheses Ho: the abundance of orchid bees species is distributed uniformly around the year; H1: the bees not distributed uniformity (Morellato *et al.* 2000).

The sample-size coverage-based rarefaction and extrapolation curves for Hill numbers were constructed according to Chao *et al.* (2014)

in the tool online propose by Chao *et al* (2016), to compare sample sufficiency to all sites sampled. The estimator coverage (Ec) was used for this analysis; the value can range from 1 to 0, with value 0 when the sampling was insufficient and 1 value when the sample is satisfactory.

Results

A total of 3019 male Euglossini bees from 42 species belonging to all five genera of the tribe were captured in the three study areas. The most representative groups were the genera *Euglossa* (61%) and *Eulaema* (33.22%). The most abundant species were *El. cingulata* (Fabricius) (26.37%), *Eg. cordata* (Linnaeus) (22%), *Eg. piliventris* Guérin (9.18%), and *Eg. viridis* (Perty) (6.06%). Together these species made up 63.67% of the sample (Table 1).

In the collections using insect nets (site MZ), 68.26% belonged to the genus *Euglossa*, 26.77% to *Eulaema*, 3.95% to *Eufriesea*, 0.68% to *Exaerete*, and 0.34% to *Aglae*. The bait trap results for the CP1 site showed that 56.02% of the bees were *Euglossa,* 37.97% were *Eulaema*, 2.63% were *Eufriesea*, 2.82% were *Exaerete*, and 0.56% were *Aglae*. At the CP2 site, the most abundant genera were *Euglossa* (52.89%), *Eulaema* (40.04%), *Eufriesea* (4.22%), *Aglae* (1.47%), and *Exaerete* (1.37%).

All parameters of Hill numbers showed the complete to partial overlapping of the confidence intervals. However, the main difference found was the species composition between the sites. The rarefaction and extrapolation analysis based on the Hill numbers for the parameter q =0 did not showed difference in the species richness between CP2 and MZ sites, while CP1 presented values lesser for this parameter (Hill numbers q=0: MZ= 40; CP2= 38; CP1=32) (Figure 2). In the case of the parameter q=1, which is equivalent to the exponential of the familiar Shannon

measure of diversity, the site CP2 was most diverse than MZ and CP1 sites (Hill numbers q=1: CP2=13.07; MZ= 12.39; CP1=12.15), with the latter showing overlap of confidence intervals. Already the parameter q=2 (Simpson index), showed similar patterns of community distribution, with the closest MZ and CP2 communities in relation to CP1 (Hill numbers q=2: MZ= 7.05; CP2=6.94; CP1=6.66) (Table I).

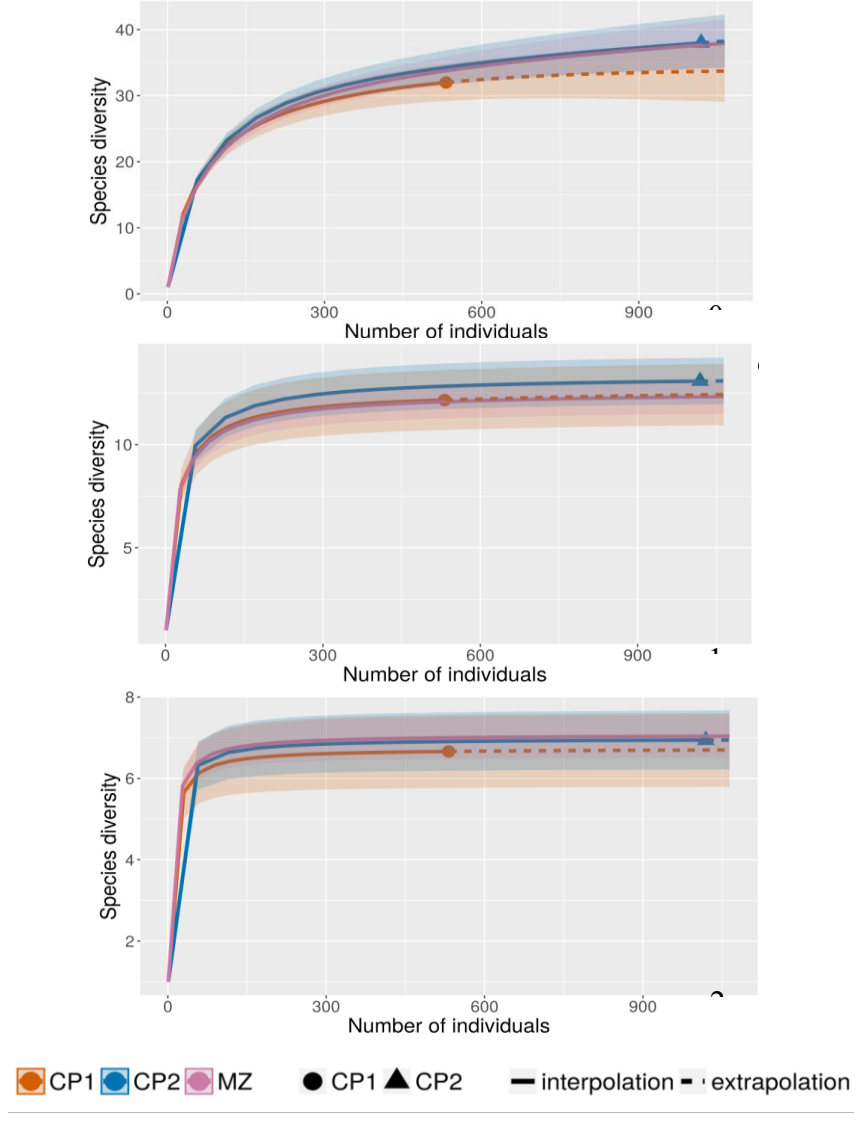


Figure 2 Comparison of sample-size-based rarefaction (solid lines) and extrapolation (dashed curves), to orchid bees captured in the three

different sites in the *Reentrâncias Maranhenses Protection area* - RM (CP1: orange, CP2: blue, and MZ:). The species diversity for Hill numbers of order *q*=0 (left panel), q=1 (middle panel), and q=2 (right panel). The 95% confidence intervals (orange-blue-purple regions) were obtained by a bootstrap method based on 1000 replications.

The estimator coverage showed the overlap of values for all sites MZ (Ec= 0.995), CP (Ec= 0.994) and CP1 (Ec = 0.992), it showed that the sampling effort employed in these areas was sufficient to sample a large part of the communities (Figure 3). The values obtained for its estimators showed that the sampling had 99% success in recognizing the orchid bee community on the RM.

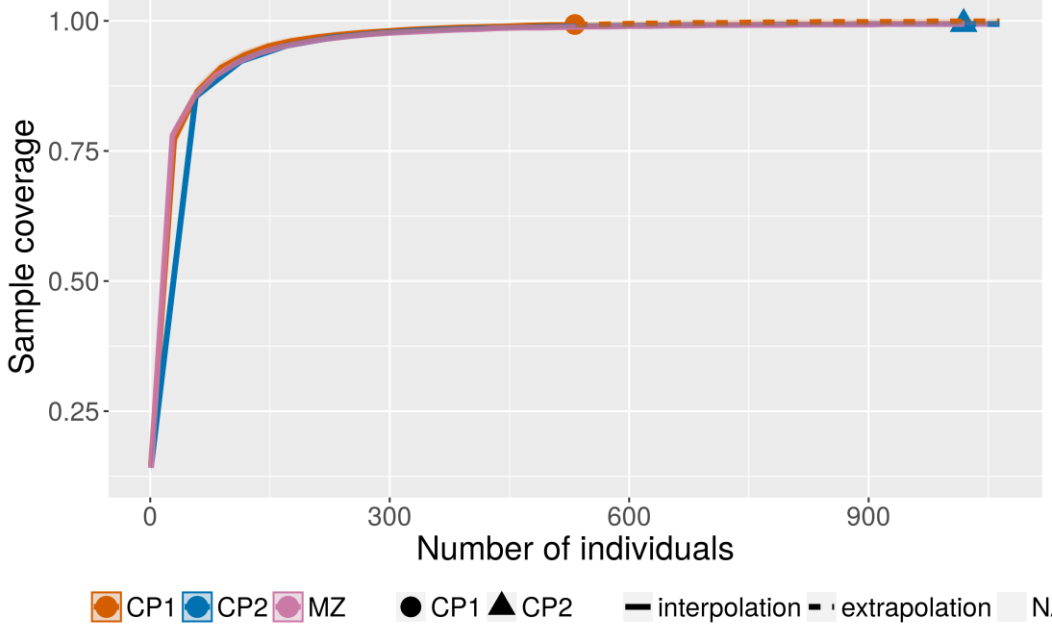


Figure 3 Sample completeness curve based on rarefied samples (solid lines) and extrapolated samples (dashed line). The Hill numbers of order *q*=0 was used with 95% confidence intervals for orchid bees sampled in sites CP1, CP2 and MZ in the *Reentrâncias Maranhenses Protection Area* - RM.

Table I Diversity profile based in Hill numbers (parameters $q = 0$ to 2) for orchid bees captured using insect nets (MZ) and bait traps (CP1 and CP2) at three study sites in the municipalities of Cururupu and Mirinzal, western coast of Maranhão, Brazil. * New records for the *Maranhense* Amazon updated from Rebêlo e Silva (1999).

Species	MZ (%)	CP1 (%)	CP2 (%)	Total
Ag. caerulea Lepeletier & Serville, 1825*	5 (0.34)	3 (0.56)	15 (1.47)	23 (0.76)
Ef. nordestina (Moure, 1999)	1 (0.07)			1 (0.03)
Ef. concava (Friese, 1899)*			1 (0.1)	1 (0.03)
Ef convexa (Friese, 1899)*	12 (0.82)	2 (0.38)	3 (0.29)	17 (0.56)
Ef. elegans (Lepeletier, 1841)	2 (0.14)		2 (0.2)	4 (0.13)
Ef. ornata (Mocsáry, 1896)	4 (0.27)	1 (0.19)	9 (0.88)	14 (0.46)
Ef. pulchra (Smith, 1854)	3 (0.2)	1 (0.19)	14 (1.37)	18 (0.6)
Ef. superba (Hoffmannsegg, 1817)	1 (0.07)	1 (0.19)	1 (0.1)	3 (0.1)
Ef. surinamensis (Linnaeus, 1758)	17 (1.16)			17 (0.56)
Ef. vidua (Moure, 1976)*	18 (1.23)	9 (1.69)	13 (1.28)	40 (1.32)
Eg. amazonica Dressler, 1982*	21 (1.43)	6 (1.13)	9 (0.88)	36 (1.19)
Eg. augaspis Dressler, 1982	3 (0.2)		1 (0.1)	4 (0.13)
Eg. avicula Dressler, 1982	11 (0.75)	3 (0.56)	4 (0.39)	18 (0.6)
Eg. chalybeata Friese, 1925	1 (0.07)	3 (0.56)	6 (0.59)	10 (0.33)
Eg. cognata Moure, 1970	12 (0.82)	8 (1.50)	23 (2.26)	43 (1.42)
Eg. cordata (Linnaeus, 1758)	384 (26.16)	120 (22.56)	162 (15.9)	666 (22.06)
Eg. decorata Smith, 1874	3 (0.2)			3 (0.1)
Eg. despecta Moure, 1968*	34 (2.32)	19 (3.57)	27 (2.65)	80 (2.65)

Species	MZ (%)	CP1 (%)	CP2 (%)	Total
Eg. fimbriata Moure, 1995	1 (0.07)	4 (0.75)	6 (0.59)	11 (0.36)
Eg. hemichlora Cockerell, 1917*	10 (0.68)		2 (0.2)	12 (0.4)
Eg. ignita Smith, 1874*	29 (1.98)	13 (2.44)	15 (1.47)	57 (1.89)
Eg. imperialis Cockerell, 1922	1 (0.07)	2 (0.38)	1 (0.1)	4 (0.13)
Eg. liopoda Dressler, 1982	32 (2.18)	6 (1.13)	11 (1.08)	49 (1.62)
Eg. lugubris Roubik, 2004*	8 (0.54)	2 (0.38)	4 (0.39)	14 (0.46)
Eg. magnipes Dressler, 1982*	1 (0.07)		1 (0.1)	2 (0.07)
Eg. modestior Dressler, 1982	31 (2.11)	36 (6.77)	54 (5.3)	121 (4.01)
Eg. mourei Dressler, 1982*	9 (0.61)		3 (0.29)	12 (0.4)
Eg. securigera Dressler, 1982	32 (2.18)	20 (3.76)	15 (1.47)	67 (2.22)
Eg. townsendi Cockerell, 1904	13 (0.89)	2 (0.38)	8 (0.79)	23 (0.76)
Eg. occidentalis Roubik, 2004*	1 (0.07)		1 (0.1)	2 (0.07)
Eg. piliventris Guérin, 1844	150 (10.22)	30 (5.64)	97 (9.52)	277 (9.18)
Eg. parvula Dressler, 1982*	27 (1.84)	4 (0.75)	11 (1.08)	42 (1.39)
Eg. intersecta Latreille, 1817*	47 (3.2)	10 (1.88)	46 (4.51)	103 (3.41)
Eg. viridis (Perty, 1833)	141 (9.6)	10 (1.88)	32 (3.14)	183 (6.06)
El. bombiformis (Packard, 1869)	12 (0.82)	13 (2.44)	15 (1.47)	40 (1.32)
El. cingulata (Fabricius, 1804)	323 (22)	154 (28.95)	319 (31.31)	796 (26.37)
El. nigrita Lepeletier, 1841	12 (0.82)	18 (3.38)	11 (1.08)	41 (1.36)
El. pseudocingulata Oliveira, 2006*	33 (2.25)	10 (1.88)	51 (5)	94 (3.11)
El. meriana (Olivier, 1789)	13 (0.89)	7 (1.32)	12 (1.18)	32 (1.06)

Species	MZ (%)	CP1 (%)	CP2 (%)	Total
Ex. frontalis (Guérin, 1844)	6 (0.41)	7 (1.32)	9 (0.88)	22 (0.73)
Ex. lepeletieri Oliveira & Nemésio, 2003*		1 (0.19)		1 (0.03)
Ex. smaragdina (Guérin, 1844)	4 (0.27)	7 (1.32)	5 (0.49)	16 (0.53)
Total	**1468**	**532**	**1019**	**3019**
Species richness (*q*=0)	**40**	**32**	**38**	**42**
Shannon index (*q*=1)	**12.39**	**12.15**	**13.07**	
Simpson index (*q*=2)	**7.05**	**6.66**	**6.94**	

Table II Temporal variation in orchid bees activities captured in the *Reentrâncias Maranhenses Protection Area*, Brazil. Mean vector, month, length vector (R) and Rayleight test (Z) result (*p < 0.05; **p < 0.01).

Species	Sites	Mean vector	Mean moth	**Rayleigh test** Length of mean vector (r)	(Z)
Euglossa cordata	CP1	357,909°	December	0,474	26.96**
	CP2	32,415	February	0,258	10.80**
	MZ	192,347°	July	0.126	6.10**
Euglossa piliventris	CP1	122,646°	May	0.567	9.65**
	CP2	69,988°	May	0.569	31.41**
	MZ	123,482°	May	0.404	24.53**
Euglossa viridis	CP1	213,35°	August	0.568	3.23*
	CP2	11,964°	January	0.218	1.53
	MZ	201,36°	July	0.235	7.51*
Eulaema cingulata	CP1	22,885°	January	0.246	9.31**
	CP2	3,14	January	0.212	14.39**
	MZ	203,276°	July	0.406	53.19**

The Rayleigh test showed a temporal variation in the activity of the orchid bees in the RM, with all species concentrated in the rainy season. *Euglossa cordata* was abundant the whole year (Figure 4 A, E, and I), mainly in the rainy season (R=0.474; Z=26,96; p<0.001). *Euglossa piliventris* concentrated high activity between March and May, with highest values in May (R=0.569; Z=31.41; p<0.001) (Figure 4 B and J). *Euglossa viridis* showed activity peaks in August (R=0.568; Z=3.23; p<0.05) (Figure 4C). In case of *El. cingulate,* the higher activity was concentrated in January (R=0.246; Z=9.31; p<0.001).

The bait types that attracted the largest numbers of specimens were beta-ionone (41.8%, 24 sp.), eucalyptol (31.53%, 33 sp.), methyl salicylate (6.53%, 21 sp.), vanillin (6.16%, 22 sp.), and eugenol (5.37%, 15 sp.) (Fig S1).

The results showed that 0.9% of euglossine males carried pollinaria attached to their bodies. These included *El. cingulata* bees (n = 25) with *Catasetum macrocarpum* Rich. ex Kunth pollinaria and *Eg. securigera* Dressler, 1982 bees (n = 3), which had *Catasetum barbatum* (Lindl.) Lindl pollinaria attached to them.

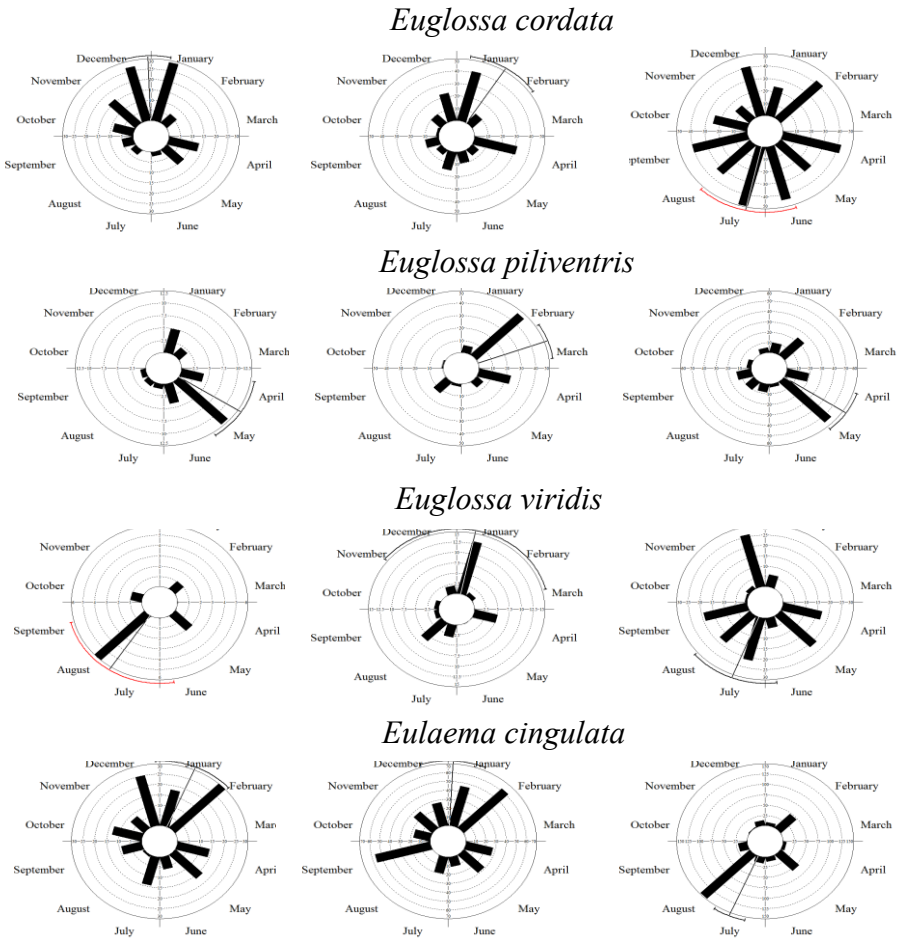


Figure 4 Temporal variation of the most abundance orchid bees captured in the CP1 (A, B, C, D, and E), CP2 (E, F, G, H and I) MZ (I, J, L, M, and N) sites in the *Reentrâncias Maranhenses Protection Area*, Brazil. From August 2015 to July 2016. The line at the top of the vector stands for standard deviation. Except for *Euglossa vidiris* P=0.218, the other species were present all year (see Table).

Discussion

Our data indicate that the region contains a high richness of orchid bee species, which is related to the presence of large areas of remnant open Ombrophylous Forest. The connectivity of these environments by riparian forests and "cocais forest" (palm forest) is another factor that may have allowed the transit of several forest-dependent species (Silva 2009; Martins *et al.* 2016, 2018). With all these aspects that favor the diversity of organisms intrinsically forest, López-Uribe *et al.* (2014) suggest that these areas were Pleistocene refuge areas for these bees in the past, which is corroborated by our data

The Maranhão is home to 56 species of euglossine bees (Rebêlo and Silva 1999; Silva and Rebêlo 2009; Silva 2012; Martins *et al.* 2016, 2018, Ferreira *et al.* 2019). Although our data only represented the *Reentrâncias Maranhenses Protection Area*, the highest species richness found (42 sp.) represents a value that is very close to that recorded in studies that

covered the whole state, which highlights the discrepancies in the knowledge about euglossine fauna in this federative unit of Brazil. The second region with the highest number of registered species for Amazon forest was observed by Nemésio and Rasmussen (2014) in the Tarapoto, region in northeastern Peru, with 53 euglossine species (Nemésio and Rasmussen 2014).

The euglossine are excellent pollen carriers that can cover about 23 km in continuous forest and fragmented forest (Janzen 1971, Botsh *et al*. 2017), aspects such as the sampling scale (1km, 600m, and 300m), the presence of the same forest matrix and simultaneous sampling can have influenced our date since there were no differences in Hill numbers analysis between the sites sampled, with difference found for the species composition only between the sites. In a similar way to our data found in the RM, Botsh *et al.* (2017) used McPhail traps to sample orchid bee communities in the Amazon forest fragments distant between 0.3 and 17.4 km from the continuous forest environment did not found differences in the abundance and species richness. These authors found difference only in the species composition and equability between fragment forest and continuous forest, that they attribute such differences to the edge effect of fragments forest and the presence of a higher variety of habitats within the continuous forest.

Although the values of the parameters *q* of the Hill numbers diversity profile did not show significant differences between the communities sampled with bait traps (CP1 and CP2) and insect net (MZ). The species compositions between these sites were different, mainly in relation to the rare species such as *Ef. nordestina*, *Ef. surinamensis*, *Eg. decora*, which were present only at the site MZ where was used the insect net method. Studies suggest that bait traps should be used as a complementary method (Nemésio and Morato 2006, Mattozo *et al.* 2011). Mattozo *et al.* (2011) suggested that the purpose of the study should be taken into account if bait traps are to be used effectively, because this method allows the collector to sample large areas at the same time. In addition, the combination of insect nets, bait traps, and eight different baits may have contributed to the identification of a significant proportion of the local orchid bees shown by the high values of the estimator coverage (Nemésio and Morato 2006; Storck-Tonon *et al.* 2009).

An interesting aspect revealed in our data is the contrast in the relation to the low abundance of *El. nigrita* and the high representativeness of *El cingulata*. These two species are widely distributed in the Neotropical region and sometimes there are overlapping in open areas such as Cerrado (Silva 2012, Martins *et al* 2018), but there is a difference in their degree of association with forest environments. While *El. nigrita* is more associated with open formations (Martins *et al* 2018) and disturbed habitats (Peruquetti *et al* 1999), in environments of the

Amazon forest where there is a predominance of *Ex smaragdina*, the cleptoparasitic species of this species (Nemésio and Silveira 2006a), *El nigrita* show lower abundance.

In contrast, *El cingulata* shows a higher degree of dependence on forest habitats and forest edges (Nemésio and Silveira 2006b), thus showing high representativeness in these environments observed in this study. The highest values of parameter q=2 (Simpson index) to the sites CP2 and MZ are directly associated with *El cingulata* and *Eg cordata* abundance in these environments.

Another reason for the high species richness revealed in this study is the frequent periodic sampling over one year, which allowed the capture of highly seasonal species, such as *Ef. nordestina*, *Ef. concava*, *Ef. convexa*, *Ef. elegans*, *Ef. ornata*, *Ef. pulchra*, *Ef. superba*, *Ef. surinamensis*, and *Ef. vidua*, that might not otherwise have been captured. For example, only two *Eufriesea* species were found by other studies in the Amazonian domain that sampled for only a few months (Oliveira and Campos 1996; Silva and Rebêlo 1999; Santos Júnior *et al.* 2014; Antonini *et al.* 2017). A long-term sampling protocol in more preserved and heterogeneous areas may in the future reveal the existence of Euglossini species not yet registered in the region.

Our data point out the existence of fluctuation in the activity of the most abundant orchid bee species in the *Reentrâncias Maranhenses* Protection Area, indicating seasonal variation in the bees activities, which was most representative of the rain season, corroborated with other studies in Amazon forest (Brito and Rêgo 2001; Oliveira 1999; Vilhena *et al.* 2017). There was uniformity in the distribution of *El. cingulata* and *Euglossa cordata* throughout the year, while *Eg. viridis* and *Eg. piliventris* showed punctual activity during the rainy season.

Most studies that investigate bait attractiveness using aromatic baits point to eucalyptol as being more attractive and that its use resulted in higher values for both abundance and species richness (Silva and Rebêlo 1999; Storck-Tonon *et al.* 2009). Our data corroborated with these observations regarding species richness, but specimen abundance in the eucalyptol traps was low. Beta-ionone attracted a higher number of individuals, and this suggests that the species *El. cingulata*, *Eg. cordata*, and *Eg. viridis* have a preference for this bait type. Beta-ionone baits have rarely been employed in studies conducted across the Amazonian domain (Vilhena *et al.* 2017). In our study, this bait was very attractive, especially for *El. cingulata*, which is a species with a wide geographic distribution and is found in the open vegetation environments of the Cerrado and the humid forest environment to the southeast of the country (Nemésio 2009).

Previous studies have suggested that *Eg. viridis* is a species with a disjointed distribution because it has been found in the Amazon rainforest and the Atlantic forest (Giangarelli and Sofia 2011). According to Nemésio (2009), this species is not particularly attracted by aromatic

compounds. However, it was frequently found in the beta-ionone and methyl salicylate bait traps in this study. This fact may change current knowledge about the distribution of this species as it may also be found in the intermediate areas between those two biomes.

The presence of specimens carrying orchid pollinaria in the study area demonstrates the contribution made by this group of bees to the pollinating services of the region (Silva *et al.* 1999). However, Dressler (1982) points out that the simple presence of pollinaria attached to Euglossini bees does not guarantee that it comes from local orchid species. However, when pollinaria were found on RM bees, several individuals of *Catasetum* spp. were observed flowering in the areas close to the collection points.

Our data demonstrate the high euglossine diversity in RM and reaffirms the relevance of sampling throughout the year as it allows researchers to verify the complete phenological patterns of euglossine species that might not be found in just a few days of sampling. In addition, the use of the two main sampling methodologies applied in bee studies was complimentary because the traps captured a considerable number of the large species, but the insect net method helped in catching smaller species. This allowed a more complete sample of the community to be collected.

Our data show that the coast of Maranhão maintains favorable conditions that support a high diversity of orchid bees that may have been established millions of years ago (Pleistocene). The RM conservation is important because it shelters this high richness of euglossine bees, thus preserving the pollination services responsible for maintaining this rich and diverse environment. However, human actions such as deforestation, forest fires, and habitat fragmentation may gradually change this scenario

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Contributions

The authors RRDP, GCC, and DCM participated in the field activities; DCM performed the data analysis; DCM and JESJ wrote the article, and all authors participated in the reviews and approved the final manuscript.

Conflict of interest

We declare that there is no conflict of interest regarding the study presented in this paper.

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Supplementary Material

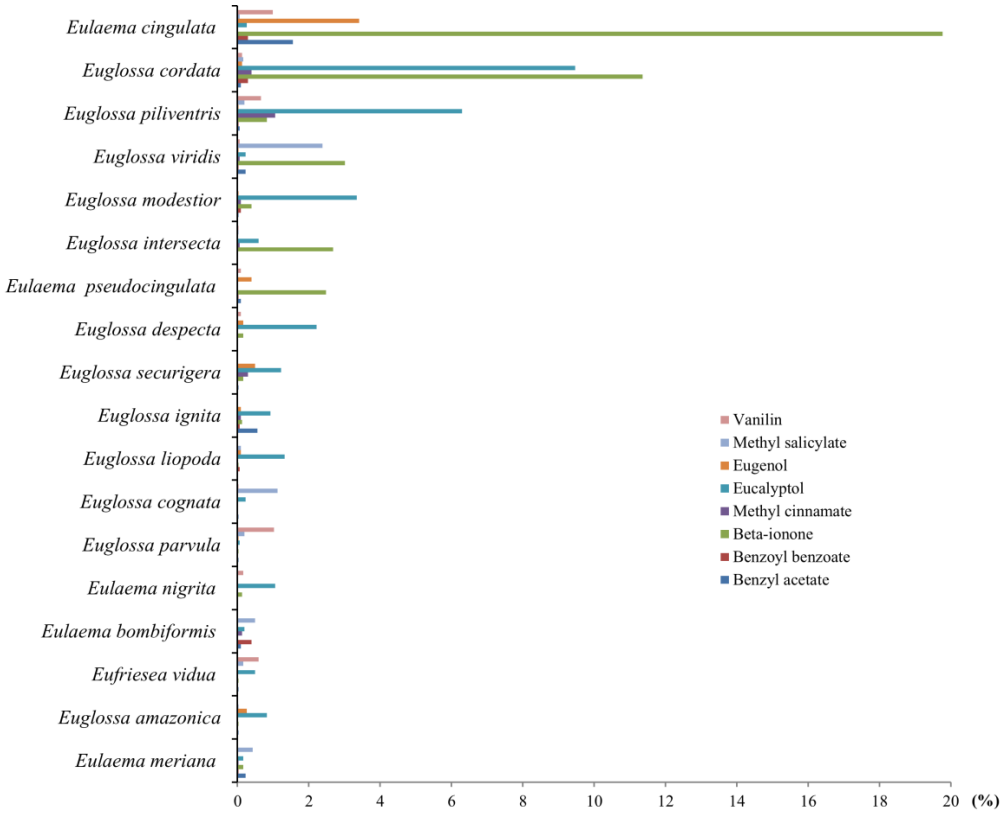

Figure 1S Most representative species of orchid bees attracted by eight aromatic baits (benzyl acetate, methyl cinnamate, benzyl benzoate, beta-ionone, eucalyptol, eugenol, methyl salicylate, and vanillin) in the dense evergreen forest located in the municipalities of Cururupu and Mirinzal, Maranhão, Brazil

7. CONCLUSÕES

O DNA é uma ferramenta importante para se entender a história evolutiva das populações de abelhas e traçar estratégias que permitam a preservação dos ambientes naturais em que as espécies habitam. Com isto em mente, no Capítulo I conseguimos identificar agrupamentos genéticos contrastantes mesmo para espécies evolutivamente relacionadas como *Eulaema nigrita* e *Exaerete smaragdina*, contudo estes mesmo padrões nos permitiram encontrar na área da Mata Atlântica, nos estados do Espírito Santo para *El. nigrita,* e Pernambuco para *Ex. smaragdina* como potenciais refúgios às oscilações do clima no Pleistoceno. Estas áreas podem ter permitido aumento no tamanho efetivo das populações destes organismos revelados nas análises de BSP e ainda expansões populacionais verificadas na rede de haplótipos, testes de neutralidade e demais análises para ambas as espécies.

Outro dado interessante para este estudo foi a localização de um agrupamento (*cluster*) com elevada diversidade genética para *El. nigrita* na Área de Transição do Nordeste, o que corrobora com resultados encontrados por Lópes-Uribe *et al.* (2014). O reconhecimento destas áreas como potenciais refúgios pleistocênicos, vem agregar maior importância para a conservação destes polinizadores, uma vez que estes ambientes permitiram à sobrevivência de várias espécies às alterações durante o Pleistoceno.

Nossos dados indicam que o Maranhão abriga uma elevada diversidade de espécies e condições que possibilitam a manutenção da variabilidade genética das populações destas abelhas, pois o estado mantem uma grande quantidade de remanescentes florestais em domínio de Cerrado e Floresta Amazônica que conectados através de formações riparias e matas de cocais permitem o transito de espécies dependentes de florestas, como é o caso de várias espécies consideradas raras em nossas amostragens (exemplo *Aglae caerulea*, *Eufriesea vidua*, *Euglossa cognata*) nos Capítulos I e II. Portanto, estes aspectos podem ter influenciado a elevada diversidade tanto genética, como ecológica destes organismos intimamente relacionados a cobertura vegetal como são os Euglossini e corroboram a hipótese de López-Uribe *et al.* (2014) sobre a existência de um refúgio pleistocênico na costa do Maranhão. Pesquisas futuras com outras espécies podem revelar mais pistas sobre a dinâmica de colonização de espécies amazônicas nestas áreas.