UNIVERSIDADE FEDERAL DE SÃO CARLOS

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA EVOLUTIVA E BIOLOGIA MOLECULAR

HISTÓRIA NATURAL, BIOGEOGRAFIA E A GENÉTICA DE POPULAÇÕES DE *Partamona rustica*, UMA ABELHA ENDÊMICA DE FLORESTAS SECAS DO BRASIL

ELDER ASSIS MIRANDA

SÃO CARLOS – SÃO PAULO – BRASIL

ABRIL DE 2016

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Tese apresentada à Universidade Federal de São Carlos, como parte das exigências para obtenção do título de Doutor em Genética Evolutiva e Biologia Molecular, área de concentração: Genética Evolutiva.

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SÃO CARLOS – SÃO PAULO – BRASIL

ABRIL DE 2016

Ficha catalográfica elaborada pelo DePT da Biblioteca Comunitária UFSCar
Processamento Técnico
com os dados fornecidos pelo(a) autor(a)

Miranda, Elder Assis
M672h História natural, biogeografia e a genética de populações de Partamona rustica, uma abelha endêmica de florestas secas do Brasil / Elder Assis Miranda. -- São Carlos : UFSCar, 2016.
116 p.

Tese (Doutorado) -- Universidade Federal de São Carlos, 2016.

1. Abelhas sem ferrão. 2. Filogeografia. 3. Florestas secas. 4. Genética de populações. 5. Genética familiar. I. Título.

UNIVERSIDADE FEDERAL DE SÃO CARLOS

Centro de Ciências Biológicas e da Saúde
Programa de Pós-Graduação em Genética Evolutiva e Biologia Molecular

Folha de Aprovação

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"Tudo tem o seu tempo determinado, e há tempo para todo o propósito debaixo do céu." Eclesiastes 3:1

Dedico este trabalho aos meus pais, Emília e Elezenisio, aos meus irmãos, Elton e Emile, e a minha linda esposa, Jamile. A eles, pelo amor, atenção, apoio e paciência.

AGRADECIMENTOS

Ao Deus designer da vida, único, vivo e eterno, pela graça de poder concluir mais este ideal.

À minha esposa, Jamile Miranda, pelo companheirismo, por ser meu porto seguro, pelo apoio incondicional durante toda minha carreira profissional, pelas renúncias em prol dos nossos sonhos e pelas palavras certas nos momentos difíceis.

À minha família, minha mãe Emília, meu pai Elezenisio e aos meus irmãos, Elton e Émile, por terem me dado o apoio e o suporte necessários para chegar até aqui – Muito obrigado!

Ao meu orientador, Dr. Marco Antonio Del Lama, pelos muitos ensinamentos, pela amizade, pela enorme paciência, pelos muitos conselhos e pela presença e atenção, constantes, durante estes quatro anos – Muito obrigado, Professor Marco, "vamos em frente, sempre e com sangue nos olhos"!

Ao programa de Pós-graduação em Genética Evolutiva e Biologia Molecular e à FAPESP, pela bolsa de Doutorado (Processo 2012/23342-1).

Ao Amigo e parceiro de trabalhos, Antônio Carvalho, pelo apoio, amizade, pelas discussões sobre a filogeografia e pelos bons momentos de "resenhas" que tornaram os dias mais alegres. Muito obrigado Tony, "tudo nosso"!

Aos colegas do Laboratório de Genética Evolutiva de Himenópteros (LGEH), especialmente Diego Moure, Pedro Cardoso, Tecavita Cardoso, Diana Machado, Camila Sabadini, Mariana Dessi, Antônio Bragato, Natália Cerântola, Gabriele Freiria, Marcella Peixoto, Simone Poppi, Paula Goria, Kátia Ferreira e Isabel Godoy; aos colegas do PPGEV, Carolina Machado, Bruno Saranholi e Manolo Perez, e aos funcionários da UFSCar, Gallo e Ivanildes Menezes.

Aos parceiros de trabalhos, Henrique Batalha, Carlos Congrains, Aline Cândida e Cláudia Inês, pelas discussões e participação em trabalhos desta tese.

Ao ICMBio, pela licença de coleta. A todos que me ajudaram nas muitas expedições de coleta pelo Brasil, em especial ao meu pai Elezenísio Miranda e aos amigos Bruno Santos, Tiago Cunha, Sr. Eduardo e Raimundo Teodoro (Milagres-BA), Cosme e Gisley (Iramaia-BA), Sr. Juscelino, Diego e Alex Fabio Lima de Melo (Macajubas-BA e Ruy Barbosa-BA), Francisco Matias (Contendas do Sincorá-BA), Itamar e Gilberto (Serra do Ramalho-BA), Antônio (Botuporã-BA), Fernando Mendes (Lontra-MG), Pedro Silva e Claudinei (Ituaçu-BA), Aparecido (Macaúbas-BA), Delissandro Carneiro e Alan (Tanque Novo-BA), Gilson (Boa Vista do Tupim-BA), Francisco (Cocos-BA), Davi Nogueira e Thiago Mahlmann (Manaus-AM), Leomir Campos (Cravolândia-BA), Tiago Nascimento (Apuarema-BA), Ailton (Jequié-BA), Airton Carvalho e Thiago Felipe (Mossoró - RN), Rodrigo Aranda (Campo Grande-MS), Jânio Felix, Anderson Vieira e Cláudia Inês (Fortaleza-CE), José Silva (Belterra-PA), José Carlos Gadelha e Sian Gadelha (Porto Velho-RO).

Aos meus amigos, Wellington Silva, Bruno Santos, Henrique Galdino, Marcelo Campos, Galber Brito, Marcos Canário, Filipe Sarmento, Tiago Nascimento, Gildomar Junior, Jhone Cruz, Isac dos Santos e Rafaele Cruz, que apesar da distância, sempre me deram apoio.

À Acaci Silva, minha segunda mãe, pelo apoio e intercessão, constantes, durante estes quatro anos; aos meus tios e tias, primos e primas; aos meus cunhados, Jeferson, Gisley e Geisa, aos meus sogros, Dilza e Paulino, pelo apoio.

Ao grupo “panela afinada”, pelos bons momentos juntos e pela atenção durante este tempo em São Carlos, em especial, ao casal Fernando e Débora Paulovich, Davi e

Débora Diniz, Thiago e Talita Bianchi, Paulo e Dulce, Heber e Maria Isabel, Benvindo e Maressa, e André Monteiro.

A todos os Professores e mestres que passaram pela minha vida, desde os primeiros anos, passando pelo ensino médio, pela UESB e UFSCar. Certamente, todos vocês contribuíram de forma direta para que eu chegasse até aqui.

Enfim, agradeço a todos os que de alguma forma contribuíram para que este ideal se tornasse realidade.

SUMÁRIO

RESUMO

Miranda, Elder Assis. D.S. Universidade Federal de São Carlos, São Carlos, abril de 2016. História natural, biogeografia e a genética de populações de *Partamona rustica*, uma abelha endêmica de florestas secas do Brasil**. Orientador: Dr. Marco Antonio Del Lama.**

Partamona rustica é uma abelha sem ferrão endêmica de florestas secas do Brasil. Apesar da sua importância como polinizadora e da sua localização em áreas ameaçadas, como a Caatinga e o Cerrado, pouco se sabe a respeito da sua história natural, biogeografia, genética de suas populações, bem como da sua história evolutiva. Esta tese é apresentada em quatro capítulos, os quais apresentam objetivos específicos que visam reduzir estas lacunas em nosso conhecimento acerca de *P. rustica*. No Capítulo I, buscou-se investigar alguns aspectos da história natural de *P. rustica*. Os resultados revelaram que o principal substrato de nidificação utilizado pela espécie são termiteiros da espécie *Constrictotermes cyphergaster*, que *P. rustica* é visitante floral de pelo menos 62 tipos de plantas, e se ditribui em áreas de Caatinga e Cerrado entre o norte de Minas Gerais e o sudoeste da Bahia, contornando a Chapada Diamantina. No Capítulo II foram utilizadas as espécies *P. rustica* e *Partamona helleri* como modelos para testar a hipótese de que, nas abelhas sem ferrão, a ocupação de uma determinada área é realizada por um número reduzido de fêmeas fundadoras; desta forma, cada evento de ocupação pode, eventualmente, apresentar as condições de efeito fundador. Os resultados deste estudo revelaram um ou poucos haplótipos por localidade e elevada diferenciação entre as populações analisadas, dados que suportaram a hipótese testada. Além disto, foram apontadas algumas implicações para os futuros estudos, dentre elas, a necessidade de mudança do foco atual dos estudos relacionados à estrutura genética das

populações de abelhas, os quais, via de regra, apenas estimam o nível de diferenciação interpopulacional, para estimativas das relações genéticas entre rainhas e machos que fundaram as colônias de uma dada população. No Capítulo III foi estimado o parentesco genético entre operárias de *P. rustica*, buscando avaliar o papel dos machos na dispersão e fluxo gênico entre colônias e populações. Os resultados mostraram baixos índices de parentesco genético médio entre operárias de colônias da mesma localidade e parentesco ainda menor entre operárias de colônias de diferentes localidades, sugerindo que apesar da ocupação das áreas analisadas ter se dado por poucas fêmeas, a dispersão promovida pelos machos reduz o parentesco e, consequentemente, o risco de acasalamentos endogâmicos, nocivos para os himenópteros, devidos ao sistema de determinação complementar do sexo (*csd*). No Capítulo IV foi utilizada uma abordagem integrada para reconstruir a história evolutiva de *P. rustica* num contexto espaço-temporal. Os resultados apontaram para a formação de dois grupos (grupo leste e oeste), separados pelo Vale do Rio São Francisco, que apresentaram divergência datada ao final do Pleistoceno. Os resultados ainda indicaram o grupo oeste como sendo ancestral ao grupo leste. Ademais, tanto os resultados das análises genéticas quanto da paleomodelagem são compatíveis com uma história de populações de tamanho constante. Os dados apresentados nesta tese poderão ser utilizados para nortear estratégias de conservação e manejo da espécie, bem como dos biomas Caatinga e Cerrado; ampliam o conhecimento acerca da biologia e da genética das abelhas sem ferrão, podendo ser úteis na criação racional destas abelhas e reforça-se a necessidade de novas e mais detalhadas investigações sobre as mudanças climáticas Pleistocênicas e seus efeitos na diversificação da biota endêmica das florestas secas da região Neotropical, principalmente, novos estudos envolvendo a grande diversidade de abelhas existente nesta região.

ABSTRACT

Miranda, Elder Assis. D.S. Universidade Federal de São Carlos, São Carlos, Abril 2016. Natural history, biogeography and population genetics of *Partamona rustica*, an endemic stingless bee from dry forests in Brazil**. Advisor: Dr. Marco Antonio Del Lama.**

Partamona rustica is a stingless bee endemic to dry forests in Brazil. Despite its importance as a pollinator and its occurrence in endangered areas, such as the *Caatinga* (scrubland forest) and *Cerrado* (savanna) biomes, its natural history, biogeography, population genetics and evolutionary history are poorly known. This PhD Dissertation is presented in four chapters addressing specific aims to reduce the gaps in knowledge regarding *P. rustica*. In Chapter I, aspects of the natural history of this bee were investigated. The results revealed that *P. rustica* occurs from the northern portion of the state of Minas Gerais to the southern portion of the state of Bahia in the *Caatinga* and *Cerrado* biomes, contouring the plateaus and hills of *Chapada Diamantina* by the east. It is a floral visitor of at least 62 kinds of plant and its nests are built in arboreal termite nests constructed by *Constrictotermes cyphergaster.* In Chapter II, the hypothesis was tested that populations of stingless bees, such as *P. rustica* and *Partamona helleri*, have a high degree of differentiation when analyzed for mitochondrial genes due to the fact that the occupation of a site is performed by a small number of founding females. Therefore, each occupation event may exhibit a founder effect. The results revealed few haplotypes per population as well as a high degree of genetic differentiation, which supports the hypothesis. Implications for future studies were also indicated, such as the need to change the current focus of studies related to the genetic structure of bee populations, which usually estimate the degree of inter-population differentiation, to

estimates of genetic relatedness among queens, workers and males who founded the colonies of a given population. In Chapter III, the genetic relatedness among workers of *P. rustica* was estimated to evaluate the role of males in dispersal and gene flow among colonies and populations. The results revealed a low degree of average genetic relatedness among workers of colonies from the same locality and even lower relatedness among workers of colonies from different localities, suggesting that, although the areas analyzed were occupied by few females, dispersal promoted by males reduces relatedness and, consequently, the risk of inbreeding, which is harmful to hymenopterans due to their complementary sex determination system (locus *csd*). In Chapter IV, we used an integrative approach to reconstruct the evolutionary history of *P. rustica* in a spatiotemporal framework. The results identified two groups: one to the west and the other to the east of the São Francisco River Valley, which putatively diverged during the late Pleistocene, and indicated the western group as ancestral to the eastern group. The results also indicate that the inferences from both the analysis of genetic data and spatial distribution modelling are compatible with a history of populations of a constant size. The data presented in this thesis can be used to guide conservation and management strategies concerning the species as well as the *Caatinga* and *Cerrado* biomes, broaden knowledge on the biology and genetics of stingless bees, which can be useful in beekeeping and bee management, as well as reinforce the need for more detailed investigations into Pleistocene climate changes and the effects on the diversification of the endemic biota in Neotropical dry forests, especially studies involving the considerable diversity of bees in this region.

INTRODUÇÃO GERAL

INTRODUÇÃO GERAL

A abelha sem ferrão *Partamona rustica* ocorre em florestas secas do Brasil, distribuindo-se em regiões de campos Cerrado no norte de Minas Gerais e áreas de Caatinga no sudoeste da Bahia (Camargo e Pedro, 2003) – biomas ameaçados e alvos de fortes pressões antrópicas (Leal *et al*. 2005; Myers *et al*. 2000). Essa abelha faz parte do clado cupira e possui o hábito de nidificar em termiteiros arborícolas externos da espécie *Constrictotermes cyphergaster* (Camargo e Pedro, 2003; Miranda *et al*., 2015). Como outras abelhas, apresenta importante papel como polinizador, sendo visitante floral de, pelo menos, 62 diferentes espécies de plantas (Miranda *et al*., 2015). Apesar da ocorrência restrita em dois biomas muito ameaçados e da sua importância como polinizador, pouco se conhece sobre a sua história natural, biogeografia, aspectos ecológicos, genética de suas populações, bem como a sua história evolutiva.

Esta tese é apresentada em quatro capítulos, os quais apresentam objetivos específicos que visam reduzir estas lacunas em nosso conhecimento acerca de *P. rustica.* Estes capítulos são apresentados em forma de artigos, em que a estrutura de cada um segue as normas pré-estabelecidas pelas revistas nas quais o capítulo se encontra publicado ou será submetido à publicação. A seguir, discutiremos, de forma resumida, as ideias de cada capítulo.

Capítulo I - Natural history and biogeography of *Partamona rustica,* an endemic bee in dry forests of Brazil

Neste capítulo, são investigados alguns aspectos da história natural de *P. rustica*, tais como as plantas visitadas por ela, substratos onde esta abelha constrói seus ninhos e possíveis predadores. Ademais, são investigados alguns aspectos biogeográficos, como

a distribuição geográfica, elevação e clima das áreas ocupadas por esta abelha. Finalmente, frente às ameaças iminentes, é apontada a importância de se concentrar esforços de conservação das áreas de florestas secas do Brasil, bem como das espécies endêmicas destas áreas. Este manuscrito se encontra publicado na revista *Insectes Sociaux* (doi:10.1007/s00040-015-0400-z).

Capítulo II - Fine scale analysis of the genetic structure of stingless bee populations assessed by mtDNA using species of *Partamona* as models

Neste capítulo, é testada a hipótese de que, nas abelhas sem ferrão, a ocupação de uma determinada área é realizada por um número reduzido de fêmeas fundadoras; desta forma, cada evento de ocupação pode, eventualmente, apresentar as condições de efeito fundador. Para testar esta hipótese, foram utilizadas populações de *P. rustica* como modelo para discutir a ocupação de áreas naturais restritas por fêmeas destas abelhas. Além disto, populações da espécie *Partamona helleri* também foram utilizadas para avaliar como ocorre o processo de ocupação de áreas naturais e antropizadas, por fêmeas destas abelhas. Neste manuscrito, são apontadas ainda algumas implicações da hipótese testada para os futuros estudos com abelha sem ferrão.

Capítulo III - Parentesco genético, estrutura sociogenética e das populações de *Partamona rustica*, acessados por marcadores SSR: implicações frente ao sistema *csd*

Este capítulo é complementar ao anterior, pois busca verificar algumas das implicações propostas pela hipótese de ocupação de áreas por *Partamona*. Nesse sentido, foi estimado o parentesco genético entre operárias de *P. rustica* da mesma colônia, buscando avaliar a estrutura sociogenética na espécie; bem como o parentesco entre operárias de diferentes colônias da mesma localidade e entre operárias de colônias

de localidades distintas, visando avaliar o papel dos machos na promoção do fluxo gênico, tendo em vista as implicações do processo de ocupação de áreas por fêmeas da espécie frente ao sistema *csd*. Para tanto, foram utilizadas como modelo colônias de localidades que foram ocupadas por uma ou poucas fêmeas (que apresentaram apenas um haplótipo), e uma localidade na qual foi observado o maior número de haplótipos (como controle). As análises possibilitaram inferir sobre a estrutura sociogenética e o papel dispersor dos machos para evitar endogamia, reduzindo as chances de homozigose no loco sexual.

Capítulo IV - Phylogeography of *Partamona rustica* (Hymenoptera, Apidae), an endemic stingless bee from the Neotropical dry forest diagonal

Neste capítulo, foi utilizada uma abordagem integrada para reconstruir a história evolutiva de *P. rustica* num contexto espaço-temporal, investigando a diversidade e a estrutura genética de suas populações e o papel das mudanças climáticas Pleistocênicas sobre a demografia histórica de suas populações. Ademais, buscou-se identificar áreas de refúgio específicas para a espécie durante o Pleistoceno tardio e foi aplicada *Approximate Bayesian Computation* (ABC) para testar possíveis cenários de origem e dispersão das populações a partir da sua atual distribuição geográfica.

Capítulo I

Natural history and biogeography of *Partamona rustica,* an endemic bee in dry forests of Brazil

Artigo publicado no periódico *Insectes Sociaux*

(doi:10.1007/s00040-015-0400-z)

Natural history and biogeography of *Partamona rustica,* an endemic bee in dry forests of Brazil

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Abstract

Partamona rustica is a stingless bee that occupies dry forests of Brazil, such as the *cerrado* (savanna-like biome) and *caatinga* (xeric shrubland and thorn forest), ranging from the northern portion of the state of Minas Gerais to the southwestern portion of the Bahia state. As this bee is endemic to these environments and its biology is poorly known, the aim of the present study was to investigate aspects of its natural history and biogeography. Moreover, the potential distribution of the species is analyzed through ecological niche modeling. Sampling was performed in the full range of distribution of the species. Nesting sites for 145 colonies, plants visited, and termite nests hosts were identified. Most of the termite nests in which the species nested were active (83%) and

corresponded to *Constrictotermes cyphergaster*. Pollen analyses revealed 62 pollen types from 30 families of angiosperms visited by *P. rustica*, the most common of which were from the families Leguminosae-Mimosoideae (11.1%) and Myrtaceae (7.9%). *Partamona rustica* occurs in areas at 500 ± 187.30 m above sea level with semi-arid climates. The climatic niche model revealed a very realistic range for *P. rustica*, contouring the *Chapada Diamantina* National Park by the east, where there are no records of the species. Given the ecological importance of bees as pollinators, the intense pressure exerted by bee hunters and environmental changes caused by human activities, the present findings underscore the importance of concentrating efforts on the conservation of dry areas and the endemic species that inhabit these forests.

Keywords: Conservation, *Constrictotermes cyphergaster,* ecological niche modeling, nesting sites, pollen analysis, stingless bee.

Introduction

Brazil has a considerable diversity of bees, with 1678 species distributed among the families Apidae (n = 913), Halictidae (n = 251), Colletidae (n = 104), Andrenidae (n = 82), and Megachilidae (n = 328) (Silveira *et al*. 2002). The stingless bees are an important group, with 417 species considered valid in the Neotropical region (Camargo and Pedro 2013).

Stingless bees of the genus *Partamona* Schwarz, 1939 have a broad geographical distribution, occurring from southern Mexico to southern Brazil (Camargo and Pedro 2003). *Partamona rustica* Camargo and Pedro 2003 is an endemic bee to the Brazilian biomes known as the *cerrado* (savanna) and *caatinga* (xeric shrubland and thorn forest), ranging from northern portion of the state of Minas Gerais to the southwestern portion of the state of Bahia and nesting in active arboreal termite nests (Camargo and Pedro

2003). The lack of studies on nesting sites, plants visited, natural enemies, and environmental constraints on its geographic distribution means that little is known of the natural history of *P. rustica*.

Bees play an important role as pollinators and are therefore key elements in the maintenance and conservation of natural ecosystems (Slaa *et al*. 2006; Ollerton *et al.* 2011; Wratten *et al.* 2012). Studies on plants and animals in dry forests in Brazil have shown that these environments harbor high levels of endemism, species richness and insect-plant interactions (Klink and Machado 2005; Zanella and Martins 2008; Giannini *et al.* 2013). Therefore, investigations involving organisms in these forests are important, especially due to the fact that such organisms have been largely excluded from discussions on conservation (Klink and Machado 2005).

The *caatinga* biome is a highly exploited, modified environment that accounts for nearly 11% of Brazil, with only 53% of its original area intact (MMA 2014; Giulietti *et al*. 2006; Castelletti *et al*. 2003). The *cerrado* biome accounts for more than 20% of country, with only 20% of its original area intact. These changes are associated with agricultural needs, which lead to deforestation and pose serious threats to biodiversity (Myers *et al*. 2000).

Knowledge on the geographical distribution of a given species is an initial step to the establishment of conservation policies. Given the difficulty in determining a realistic distribution range for any organism, ecological niche modeling constitutes a viable alternative for the construction of potential distribution maps. The aim of this method is to determine non-randomized relationships among sampling points and bioclimatic data by building models that depict a potential geographic distribution with the greatest likelihood of finding favorable environmental conditions for a species to occur (Corsi *et al*. 1999; Peterson *et al*. 2011; Svenning *et al*. 2011).

Habitat fragmentation, competition for nesting sites due to deforestation**,** and predation by bee hunters are serious pressures imposed on stingless bees, which could result in the decline or also extinction of populations of these bees (Klein *et al.* 2007; Knight *et al.* 2009). Information on the natural history and biogeographic aspects of a given species provide the basis for studies on the interactions between bees and plants and assist in the establishment of management and conservation policies (Silva *et al*. 2010; Giannini *et al*. 2013).

The aim of the present study was to investigate aspects of the natural history and biogeography of the stingless bee *P. rustica* and establish the potential geographic distribution of this species using ecological niche modeling.

Materials and Methods

Study area and sampling efforts

Adult *P. rustica* were sampled between May 2012 and January 2014. Thirty-two sites were visited in southwestern Bahia state and northern Minas Gerais state. *Partamona rustica* was found at 11 of these sites (1 to 11 in Table 1 and in Figure 1), totalizing 145 nests (mean = 13 per site). However, no nests were found at 21 sites (12 to 21, red triangles in Figure 1): Santo Estevão, Itatim, Itaberaba, Amargosa, Brejões, Cravolândia, Irajuba, Maracás, Jequié, Barra da Estiva, Mucugê, Andaraí, Anagé, Vitória da Conquista, Bom Jesus da Lapa, Serra do Ramalho, Carinhanha, Guanambi (in Bahia), Espinosa, Janaúba, Januária (in Minas Gerais).

Table 1. Sites at which *Partamona rustica* nests were sampled.

	Localities	Abbreviation	S	W	Altitude	N
1	Ruy Barbosa-BA	RBA	-12.2279	-40.3109	314	12
2	Boa Vista do Tupim-BA	BVT	-12.5021	-40.4709	328	14
3	Milagres-BA	MIL	-12.9311	-39.7197	365	20
4	Iramaia-BA	IRA	-13.8617	-40.0814	392	15
5	Manoel Vitorino-BA	MVT	-13.9393	-40.5634	256	16
6	Contendas do Sincorá-BA	CSI	-13.7937	-41.0165	300	12
7	Ituaçu-BA	ITU	-13.8823	-41.3246	586	11
8	Tanque Novo-BA	TNO	-13.5949	-42.5211	900	14
9	Macaúbas-BA	MAC	-12.243	-40.2499	756	11
10	Cocos-BA	COC	-14.1579	-44.4031	587	10
11	Conego Marinho-MG	CMA	-15.3143	-44.3819	632	10
Total						145

N = number of nests sampled

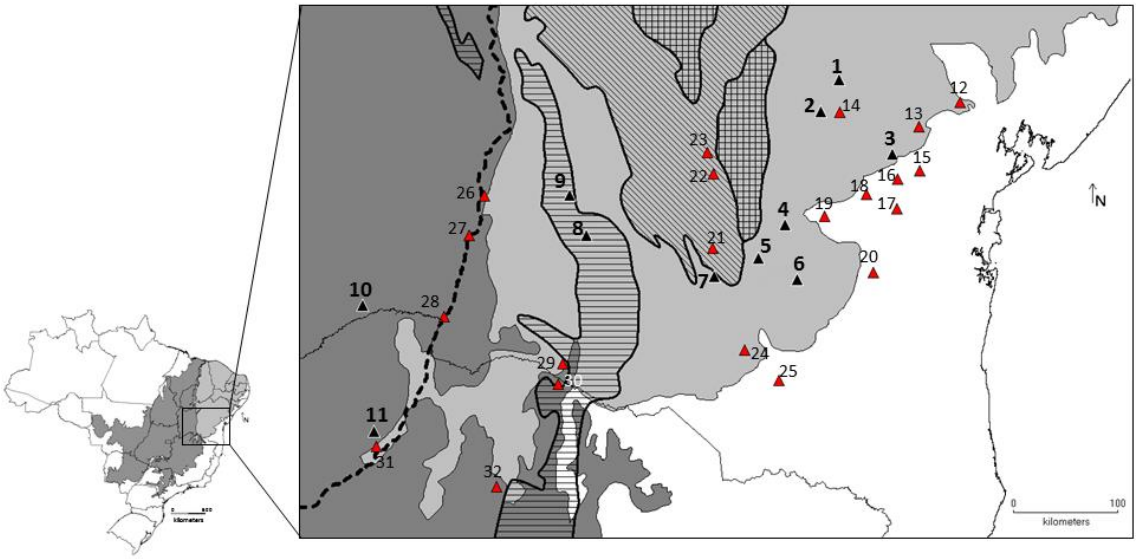

Figure 1. Map illustrating sites visited during field expeditions. Black triangles represent 11 sites with active *P. rustica* colonies (Table 1). Red triangles (12 to 32) refer to sites where the species was not found. Plateaus and hills of *Chapada Diamantina* are represented by diagonal lines. *Serra do Espinhaço* is represented by horizontal lines. *Chapada do Rio Irecê* and *Utinga* is in grid shape. São Francisco River is indicated by the dotted line.

Data collection

Nests were located through active searches of at least 16 hours per site, in order to standardize sampling efforts. The nests were photographed, geo-located, and measured and the site was characterized (identification of the substrate in which the termites were found). Five workers were collected from each nest and kept in dry conditions for identification, and 15–20 workers were preserved in 70% alcohol for pollen analysis. Fifteen termites were also sampled and preserved in 70% alcohol for taxonomic identification.

In addition, 37 farmers, beekeepers or bee hunters from 27 sites were informally interviewed to determine their knowledge on the occurrence, exploration, and conservation of *P. rustica*, based on three questions: i) “Do you know the "cupinheira" stingless bee (*Partamona rustica*)?”; ii) “Have you ever used or removed honey, wax or pollen from the nests of this bee?” ii) “Have you seen this bee nests recently?” The answers were recorded and tabulated.

Pollen analysis

Five *P. rustica* nests were selected at random from each of the 11 sites sampled. From each nest, the pollen load of 15-20 adult workers (about 825 workers in all) was extracted for analysis. In the laboratory, the workers were placed in test tubes, which were shaken gently. The workers were then removed and the remaining material was centrifuged for 15 minutes at 2000 rpm (Silva *et al*. 2014). The alcohol was discarded and glacial acetic acid (4 mL) was added to the pellet. The material was acetolyzed (Erdtman 1960) and placed on slides (two per sample) prepared with Kisser gelatin. Pollen types were analyzed by comparisons to material in the collection of the Palynology Laboratory of the Biology Department of the Faculdade de Filosofia,

Ciências e Letras de Ribeirão Preto of Universidade de São Paulo (FFCLRP - USP), and the specialized literature (Silva *et al*. 2010; Silva *et al*. 2014; Bauermann *et al*. 2013). Voucher specimens were deposited in the aforementioned collection.

Pollen analyses were conducted with the aid of a binocular microscope. Digital pictures of the pollen grains were obtained using a Leica DFC280 photodocumentation system. Quantitative analyses were conducted to evaluate the richness of the first 400 pollen grains of each sample (Montero and Tormo 1990). Shannon-Wiener diversity (H') (Shannon and Weaver 1949) and uniformity per site (J') (Pielou 1966) were estimated using the PAST program (Hammer *et al.* 2001), to evaluate the trophic niche of *P*. *rustica*.

Ecological niche modeling

A potential distribution model for *P*. *rustica* was constructed using the maximum entropy algorithm implemented in MaxEnt3.3.3k (Phillips *et al*. 2006; Phillips and Dudík 2008). The model was obtained after different tests using 21 occurrence records and 19 bioclimatic variables obtained from Worldclim (http://www.worldclim.org). Two important databases on the occurrence of Meliponini in Brazil were consulted to compile the complete records of occurrence of *P*. *rustica*. One was the Moure Collection of the Federal University of Paraná and the other was the Camargo Collection of FFCLRP - USP. Ten sites at which *P*. *rustica* was previously sampled were identified (Table S1). For samples on which no coordinates were available, geo-location was performed based on the sample labels using Google Earth. The occurrence data for the species was supplemented with the 11 sites at which active nests were found in the present study (Table 1).

Ecological niche modeling was conducted with grids edited in DIVA-GIS 7.5.0.0 (http://www.diva-gis.org/) containing the minimum area for the known range of occurrence of the species to prevent the effect of possible bias in the output of the model resulting from background extension and niche expansion (Anderson and Raza 2010). The potential distribution map was edited in Arc-Gis 10.1.

Area Under the Curve (AUC) values higher than 0.9 are considered reliable in recent analyses (Giannini *et al*. 2012; Giannini *et al*. 2013). However, alternative analyses may be more efficient when calibrating and improving niche models. Such analyses can be carried out using different regularization multiplier values ranging from 0.5, 1, 1.5, … to 4, with two sets of MaxEnt features (quadratic + product + threshold + hinge and quadratic + product + hinge), totaling 16 calibrations. These combinations were employed in the present study based on the amount of sampling sites for *P. rustica*. It is expected that such combinations of features generate different AUC values and the 10 percentile training omission ($OR_{10\%}$). The latter parameter indicates the likelihood of finding favorable conditions for the species to occur when using the lowest value attributed to any of the 90% occurrence records with the highest scores. In summary, the most reliable potential distribution model for the species is expected to present a high AUC value and the lowest $OR_{10\%}$ value. Moreover, 30% of the occurrence sites (n=6) were randomly chosen to test the predictability of the models for determining non-sampled areas where the species could occur (Rosauer *et al*. 2009).

Results and Discussion

Characterization of nests and sites used by *P. rustica*

The entrances of the *P. rustica* nests were located mostly in the lower-middle portion of the termite nests. The conical shape of the entrances is simple compared to

other species of this genus, such as *Partamona ailyae* Camargo (1980), *Partamona helleri* Friese (1900) and *Partamona gregaria* Pedro and Camargo (2003) (Camargo and Pedro 2003), measuring 3.0 cm in entrance, built with mud and slightly detached from the surface of the substrate. Unlike other sites, in which the mud of the entrances had the same color as the nests, the nests at Manoel Vitorino had reddish mud at the entrances (Figures 2A and 2B).We also observed color variation in the wax envelope that ranged from reddish in Tanque Novo and Cocos to brown in Iramaia, Ruy Barbosa, Macaúbas and Boa Vista do Tupim (Figures 2C and 2D).

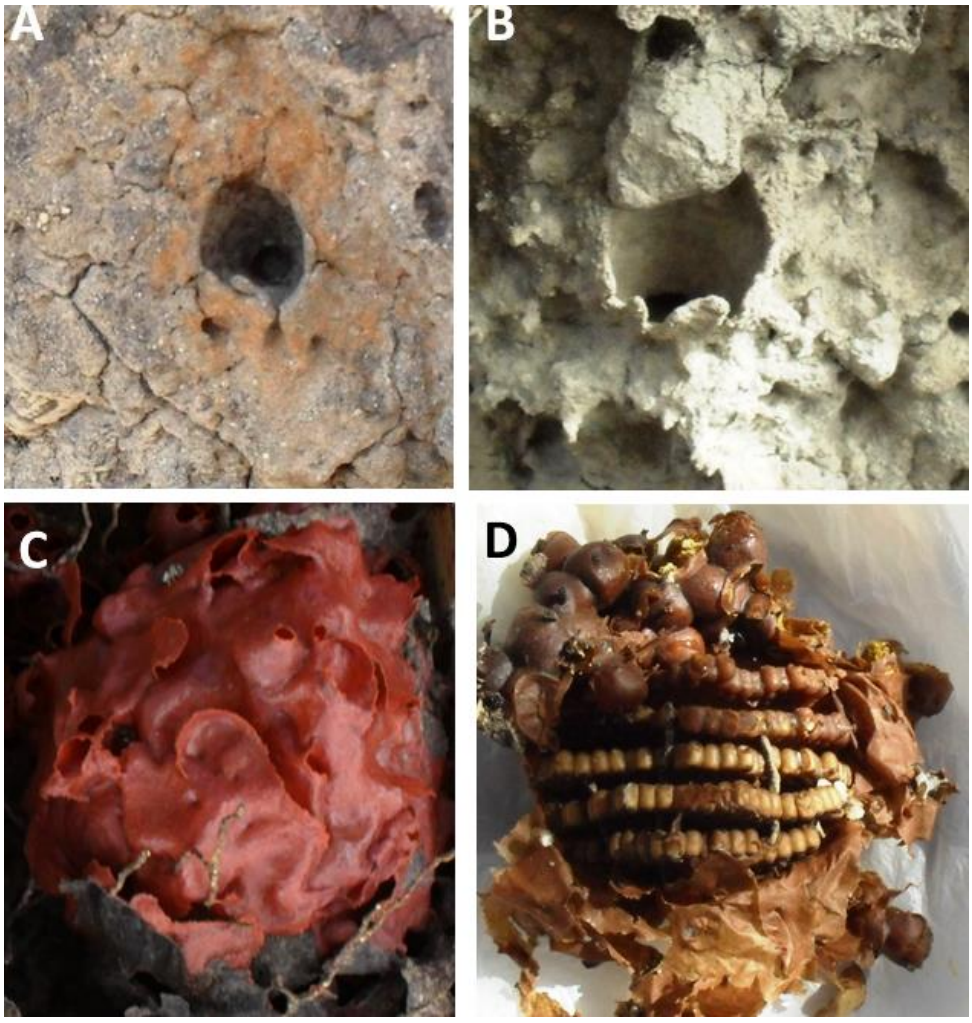

Figure 2. Different coloration recorded for nest entrances (A and B) and envelopes (C and D) of *P. rustica.*

Most termite nests with colonies of *P. rustica* were active (83%; n = 144). One colony was found in a crack in the wall of a house made of mud in Macaúbas, Bahia. This demonstrates that *P. rustica* builds nests preferentially in active arboreal termite nests, but not exclusively.

Nearly all colonies of this stingless bee were associated with *Constrictotermes cyphergaster* Silvestri (Isoptera, Termitidae) (90%; n=144). Only two colonies were associated with nests of *Nasutitermes* spp. (Ruy Barbosa and Boa Vista do Tupim). The genus *Constrictotermes* is endemic to South America, with five known species, most of which build arboreal nests (Godinho *et al*. 1989; Melo and Bandeira 2004). *C. cyphergaster* is commonly found in the central savanna of Brazil as well as Paraguay, Bolivia and northern Argentina, but can also be found in the Atlantic Forest (Araújo 1970; Constantino 1998). Studies report the occurrence of *C. cyphergaster* in the *caatinga*, which is the main species of termite that builds conspicuous nests in this biome (Godinho *et al*. 1989; Melo and Bandeira 2004). Lorenzon *et al*. (1999) report the association of the bee genus *Partamona* with this species of termite.

The present data suggest a strong preference by *P. rustica* to build nests in the nests of *C. cyphergaster*, although nests of genus *Nasutitermes* were found at all the sites analyzed. Studying the nesting ecology of *P. rustica* and *P. cupira* in Milagres (state of Bahia, Brazil), Barreto and Castro (2007) also found this preference, despite the presence of other arboreal termite nests in the area, as 90% (n = 28) of the stingless bee colonies were built in nests of *C. cyphergaster*. In this study, only one nest of *P. rustica* was associated with another termite species (*Nasutitermes corniger* Motschulsky).Termite nests hosting colonies of *P. rustica* had a mean length and circumference of 71.22 ± 13.17 cm and 139.95 ± 25.26 cm, respectively. Barreto and

Castro (2007) report a similar mean length (63.45 ± 24.04 cm; n = 25) in the nests of *C. cyphergaster* hosting colonies of *P. rustica* and *P. cupira*.

Termite nests were mostly attached to *umburana* (*Commiphora leptophloeos* Mart. J. B. Gillett) and *umbuzeiro* (*Spondias tuberosa* Arruda) trees, with 44 and 13 records, respectively. Barreto and Castro (2007) reported the same preference. According to the authors cited, species of the genus *Partamona* build nests in preexisting cavities made by the cactus parakeet (*Aratinga cactorum* Kuhl, 1820) in the *caatinga*. However, although termite nests with such hollows were found during the field trips in the present study, no nesting activity of *P. rustica* in cavities made by this bird was observed.

Trophic resources and plants visited by *P. rustica*

The pollen analysis revealed 62 types (Table S2), with 17.0 ± 6.0 pollen types per site. Pollen from *Anacardium sp.* was found at 10 of the 11 sampled sites, while pollen from *Senna sp.*, *Mimosa tenuiflora* (Wild.) Poir. and *Syagrus* sp. were found at 9, 8 and 8 sites, respectively (Table 2). These results may be attributed to the fact that these plants are found throughout the *caatinga* biome and some flower both in dry and rainy periods (Maia-Silva *et al*. 2012).

Table 2. Number of pollen types collected by *Partamona rustica* per site, pollen diversity (H'- Shannon index) and pollen uniformity (J'). The results were obtained based on the analyses of pollen collected from workers at 11 sites in the states of Bahia and Minas Gerais. The sites are described in Table 1.

Localities	**BVT**	**COC**	**CMA**	**CSI**	**IRA**	**ITU**	**MAC**	**MVT**	**MIL**	**RBA**	**TNO**
Number of pollen types	19	16	28	14	8	7	11	15	15	27	27
H´	1.089	2.058	2.731	1.094	1.018	1.123	1.835	1.743	2.174	2.304	2.411
J´	0.376	0.780	0.838	0.426	0.522	0.577	0.835	0.679	0.823	0.707	0.758

Although flowers of the genus *Senna* have poricidal anthers, it is common to find pollen from species of this genus among the material collected by stingless bees that do not vibrate, as observed for *Scaptotrigona depilis* Moure (1942) and *S.* aff. *depilis* (Ferreira *et al.* 2010; Faria *et al.* 2012). Novais *et al.* (2013) also observed pollen from *Senna* in pots and honey of *Tetragonisca angustula* from *caatinga* sites, reinforcing the importance of these plants species in the diet of this bees. They obtain pollen by plundering material that falls on the petals after vibrations made by other bees and by accessing the resource through openings made by species of *Trigona* on the anthers of flowers. The same occurs with other poricidal anther plant species of the genera *Solanum, Tibouchina* and *Miconia* (Marques-Souza *et al.* 2007; Ferreira *et al.* 2010; Faria *et al.* 2012).

The greatest pollen diversity was found in colonies from Conego Marinho (CMA), Tanque Novo and Ruy Barbosa (TNO and RBA, respectively) (H'_{CMA} = 2.73; H'_{TNO} = 2.41 and H'_{RBA} = 2.30). The lowest degree of pollen diversity was found in colonies from Iramaia and Ituaçu (IRA and ITU) (H'_{IRA}= 1.02 and H'_{ITU} = 1.12) (Table 2). These findings may be explained by the fact that surveying in CMA, TNO and RBA was conducted in the rainy season and these regions are composed of different ecotones, such as transitional areas of the *caatinga*, *cerrado* and seasonal semi-deciduous forests. On the other hand, surveying was conducted in the dry season at IRA and ITU, when few plant species flower.

Different degrees of pollen diversity were found in samples from CMA, Macaúbas (MAC) and Milagres (MIL) (Table 2). However, similar uniformity was found at these sites (J' = 0.838; J' = 0.835 and J' = 0.823, respectively). This indicates that colonies of *P. rustica* from these sites did not exhibit selectivity for a given source

of pollen, i.e., there was no dominant pollen type (when a pollen type represents more than 45% of total). Dominance was only recorded at IRA (*Anacardium sp*., 81.3%), ITU (*Pityrocarpa moniliformis* (Benth.) Luckow and Jobson, 60.7%), Boa Vista do Tupim (BVT; *Myrcia* sp., 67.5%) and Contendas do Sincorá (CSI; *Mimosa caesalpiniaefolia* Benth (69.3%) (Table S2). Among these four sites, surveying was conducted mostly in the dry period. At CSI, surveying was conducted in the rainy season, when *M. caesalpiniaefolia* flowers (Maia-Silva *et al*. 2012). It is therefore evident that *P. rustica* is a generalist regarding food sources, as described for most stingless bees studied (Ramalho *et al.* 1990; Marques-Souza *et al.* 2007; Ferreira *et al.* 2010; Faria *et al.* 2012; Aleixo *et al.* 2013).

Factors such as nest proximity, high protein content and the dominance of flowering plant species may explain the high frequency of plants visited by bees (Kleinert *et al*. 2009). Antonini *et al*. (2006) found that *Melipona quadrifasciata* Lepeletier (1836) visited only 19.3% of flowering plants in its habitat. Despite the generalist classification (Faria *et al*. 2012; Aleixo *et al*. 2013), this finding may be attributed to the fact that these bees concentrate foraging activities in plants close to the nests that offer greater yields, as predicted by the theory of optimal foraging (MacArthur and Pianka 1966).

The pollen analysis revealed that *P. rustica* visited 30 families of plants (Table S2). The most visited families were Leguminosae-Mimosoideae (11.1%) and Myrtaceae (7.9%), which were present at all sites. Previous studies also describe these plant families as the most visited by bees from the tribes Meliponini and Apini (Cortopassi-Laurino and Ramalho 1988; Imperatriz-Fonseca *et al*. 1989; Ramalho *et al*. 1990; Faria *et al*. 2012; Aleixo *et al*. 2013). According to Ramalho *et al*. (1990), social bees are expected to visit species of Leguminosae, Malvaceae and Myrtaceae more frequently.

Moreover, species of family Myrtaceae have flowers with many stamens and longitudinally opened anthers, which facilitates the acquisition of pollen (Ferreira *et al*. 2010).

Information on plant species that bees visit allows the determination of niche overlap and bee-plant interactions, which can contribute to the drafting of management and conservation policies (Eltz *et al*. 2001; Silva *et al*. 2010). Thus, analyses were performed to determine the most common pollen sources for *P*. *rustica* at all sites.

Biogeographical aspects and potential distribution of *P. rustica*

Partamona rustica was found at a mean altitude of 500 ± 187.30 m. The highest altitude was recorded at TNO (900 m) on the Espinhaço hills and the lowest was recorded at Manoel Vitorino (256 m), both in the state of Bahia (Table 1).The species apparently occurs in semi-arid environments.

The calibration of the model indicated that the QPTH-1 (1 refers to the regularization multiplier) set of features is the ideal combination for predicting potential areas with favorable conditions for *P*. *rustica* (AUC = 0.949; $OR_{10\%}$ = 0.493) (Figure S1). This set of features was used to construct the potential distribution map for the species (Figure 3). Suitable areas for the occurrence of *P*. *rustica* were identified in the *Cerrado* biome of northwestern Minas Gerais to the *caatinga* biome in southwestern Bahia, contouring the plateaus and hills of the Chapada Diamantina and Chapada do Rio Irecê and Rio Utinga by the east (Figure 3). Indeed, there are no records of the species in these areas in museum collections and no specimens were found during surveys conducted in Mucugê, Andaraí and Barra da Estiva (state of Bahia) between December 2012 and January 2013.

The data indicated that the environmental conditions, such as low temperatures in winter and rock vegetation in the Chapada Diamantina National Park, constrain the occurrence of *P. rustica*. Moreover, the termite mounds in which *P. rustica* nests have a low potential of occurrence in this region (Schmidt 2007). These data corroborate the niche modeling results for *P. rustica* and may explain the absence of the species in such locations.

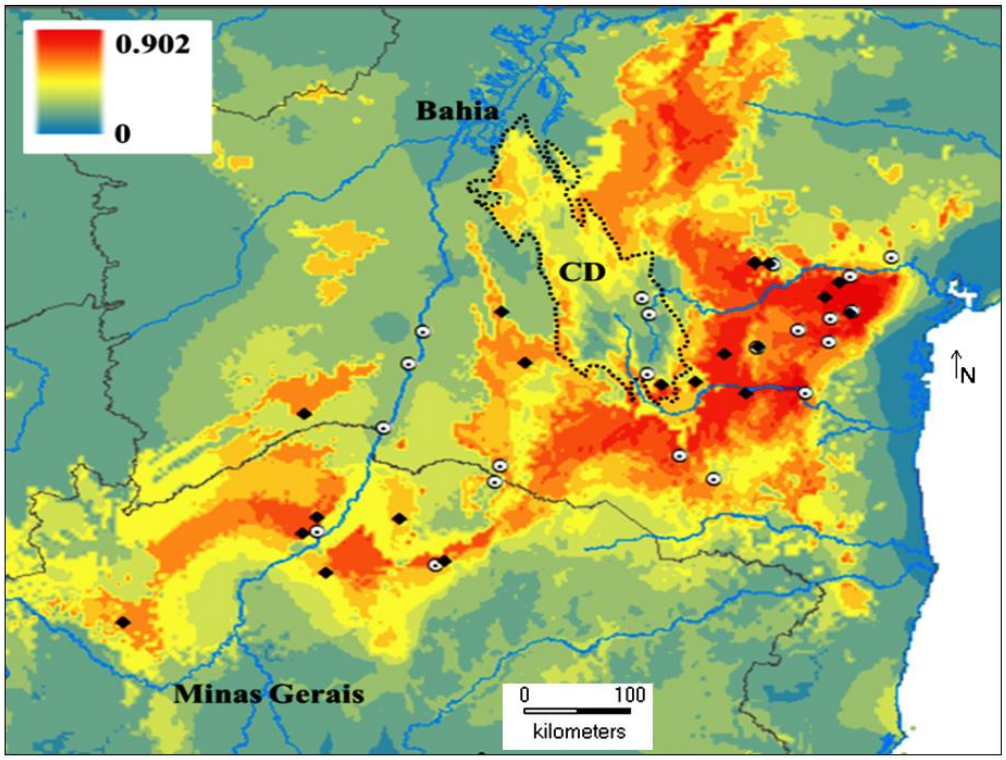

Figure 3. Potential geographic distribution of *P. rustica*. *Black diamonds* represent the 21 records for the species used to build the potential distribution map through niche modeling. White circles with a black point indicate sites at which no *P. rustica* colonies were found. *Legend* refers to the likelihood of finding suitable conditions for the species to occur. *Chapada Diamantina* (CD) is shown as an area of low potential for the occurrence of the species.

According to Pedro and Camargo (2003), *P. rustica* is endemic from northern Minas Gerais to Espinhaço hills in Maracás in the state of Bahia. In the present study, however, colonies were found in Ruy Barbosa and Boa Vista do Tupim, which are 150 km to the north of Maracás, demonstrating a larger distribution range for *P. rustica.* The species has its distribution discontinued by the São Francisco River in northern Minas Gerais (Figure 1).

A case of predation, imminent dangers, and conservation

A colony of *P. rustica* was found being attacked by the ant *Camponotus rufipes* Fabricius (1775) in Boa Vista do Tupim. Besides feeding on honey and pollen, this ant also predated the larvae of the bees. According to Nogueira-Neto (1953), some ants build nests in preexisting hollows in hives, resulting in little injury to the bees. It is also possible that ants may drive off other predators, secondarily defending the bee colony (Nogueira-Neto 1953). In the present study, however, ants were only found predating the *P. rustica* colony.

The field observations suggest that bee hunters are the most serious threat to *P. rustica*. These hunters use honey, pollen and wax for commercial and pharmacological means as well as for folk remedies. Due to the honey, non-aggressive nature and the ease of locating *P. rustica* nests, the species has been intensively exploited and nests have become rare or even absent from some areas of its distribution. Indeed, no nests were found at previously sampled sites, such as Maracás, Anagé, Vitória da Conquista, Jequié, Guanambi, Amargosa, Iaçu and Itaberaba (in the state of Bahia) and Espinosa (in the state of Minas Gerais). The niche model predicted some of these areas as highly suitable for the species to occur, but the intense human pressure is likely the main cause of the current situation.

Most (70%) of the farmers, beekeepers and bee hunters interviewed, knew *P. rustica*, 88% of these interviewees had used resources obtained from this species (honey, wax or pollen), and most had seen the species rarely (58%) or had not seen (31%) this bee nests currently. Exploitation has reduced the number of swarms, and colonies have become more susceptible to natural enemies, such as birds, ants, and phorids (e.g., *Pseudohypocera* spp.). Additionally, the harmful effects of bee hunters place strong pressure on stingless bees (Klein *et al.* 2007; Knight *et al.* 2009).

The intense deforestation of the *caatinga* biome also likely exerts an influence on the decline in the population size of these bees, as reported by Neves and Castro (2006) for *Melipona mandacaia* Smith (1863). Indeed, stingless bees are becoming increasingly rare in some semi-arid regions, as deforestation diminishes the density of trees in which the species nests.

In conclusion, the intense exploitation of *P. rustica* by bee hunters and the loss of habitat due to deforestation for agricultural purposes threaten populations of this endemic species, which is restricted to dry forests in Brazil. Given the ecological value of bees and the constant pressures placed on the *caatinga* and *cerrado* biomes, the present findings underscore the importance of the conservation of these environments and the endemic species such environments harbor.

Acknowledgments

The authors are grateful to the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial support (Process numbers 2011/21501-2, 2012/23342-1 and 2011/13391-2), Dr Silvia Regina de Menezes Pedro (FFCLRP - USP) and Dr Eliana Marques Cancello (MZ-USP) for the taxonomic identification of the bees and termites, respectively, Dr Carlos Alberto Garófalo (FFCLRP-USP) for the use of the

Palynology Laboratory, and all the beekeepers and other individuals from the 32 visited sites that assisted during the surveying expeditions.

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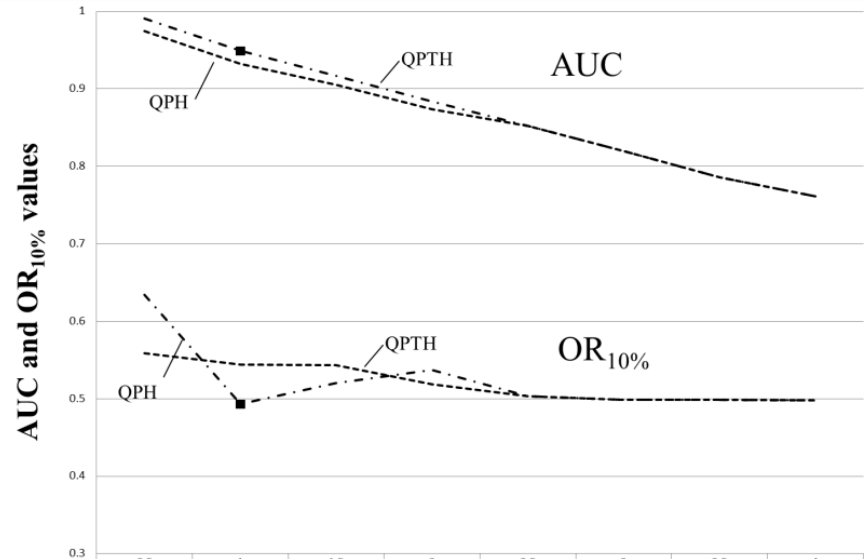
Insectes Sociaux

Supplementary material

Natural history and biogeography of *Partamona rustica,* an endemic bee in dry forests of Brazil

Miranda EA[1], Carvalho AF[1,2], Andrade-Silva ACR[3], Silva CI[4] e Del Lama MA[1]



	0.5	1	1.5	2	2.5	3	3.5	4
AUCQPTH	0.991	0.949	0.917	0.884	0.852	0.82	0.786	0.761
AUCQPH	0.975	0.932	0.905	0.874	0.852	0.82	0.786	0.761
ORQPTH	0.634	0.493	0.52	0.537	0.503	0.499	0.499	0.498
ORQPH	0.559	0.544	0.543	0.519	0.503	0.499	0.499	0.498

Figure S1. Results referring to the model calibration tests of *P. rustica*. Different parameters were tested in MaxEnt to choose the best set of features for the potential distribution model. Although the AUC value of model QPTH-0.5 is the highest (0.991), the QPTH-1 model was selected because this model had the lowest $OR_{10\%}$ value (0.493) as well as a high AUC value (0.949).

Table S1. Occurrence records obtained in collections and museums used for modeling the potential distribution of *Partamona rustica*. Geographical coordinates of these sites are indicated.

Locality	S	W
Amargosa. BA	-13.052	-39.631
Bonfinópolis de Minas. MG	-16.441	-46.110
Itaberaba. BA	-12.512	-40.351
Itamarandiba. BA	-17.932	-42.905
Itatim. BA	-12.723	-39.728
Jaíba. MG	-15.317	-43.650
Januária. MG	-15.470	-44.512
Lontra. MG	-15.897	-44.294
Maracás. BA	-13.424	-40.447
Nova Porteirinha. MG	-15.776	-43.252

Table S2. Pollen types and plant families visited by *Partamona rustica* based on analyses of material obtained from workers at 11 sites in the states of Minas Gerais and Bahia. Locality abbreviations follow Table 1.

Family	Species/Pollen types	Percentual of the pollen/localities										
		BVT	**COC**	**CMA**	**CSI**	**IRA**	**ITU**	**MAC**	**MVT**	**MIL**	**RBA**	**TNO**
Acanthaceae	*Ruellia* sp.											0.14
Amaranthaceae	*Chamissoa* sp.		0.52	0.7								
Anacardiaceae	*Anacardium* sp.	6.66	30.05	5.62	2.61	81.28		10.34	21.38	22.92	19.86	9.26
Arecaceae	*Cocus nucifera*			0.7								3.13
	Syagrus sp.		2.59		1.75	0.17	5.52	13.79	20.51	1.76	19.72	
Asteraceae	*Bidens* sp.											0.14
	Eupatorium sp.											0.14
	Vernonia sp.	0.41		2.82	1.32							
Bignoniaceae	*Handroanthus* sp.										1.56	
Bombacaceae	*Pseudobombax longiflorum*	0.82	5.18					3.45	29.91			1.23
Boraginaceae	*Cordia* sp.	0.14		17.61	1.32	0.17			0.85	7.06	3.4	
Combretaceae	*Combretum leprosum*			0.7		2.75				1.18		
Commelinaceae	*Commelina erecta*	3.14									16.17	6.13
Convolvulaceae	*Ipomoea* sp.	1.09		0.7					0.43			0.41
	Jacquemontia montana	0.14									0.14	0.14
Dilleniaceae	*Davilla* sp.				0.44							
Erythroxylaceae	*Erythroxylum* sp.		0.52	0.7			0.61					
Euphorbiaceae	*Croton* sp.				7.24		0.61		1.71	3.53	1.99	

	Joanesia sp.			4.23	12.28				1.18		
Lamiaceae	*Hyptis suaveolens*											0.27
Leg. Caesalpinioideae	*Senna* sp.	3.68	10.36	0.7	1.1			3.45	4.7	1.18	0.71	3.95
Leg.Mimosoideae	*Anadenanthera colubrina*		0.52	6.34				10.34	0.85			4.36
	Inga sp.											0.7
	Mimosa caesalpiniaefolia	0.27			69.3				6.41		0.43	2.72
	Mimosa quadrivalvis	0.14							1.28		0.14	0.14
	Mimosa tenuiflora	12.28				2.92	6.75	20.69	1.71	20	13.62	20.03
	Pityrocarpa moniliformis	0.41					60.74				4.4	
	Senegalia sp.				1.1							
Leg. Papilionoideae	*Delonix regia*			3.52								
Malvaceae	*Desmanthus* sp.					0.17					0.14	
	Machaerium sp.			11.97				3.45		3.53	0.28	1.63
	Sida cordifolia										0.28	
	Triumfetta rhomboidea		20.73	1.41				3.45				2.32
Meliaceae	*Guarea* sp.			1.41	0.22			3.45		2.94	2.13	
Myrtaceae	*Campomanesia* sp.			4.93								
	Eucalyptus sp.	0.14		2.11	0.22					3.53	0.43	3.81
	Eugenia sp.		6.2	3.52		12.37	1.84	24.14		18.24	4.11	
	Myrcia sp.	67.53			0.22				8.97		6.1	5.04
	Sysygium sp.		5.7							4.71		
Poaceae	sp1									0.59		
Polygonaceae	*Polygonum* sp.										0.85	
Rubiaceae	*Borreria verticilata*			0.7							0.14	
	Richardia grandiflora			4.23								0.27
Rutaceae	*Citrus limonea*								0.43		0.57	16.74

	Hortia sp.			0.7							
Sapindaceae	*Matayba guianensis*										1.63
	Serjania reticulata	0.27	11.92				3.45			0.28	1.09
Solanaceae	*Solanum* sp.	0.14									
Styracaceae	*Styrax camporum*		0.52	4.97							
Turneraceae	*Turnera subulata*		0.52								
Urticaceae	*Cecropia* sp.		3.63	1.41		23.93					
Indeterminate	Indet. sp1			0.7	0.17						12.13
	Indet. sp2	0.55			0.88			0.43	7.65	0.57	2.18
	Indet. sp3	0.14									
	Indet. sp4	2.05						0.43			0.27
	Indet. sp5									1.56	
	Indet. sp6									0.14	
	Indet. sp7		0.52							0.28	
	Indet. sp8			3.52							
	Indet. sp9			11.27							
	Indet. sp10			2.11							
	Indet. sp11		0.52	0.7							

Pollen class by importance: dominant (>45%), accessory (15 - 45%), reasonably important (3 - 15%), occasional (<3%).

Capítulo II

Fine scale analysis of the genetic structure of stingless bee populations assessed by mtDNA using species of *Partamona* as models

ORIGINAL PAPER

Fine scale analysis of the genetic structure of stingless bee populations assessed by mtDNA using species of *Partamona* as models

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Abstract

In this study we tested the hypothesis that populations of stingless bees, such as *Partamona rustica* and *Partamona helleri*, have a high degree of differentiation when analyzed for mitochondrial genes due to the fact that the occupation of a site is performed by a small number of founding females. Therefore, each occupation event may, eventually, exhibit a founder effect. The results showed that the populations of the two species have high haplotype and low nucleotide diversities. The haplotype network revealed few haplotypes per population as well as unique and/or private haplotypes within populations. Moreover, the populations of both species exhibited a high degree of genetic differentiation (*P. rustica* – Φ_{ST} = 0.849; P = 0.000; *P. helleri* – Φ_{ST} = 0.958; P = 0.000), which also supports the hypothesis. A positive correlation was found between the size of the sampling area and the number of haplotypes per locality (*P. rustica* – r = 0.764; P < 0.05), but not between the number of colonies and number of haplotypes per locality for either species. The common occurrence of unique and private haplotypes per site suggests that the continuity of the occupation process does not occur with the arrival of new females to these sites. Instead, the increase in the number of colonies at each site occurs basically through the reproduction of the few original colonies. Neutrality tests were non-significant for the two species, demonstrating that these results are not likely due to population bottlenecks, which lends further support to the hypothesis tested herein.

Keywords Genetic differentiation - Meliponini - Mitochondrial markers - Population genetics

Introduction

Social bees from the tribe Meliponini, known as stingless bees, are distributed throughout tropical and subtropical regions of the world, with greater diversity in Neotropical and Indo-Malayan regions (Camargo 1989). According to Camargo and Pedro (2003), 417 species have been described for the Neotropical region. More recent data indicate 244 valid species for Brazil alone, besides another 89 as-yet undescribed forms (Pedro *et al.* 2014). Stingless bees play an important ecological role as pollinators and are considered as key elements for the maintenance and conservation of natural ecosystems as well as economically important agricultural systems (Klein et al. 2007; Ollerton et al. 2011; Wratten et al. 2012). Despite such diversity and importance, little is known regarding the reproductive biology of these organisms. Understanding biological aspects can help define how these species occupy natural environments and those altered by human activities.

Stingless bees live in colonies ranging from a thousand to 180 thousand individuals (Michener 2000; Sakagami 1982; Camargo and Pedro 2003) and require specific environmental resources, such as the existence of suitable sites and materials (resins) to build nests as well as nutritional resources (pollen and nectar) (Silveira et al. 2002). Therefore, the quantity and quality of the resources offered constitute a limiting factor for the number of colonies that a given area can support, especially in the more populous colonies and populations in competition for such resources.

Colony reproduction among stingless bees occurs through swarming. The link between maternal and daughter colonies remains for some time, since the new colony depends on workers and food provided by the original colony (Nogueira-Neto 1954). Thus, reproductive females do not perform wide dispersal in the process of forming a new colony (Engels and Imperatriz-Fonseca 1990). According to Wille and Orozco (1975), new queens of the

Partamona species are philopatric, remaining in the place of origin and not flying more than 300 meters from the maternal nest during the swarming process. Solitary bees have a small foraging range and the range of pollinators is considered to be positively correlated with body size (Gathmann and Tscharntke 2002; Araújo et al. 2004; Greenleaf *et al*. 2007). Araújo et al. (2004) report that the maximum flight distance of *Partamona cupira* workers is from 1159 to 1710 meters. However, Zayed et al. (2005) point out that the distance wild bees cover during foraging trips is not necessarily related to dispersal ability and should therefore not be used to predict gene flow.

Given the dependence on environmental quality to meet their needs, their eusocial organization, form of colony reproduction and the low degree of vagility, it is possible to presume that a given area is occupied by few females from species that exhibit such traits. Thus, few and occasionally different mitochondrial lineages are expected within each population, resulting in a high degree of inter-population differentiation with regard to mitochondrial genes, as each occupation event may exhibit a founder effect, leading to the occurrence of private haplotypes.

To test this hypothesis, the proper choice of species to analyze is essential. Such species should have the following characteristics: i) not used in beekeeping activities to lend greater credibility to the results, since the colonies are not multiplied and/or transported from one place to another, like bees of the genus *Melipona*; ii) relatively large populations and easily located/collected samples; and iii) a recently revised genus to facilitate taxonomic identification. Among the groups of stingless bees in Brazil with such characteristics, the genus *Partamona* was chosen for the present investigation. With wide geographic distribution (southern Mexico to southern Brazil), this genus has 33 described species that occur in forests, on savannahs and in mountainous regions (Andes and Central American mountain ranges), with the capacity to occur at altitudes surpassing 2000 meters (Camargo and Pedro

2003). As the species of this genus occur in different environments, two with different characteristics were selected for the present study. *Partamona rustica* Camargo and Pedro (2003) from the cupira clade occurs in Brazil, ranging from the northern portion of the state of Minas Gerais to the southern portion of the state of Bahia in the dry *Caatinga* (xeric shrubland and thorn forest) and savannah biomes, nesting in arboreal termite nests constructed by *Constrictotermes cyphergaster* (Camargo and Pedro 2003; Miranda *et al.* 2015). This species was used as a model to evaluate the occupation process by females in restricted natural areas. *Partamona helleri* Friese (1900) is phylogenetically close to the cupira clade and occurs in the Atlantic Forest as well as open areas of southeastern Brazil, distributed from the state of Bahia to the state of Santa Catarina (Camargo and Pedro 2003). The nests of this species are semi-exposed and are commonly found in urban areas embedded in wall crevices, roof eaves and air conditioners as well as natural sites, such as the roots of epiphytes and abandoned nests of the bird *Phacellodomus* sp. Given its occurrence in both natural areas and those altered by human activities, this species was used to establish how the occupation process occurs by females with greater plasticity with regard to nesting sites.

The aim of the present study was to analyze genetic diversity and differentiation among *P. rustica* and *P. helleri* populations and offer demographic inferences for population groups of each species to test the occupation hypothesis for species of stingless bees. Moreover, a discussion is offered on which species of stingless bees this hypothesis can be applied.

Material and Methods

Sampling

Adult workers were collected from *P. rustica* nests in natural areas between May 2012 and January 2014. Through active searches, 11 localities were sampled, with an average of 13 nests per locality, totaling 145 nests (Fig. 1 and Table 1). In each locality, at least two

different sites with areas of 1 to 2 Km^2 were sampled. The two sites were separated by a distance of approximately two kilometers. The size of the area sampled in each locality (Table 1) was measured using Google Earth Pro (available at https://www.google.com.br/earth).

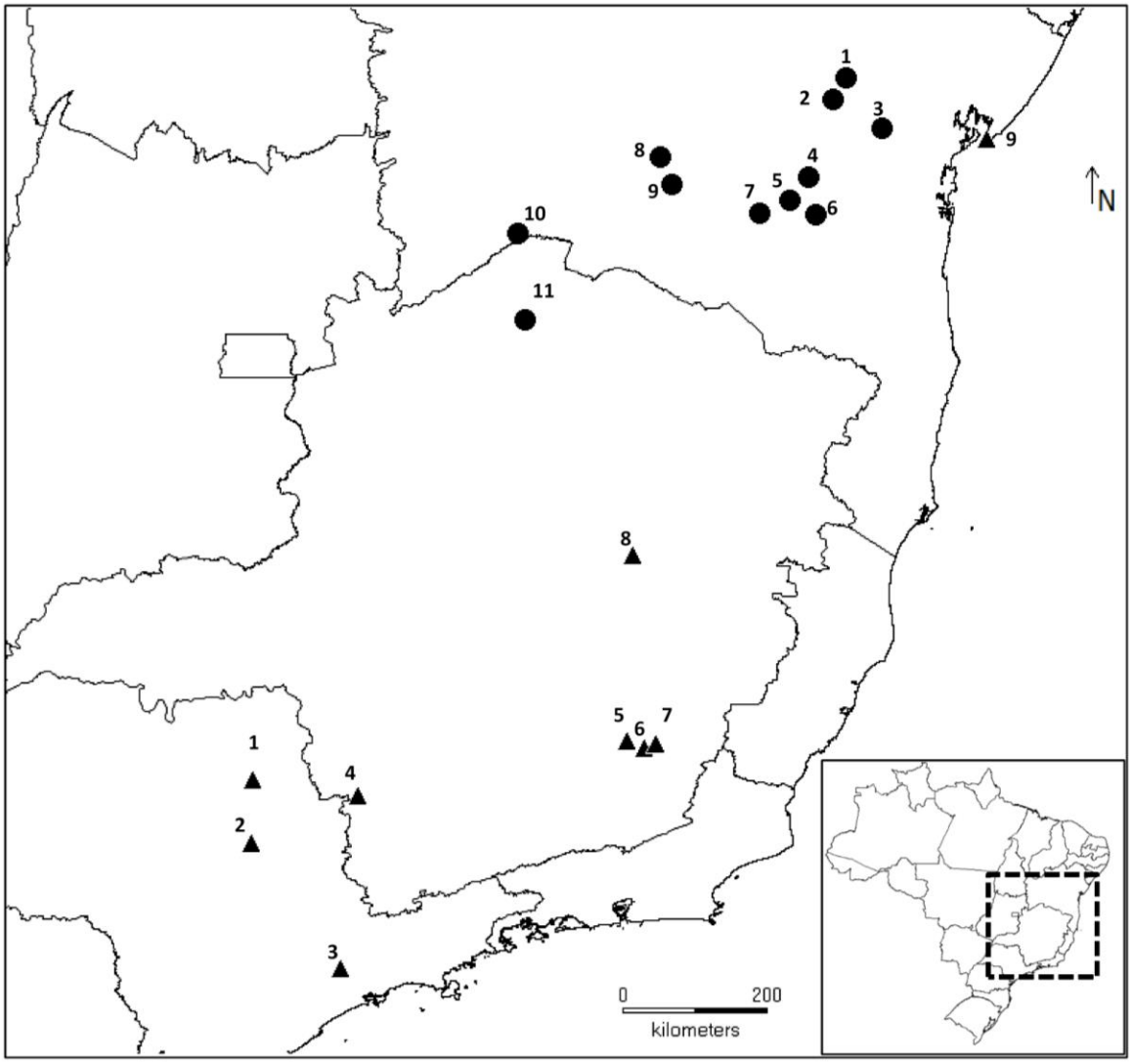

Fig. 1 Map of localities sampled. Circles represent 11 localities sampled for *P. rustica*. Triangles represent nine localities sampled for *P. helleri*. Localities are listed according to Table 1

Table 1 Localities visited and respective geographic coordinates, areas analyzed (*square kilometers*) and the number of nests (N) sampled for *P. rustica* and *P. helleri*. All *P. rustica* specimens were collected in natural environments in the state of Bahia and one natural environment in the state of Minas Gerais (Cônego Marinho). *P. helleri* specimens were collected from urban areas in the states of São Paulo, Minas Gerais and Bahia

Species		Localities	Initials	S	W	N	A
P. rustica	1	Ruy Barbosa	RBA	-12.228	-40.311	12	0.89
	2	B. Vista do Tupim	BVT	-12.502	-40.471	14	0.55
	3	Milagres	MIL	-12.931	-39.72	20	2.49
	4	Iramaia	IRA	-13.862	-40.081	15	0.11
	5	C. do Sincorá	CSI	-13.794	-41.017	12	0.2
	6	Manoel Vitorino	MVT	-13.939	-40.563	16	0.1
	7	Ituaçu	ITU	-13.882	-41.325	11	0.51
	8	Macaúbas	MAC	-12.243	-40.25	11	1.36
	9	Tanque Novo	TNO	-13.595	-42.521	14	1.22
	10	Cocos	COC	-14.158	-44.403	10	3.58
	11	Conego Marinho	CMA	-15.314	-44.382	10	2.36
					Total	**145**	
P. helleri	1	USP - Ribeirão Preto-SP	URP	-21.164	-47.857	24	-
	2	UFSCar- São Carlos-SP	USC	-21.978	-47.881	34	-
	3	USP - São Paulo-SP	USP	-23.561	-46.732	14	-
	4	Muzambinho-MG	MUZ	-21.364	-46.518	13	-
	5	Porto Firme-MG	PFI	-20.672	-43.084	8	-
	6	Viçosa – MG	VIC	-20.765	-42.869	20	-
	7	São Miguel do Anta-MG	SMA	-20.706	-42.720	17	-
	8	Rio Vermelho-MG	RVE	-18.297	-43.012	8	-
	9	UFBA - Salvador-BA	SAL	-13.005	-38.507	9	
					Total	**147**	

A total of 147 *P. helleri* nests were sampled between August 2012 and July 2004 in nine different areas: campus of the Universidade Federal de São Carlos (UFSCar) in the city of São Carlos, campuses of the Universidade de São Paulo (USP) in the cities of Ribeirão Preto and São Paulo, campus of the Instituto Federal do Sul de Minas (IFET) in the city of Muzambinho (state of Minas Gerais), campus I of the Universidade Federal da Bahia (UFBA) in the city of Salvador (state of Bahia) and four urban areas in the cities of Viçosa, São Miguel do Anta, Porto Firme and Rio Vermelho, all located in the state of Minas Gerais (Table 1 and Fig. 1).

Genetic analysis

Total DNA was extracted from one worker per colony based on the protocols proposed by Sheppard and McPheron (1991) or Waldschmidt *et al.* (1997). For *P. rustica,* the mitochondrial genes cytochrome c oxidase I (COI) and the terminal region of subunit I and beginning of subunit II of the COI gene (COI-COII region) were used. For *P. helleri,* mitochondrial genes COI and cytochrome *b* (Cytb) were amplified. For both species, the primers employed were designed for *Apis mellfera* and other insects (Table 2). All polymerase chain reactions (PCR) (25 μL) contained template DNA (50 ng), 1X of Taq buffer (Invitrogen), 250 μM of each dNTP, l.0 μM of each primer, 2.5 mM of $MgCl_2$ and 1 U of Platinum Taq DNA polymerase (Invitrogen). The PCR conditions for all gene regions were as follows: initial denaturation at 94 ºC for 5 minutes, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C and extension at 72 °C for 1 min, and completed with an additional extension step at 72 °C for 10 min. The PCR products were electrophoresed in agarose gels stained with GelRed^{TM}. The PCR product was purified using the illustra ExoProStar 1-Step Kit (GE Healthcare). The mitochondrial genes were sequenced in both directions with the same amplification primers using the BigDye v 3.0 Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Carlsbad, CA, USA) on an automated sequencer (ABI 3730 XL, Applied Biosystems).

Table 2 Primers used for PCR amplifications of mitochondrial genes

Region	***Primers***	**Reference**
COI	F: 5'-CAACATTTATTTTGATTTTTTGG-3' R: 5'-GATATTAATCCTAAAAAATGTTGAGG-3'	Dick *et al.* (2004)
Cytb	F: 5'-TATGTACTACCATGAGGACAAATATC-3' R: 5'-ATTACACCTCCTAATTTATTAGGAAT-3'	Crozier *et al.* (1991)
COI	F: 5'-GGAGATCCAATTCTTTATCAAC-3' R: 5'-GATATTAATCCTAAAAAATGTTGAGG-3'	F: Afonso (2012) R: Dick *et al.* (2004)
COII	F: 5'-TCTATACCACGACGTTATTC-3' R: 5'-GATCAATATCATTGATGACC-3'	Hall and Smith (1991)

Genetic diversity

The electropherograms were edited using the Codon Code v3.7.1 software program (CodonCode, Dedham, Massachusetts, USA). Sequences were aligned using the Clustal W algorithm with the aid of the BioEdit 7.0.9.0 program (Hall 1999). All alignments were visually inspected and corrected. Both *P. rustica* and *P. helleri* haplotype sequences were deposited in the GenBank (accession numbers KT765111 - KT765129 and KU641268 - KU641285, respectively). The number of variable sites (S), number of haplotypes (h), haplotype diversity (Hd) and nucleotide diversity (π) of each mitochondrial gene region were calculated per species as well as for all concatenated gene regions per species using the DnaSP v5.10.01 program (Librado and Rozas 2009).

Haplotype network and population structure

Haplotype networks were constructed employing a median-joining network (Bandelt et al. 1999) in NETWORK software (www.fluxus-engineering.com/sharenet.htm), using the concatenated gene regions COI and COII for *P. rustica* and COI and Cytb for *P. helleri*. Analysis of molecular variance (AMOVA) (Excoffier et al. 1992) was performed to estimate the degree of population genetic differentiation among localities using two hierarchical levels for concatenated genes for each species separately. The Φ_{ST} index was also estimated. These analyses were implemented using the ARLEQUIN 3.11 program (Excoffier et al. 2005) and significance was determined with 1000 permutations.

Spatial analysis of molecular variance (SAMOVA) was used to define possible groups of geographically homogeneous populations. This approach was implemented using the SAMOVA 2.0 program (Dupanloup et al. 2002). Population groups were not initially defined. The concatenated mitochondrial regions of *P. helleri* and *P. rustica* were used in this analysis

Pearson's correlation coefficients (r) were calculated for the analysis of correlation between the size of the sampling area in each locality and the number of haplotypes characterized for *P. rustica* in the same locality as well as between the number of colonies per locality and the number of haplotypes for the two species. Since the *P. helleri* samples came from human-modified landscapes, no determination of the correlation between the size of the sampling area and number of haplotypes was investigated for this species. These analyses were implemented using the Bioestat v.5.0 program (Ayres et al. 2007).

Demographic inference

To test for evidence of demographic expansion, the neutrality indexes Fu's Fs (Fu 1997) and Tajima's D (Tajima 1989) and the R2 population growth test (Ramos-Onsins and Rozas 2002) were implemented for each population group of each species using the DnaSP 5.10 program (Librado and Rozas 2009). The significance of these tests was determined based on 1000 coalescent simulations.

Results

Characterization of sequences and genetic diversity

A fragment of 1279 bp and 1096 pb was obtained for the concatenated genes of *P. rustica* and *P. helleri*, respectively. No indels occurred in these fragments. The species exhibited low nucleotide diversity ($\pi_{P.rustica}$ = 0.00215; $\pi_{P.helleri}$ = 0.00540) and high haplotype diversity ($Hd_{P.rustica}$ = 0.912; $Hd_{P.helleri}$ = 0.795) for the fragments of the concatenated genes (Table 3).

Table 3 Fragment size (pb), GC content, number of variable sites (S), number of haplotypes (h), haplotype diversity (Hd) and nucleotide diversity (π) for each mitochondrial gene and all concatenated regions (conc.) in *P. rustica* and *P. helleri*

sd, standard deviation

Species	Gene	size (pb)	G+C	S	h	π	Hd
P. rustica	COI	629	0.230	8	10	0.00212 ± 0.00014	0.782 ± 0.024
	COII	650	0.166	9	9	0.00217 ± 0.00024	0.660 ± 0.040
	CONC.	1279	0.198	17	21	0.00215 ± 0.00013	0.912 ± 0.012
P. helleri	COI	611	0.224	20	10	0.00653 ± 0.00036	0.795 ± 0.025
	Cytb	485	0.222	13	8	0.00399 ± 0.00047	0.745 ± 0.023
	CONC.	1096	0.223	33	10	0.00540 ± 0.00039	0.795 ± 0.025

sd, standard deviation

Haplotype network, neutrality tests and population differentiation

Figure 2 displays the relationships among the 21 haplotypes obtained for *P. rustica*. Unique and private haplotypes were found in samples from Boa Vista do Tupim (BVT), Manoel Vitorino (MVT) and Ituaçu (ITU). Two haplotypes were identified for *P. rustica* samples from four localities (RBA, MIL, IRA and CSI), three were identified for colonies from Macaúbas (MAC) and Tanque Novo (TNO) and four haplotypes were identified in the samples from Cocos (COC) and Cônego Marinho (CMA) (Fig. 2). For *P. rustica*, a positive, significant correlation was found between the size of the sampling area and number of haplotypes ($r = 0.764$; $P < 0.05$), whereas no correlation was found between the number of colonies and number of haplotypes per locality ($r = -0.465$; $P > 0.05$).

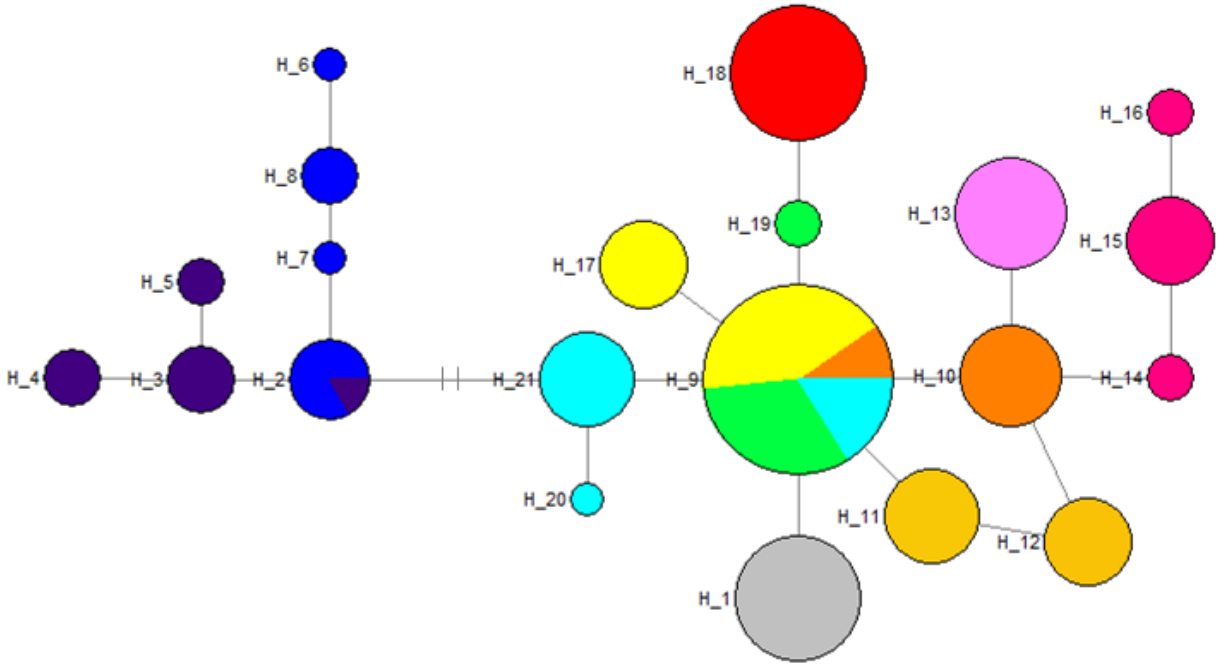

Fig. 2 Median-joining haplotype network of concatenated COI and COII (1279) for 145 samples of *P. rustica* from 11 localities. Haplotypes from MIL in yellow; MVT-red; BVT-grey; IRA-medium yellow; COC-blue; TNO-light blue; RBA-Green; CMA-purple; MAC-dark pink; ITU-light pink; CSI-orange. There was only one mutational site distinguishing all haplotypes, except between H2 and H21 (two mutation sites).

To visualize the fine-scale distribution of haplotypes at the different sites of a given locality, Fig. 3 displays the spatial distribution of haplotypes in the COC locality, which had the greatest number of haplotypes (n = 4) and largest sampling area. Three sites were sampled in this locality (S1, S2 and S3) over a total area of approximately 3.6 Km^2 (Fig. 3). These sites had one, three and two haplotypes, respectively. When analyzed on a fine spatial scale, the heterogeneous haplotype distribution indicates that the locality may have more than one occupation site.

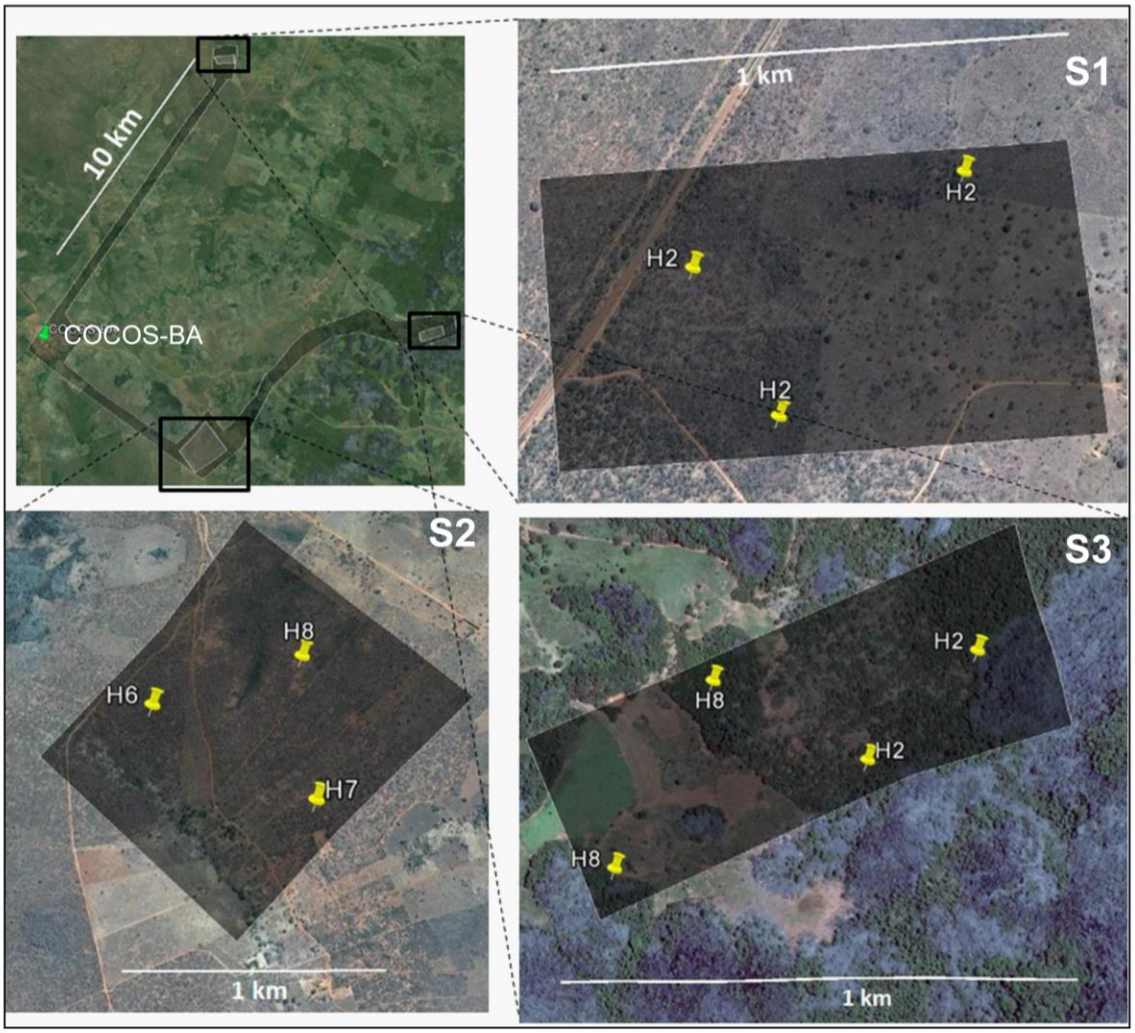

Fig. 3 Haplotype distribution for two concatenated mitochondrial gene regions in occupation sites of *P. rustica* at COC. Yellow markers represent nests sampled at sites and respective haplotypes. "S1", "S2" and "S3" represent sites sampled in each locality.

Ten haplotypes were identified in samples of *P. helleri* (Fig. 4), four of which were unique and private to different localities (USP, PFI, MUZ and SAL). Moreover, H8 was the only haplotype found in all USC (N = 34) and URP (N = 24) colonies. At three of the localities examined, samples of *P. helleri* exhibited two haplotypes (RVE, VIC and SMA). No correlation was found between the number of colonies and number of haplotypes per locality for *P. helleri* ($r = -0.153$; $P > 0.05$).

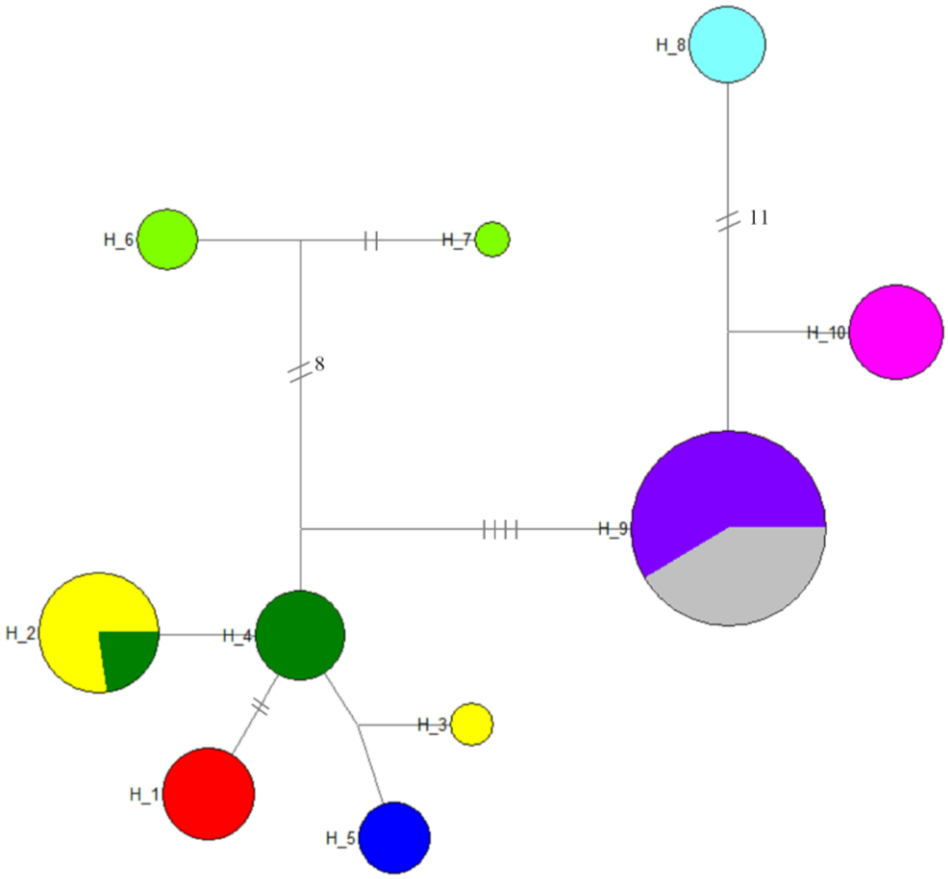

Fig. 4 Median-joining haplotype network of concatenated COI and Cytb (1096) for 147 samples of *P. helleri* from nine localities. Haplotype from URP in grey; USC-lilac; USP-pink; MUZ-red; PFI-blue; VIC-yellow; SMA-dark green; VERS-light green; SAL-light blue. Lines represent number of mutational sites between haplotypes. No line between haplotypes represents only one mutational site.

Based on the SAMOVA results, *P. rustica* populations formed two groups, the first consisting of COC and CMA colonies and the second represented by all other populations sampled (Table 1). The neutrality test values were non-significant for both of these groups (Table 4). *P. helleri* populations were clustered into three groups, the first represented by samples from Salvador (Group 1, h = 1, N = 9), the second represented by RVE, VIC, MUZ, PFI and SMA (Group 2, h = 7, N = 66) and the third represented by UFS, URP and USP (Group 3, h = 2, N = 72) samples (Table 1). The neutrality test values were non-significant for

the groups of *P. helleri* populations (Table 4). These tests were not performed for *P. helleri* Group 1 due to the fact that this group only exhibited a single haplotype.

Table 4 Neutrality tests for haplogroups of *P. rustica* and *P. helleri*

Species	Haplogroup	Fu's Fs	D	R2
P. rustica	Group 1	-1.510 (P= 0.155)	0.2248 (P= 0.626)	0.147 (P= 0.597)
	Group 2	0.091 (P= 0.563)	0.0913 (P= 0.563)	0.091 (P= 0.563)
P. helleri	Group 1	-	-	-
	Group 2	4.389 (P=0.926)	0.185 (P=0.665)	0.111 (P=0.650)
	Group 3	2,762 (P=0.930)	0.886 (P=0.836)	0.158 (P=0.822)

Finally, a degree of high differentiation was found among the *P. rustica* populations (Φ_{ST} = 0.849; P = 0.000) as well as the *P. helleri* populations (Φ_{ST} = 0.966; P = 0.000) (Table 5).

Table 5 Analysis of molecular variance (AMOVA) for 11 of populations of *P. rustica* and nine populations of *P. helleri*

Species	Variation source	d.f	Variation (%)	Φ_{ST}	P-value
P. rustica	Among localities	10	84.91	0.849	0.000
	Within localities	134	15.09		
	Total	144			
P. helleri	Among localities	8	96,66	0.966	0.000
	Within localities	138	3.34		
	Total	146			

Discussion

Genetic diversity

The low nucleotide diversity for both species analyzed indicates a high degree of similarity between the haplotypes identified. This finding may be explained by the recent origin of the two species, which therefore exhibit a lower accumulated rate of change. *Partamona rustica*

had a lower degree of nucleotide diversity in comparison to *P. helleri*, which may be explained by the fact that *P. rustica* be a species with more restricted occurrence and is endemic, whereas *P. helleri* has broader distribution, occurring in nearly all regions of the Atlantic Forest and savannahs of Brazil.

The association between the reproduction of stingless bee colonies by swarming (Nogueira-Neto 1954) and their limited flight capacity (Araújo et al. 2004) restrict maternal gene flow and consequently reduce the effective population size. Moreover, haplodiploidy and the sex determination mechanism (*csd* locus) as well as eusociality are other factors that reduce the effective population size (Zayed 2004; Jaffé et al. 2009). Thus, it has long been recognized that hymenopterans exhibit lower levels of genetic variation in comparison to diplodiploid insects (Berkelhamer 1983; Graur, 1985). Similar degrees of nucleotide and haplotype diversity have been reported for *Melipona quadrifasciata* (Batalha-Filho et al. 2010), *Partamona mulata* (Brito et al. 2013), *Plebeia remota* (Francisco et al. 2013), *Melipona subnitida* (Bonatti et al. 2014), *Tetragonula carbonaria, Tetragonula hockingsi* (Brito et al. 2014) and *Tetragonisca angustula* (Francisco et al. 2015), suggesting a similar genetic structure of the populations of these bees when assessed using mitochondrial genes.

Fine scale analysis of occupation process

The unique and private haplotypes exhibited for both species and the low number of haplotypes per locality indicated that the occupation of an area is accomplished by few founder females (maternal lineages). Even in the largest area studied (such as COC for *P. rustica*), where the highest number of haplotypes was found, differential spatial distribution of the haplotypes identified at each site was found when examined on a fine scale (Fig. 3). This result suggests that what are denominated localities in the present study may be constituted of different nesting sites by females that sometimes show different maternal lineages, resulting

in a high degree of differentiation among colonies from different localities when analyzed using mitochondrial genes. These findings were supported by AMOVA, the estimated values of which clearly point to a high degree of inter-population differentiation for both species (*P. rustica* – Φ_{ST} = 0.849; P = 0.000; *P. helleri* – Φ_{ST} = 0.958; P = 0.000). The hypothesis proposed herein is also supported by the correlation between the size of the sampling area and number of haplotypes, indicating that larger areas, as expected, have more occupation sites and therefore have a greater number of haplotypes. Furthermore, the absence of a correlation between the number of colonies and number of haplotypes per locality observed for the two species also supports this hypothesis, as it demonstrates that the increase in the number of colonies is due to reproduction by the swarming of original colonies rather than the arrival of new females to the site.

The neutrality tests for both species suggest that the few haplotypes per locality and low nucleotide diversity are not due to the effects of population bottlenecks, as all the indices had non-significant values, which lends further support to the hypothesis tested herein. Similar findings (low nucleotide diversity, low number of haplotypes per population and unique and/or private haplotypes at different localities) have been reported for *Partamona mulata* Camargo and Pedro 2003 (Brito et al. 2013) and *Melipona subnitida* Ducke (Bonatti *et al*. 2014). The authors of some of these studies suggest the results could be due to a population bottleneck effect (Brito et al. 2013; Bonatti et al. 2014). However, as rare haplotypes tend to disappear due to genetic drift and it is more likely for populations that experience bottlenecks have more common haplotypes, the hypothesis presented herein seems more appropriate for explaining the results found for these different species.

The presence of unique and/or private haplotypes, the few haplotypes per locality, the high degree of differentiation among populations of *P. rustica* and *P. helleri* and the lack of evidence of population bottlenecks support the hypothesis that the occupation of an area

occurs by one or few founder females. Furthermore, the common occurrence of unique and private haplotype in different localities appears to suggest that the progress of the occupation process does not occur through the arrival of new females to these localities. Instead, the growth of the local population occurs through the reproduction of the few original colonies for both *P. rustica* and *P. helleri*. Therefore, any occupation event may reproduce the conditions of the founder effect, which occurs when a low frequent mitochondrial lineage occupies a new place and founds a new population. The present findings also indicate that this occupation process occurs similarly in natural areas as well as areas altered by human activities. Some studies involving stingless bees report similar results (Batalha-Filho et al. 2010; Francisco et al. 2013; Brito et al. 2013; Bonatti et al. 2014; Francisco et al. 2015), indicating that this form of landscape occupation by species of *Partamona* may be also common to other stingless bees.

Implications of the hypothesis for future studies

The occupation hypothesis tested herein and supported by the present results has implications. The first concerns phylogeographic studies on stingless bees. The findings indicate that phylogeographic studies involving this group of bees should give priority to the analysis of samples from a greater number of sites than the number of colonies per site, since the occurrence of few haplotypes per locality and unique and/or private haplotypes in populations of stingless bees are common. Secondly, as the occupation of an area occurs through distinct nesting events at nearby sites (Fig. 3), it is important to determine what constitutes a natural population of these species. Given the system of sex determination in Hymenoptera, it is crucial for these species to have a mechanism to prevent inbreeding and homozygosis at the sexual locus (*csd*) to avoid the generation of diploid males that are highly harmful to colonies and populations (Beye et al. 2003; Hasselmann et al. 2008). Thus, as the occupation process is

performed by females, males must constitute the disperser sex. If this assumption is true, the acquisition of new genes by populations should occur as a result of the dispersal behavior of Meliponini males (asymmetrical sex dispersal), as observed in social insects (Fernandes et al. 2011; Cronin et al. 2013) and even bees from the tribe Euglossini in Neotropical regions (Cerântola et al. 2011; Rocha-Filho et al. 2013; López-Uribe et al. 2014).

Studies performed thus far have most often given priority to the estimation of genetic differentiation among populations of stingless bees, indicating restrictions to gene flow. However, considering the way females occupy a certain area, as described in the present study, the main aspect to investigate does not seem to have been considered properly. Studies should be designed with the purpose of demonstrating whether the magnitude of gene flow is sufficient to minimize the risks of inbred mating. Therefore, studies on the population genetics of stingless bees that nest solitarily and especially those that nest in aggregations should estimate the genetic relationships between the queens and males that founded the colonies of a given population rather than merely define the degree of inter-population differentiation (Cameron et al. 2004). It would therefore be possible to develop refined population genetics for stingless bees and define the actual genetic structure of the populations. Given the importance of this topic and the material studied, the hope is that these matters will be properly considered in the near future so as to contribute to the delineation of the best beekeeping and conservation strategies aimed at these pollinators.

Acknowledgments We are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo - (FAPESP, processes numbers 2005/60893-2, 2008/58382-8, 2011/21501-2, 2012/23342-1) for financial support, to Dra. Silvia Regina de Menezes Pedro (FFCLRP - USP) for the identification of the bees and to all beekeepers and locals that helped us during the field surveying.

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Capítulo III

Parentesco genético, estrutura sociogenética e das populações de *Partamona rustica,* acessados por marcadores SSR: implicações frente ao sistema *csd*

Parentesco genético, estrutura sociogenética e das populações de *Partamona rustica,* acessados por marcadores SSR: implicações frente ao sistema *csd*

Elder A. Miranda e Marco A. Del Lama

Resumo

As abelhas sem ferrão apresentam o sistema de determinação complementar do sexo (*csd*). Neste sistema, a heterozigose resulta em fêmeas e a homozigose para os alelos do loco transforma genótipos diplóides em machos, que geram severos custos às colônias e populações destas abelhas. Além disso, devido a características da biologia das abelhas sem ferrão como a eussocialidade, a baixa capacidade de dispersão e o sistema de enxameagem, uma dada área deve ser ocupada por poucas linhagens maternas, ou seja, poucas fêmeas fundadoras e filopátricas. No entanto, devido ao sistema *csd*, faz-se necessária a introdução de variação genética nas populações, papel que tem sido atribuído aos machos em alguns estudos anteriores. Nesse sentido, foi estimado o parentesco genético entre operárias de *P. rustica*, visando avaliar o papel dos machos na promoção do fluxo gênico entre colônias e populações. Para tanto, foram analisadas 44 colônias de *P. rustica* de três localidades, utilizando sete locos microssatélites. Nossos resultados mostraram que as colônias analisadas são monogínicas e monândrias, dado o parentesco médio estimado entre operárias da mesma colônia (r= 0,79 ±0,03). Baixos índices de parentesco médio foram observados entre operárias de colônias das localidades que apresentam haplótipos únicos e privados, demonstrando que apesar destas localidades, possivelmente, terem sido ocupadas por uma ou poucas linhagens maternas (poucas fêmeas fundadoras), o papel dispersor dos machos foi suficiente para reduzir o parentesco e minimizar os riscos de endogamia. Finalmente, nosso estudo reforça a necessidade de mudança do foco atual dos estudos relacionados à estrutura genética das populações de abelhas, os quais, via de regra, enfatizam tão somente a estimativa do nível de diferenciação interpopulacional, para estimativas das relações genéticas entre rainhas e machos que fundaram as colônias de uma dada população.

Palavras-Chave: Abelha sem ferrão, dispersão sexo-assimétrica, endogamia, fluxo gênico, machos.

Introdução

As abelhas sem ferrão, como a grande maioria dos himenópteros, apresentam o sistema de determinação complementar do sexo (*csd*). Segundo Heimpel e Boer (2008), o loco *csd* é o mecanismo mais estudado e melhor estabelecido de determinação do sexo por haplodiploidia no táxon. Neste sistema, a heterozigose resulta em fêmeas e a homozigose para os alelos do loco transforma genótipos diplóides em machos (Camargo, 1979; Beye *et al.*, 2003; Heimpel & Boer 2008; Hasselmann *et al.,* 2008). A geração de machos diplóides nestes insetos gera custos elevados, a saber, um *fitness* próximo de zero por serem estéreis ou por gerarem progênie triploide (Crozier e Pamilo, 1996; Yamauchi *et al.,* 2002; Ayabe *et al.* 2004), além de reduzir o número de fêmeas nas colônias, aumentando os riscos de extinção das mesmas (Zayed e Packer, 2005). Por estes motivos, neste sistema de determinação do sexo, os acasalamentos endogâmicos são altamente desfavoráveis, uma vez que a endogamia favorece a homozigosidade.

O Capítulo II desta tese revelou que o processo de ocupação de áreas por abelhas do gênero *Partamona* e mostraram que devido a características da biologia das abelhas sem ferrão como o tamanho de suas colônias eusociais, a baixa capacidade de dispersão e o sistema de enxameagem, uma dada área deve ser ocupada por poucas linhagens maternas, ou seja, poucas fêmeas fundadoras; resultados que foram suportados pelos únicos ou poucos haplótipos observados por localidade e pela elevada diferenciação entre as populações, quando analisadas para os genes mitocondriais. A partir destes resultados, espera-se que as fêmeas devem ser as responsáveis pela ocupação das novas áreas. Ademais, o Capitulo II ainda apontou para a possibilidade deste aspecto ser comum a outras espécies do grupo, dado os resultados similares observados em outros estudos (Batalha-Filho *et al*., 2010; Francisco *et al.*, 2013; Fernandes *et al.,* 211; Brito *et al.,* 2013; Bonatti *et al.*, 2014; Francisco *et al*,. 2015).

Assim, considerando o sistema de determinação do sexo nestes insetos, os machos devem constituir o sexo dispersor, promovendo a entrada de variação nas populações e

evitando a endogamia, caracterizando, portanto, uma dispersão sexo-assimétrica nestas abelhas, como mostrado no Capítulo II. Alguns estudos têm reportado o papel dispersor dos machos, a exemplo da análise genética das agregações de machos de *Scaptotrigona postica* realizado por Paxton (2000). Este autor sugere que os machos de *S. postica* se dispersam para longe do seu ninho e raramente se unem a congregações próximas à colônia natal. Além disto, Cameron *et al.* (2004) sugerem que a movimentação dos machos para formar agregações e o comportamento de enxameagem das fêmeas de *Trigona collina* são suficientes para evitar endogamia nas populações desta abelha.

No entanto, diante da grande diversidade de abelhas sem ferrão existente no mundo (Camargo, 1989), somente para a região Neotropical já foram descritas 417 espécies válidas (Camargo e Pedro, 2003), estudos envolvendo uma análise da estrutura genética das populações destas abelhas, reportando o papel dispersor dos machos e as suas implicações para o sistema de determinação do sexo ainda são esparsos, pois se restringem a um número reduzido de espécies.

A abelha sem ferrão *Partamona rustica* (Camargo e Pedro, 2003) faz parte do clado cupira, ocorre no Brasil em regiões de campos cerrados do norte de Minas Gerais e áreas de Caatinga no sudoeste da Bahia (Camargo e Pedro 2003; Miranda *et al.* 2015). No Capítulo II desta tese foi apresentada a estrutura populacional de *P. rustica* e demonstraram que os sítios de nidificação analisados para esta espécie foram ocupados por uma ou poucas fêmeas, dados os haplótipos únicos e/ou privados e aos poucos haplótipos observados por sítio, bem como o alto nível de diferenciação entre as populações analisadas.

Nesse sentido, foi estimado o parentesco genético entre operárias de *P. rustica* da mesma colônia, buscando avaliar a estrutura sociogenética na espécie; bem como o parentesco entre operárias de diferentes colônias da mesma localidade e entre operárias de colônias de localidades distintas, visando avaliar o papel dos machos na promoção do fluxo gênico, tendo

em vista as implicações do processo de ocupação de áreas por fêmeas da espécie frente ao sistema *csd*.

Material e métodos

Amostragem

Neste estudo, nós analisamos 44 colônias de *P. rustica* oriundas de três localidades do estado da Bahia (Brasil), sendo 16 colônias de Manoel Vitorino (MVT), 14 colônias de Boa Vista do Tupim (BVT) e 14 colônias de Tanque Novo (TNO). Analisamos uma operária adulta para cada colônia. Estas colônias correspondem às populações estudadas no Capítulo II. Analisamos as colônias de MVT e BVT porque apresentam haplótipos únicos e privados. Por outro lado, colônias de TNO foram usadas como controle, pois nesta localidade foram observados três haplótipos. Além disto, nós analisamos cinco operárias de cada colônia de MVT, totalizando 80 operárias.

Amplificação dos locos microssatélites e genotipagem

O procedimento de extração de DNA está descrito no Capítulo II. Nós utilizamos sete locos microssatélites, sendo dois descritos para *Melipona bicolor* (MBI 232, MBI 254; Peters *et al.*, 1998) e cinco descritos para *Partamona helleri* (Phel1, Phel2, Phel3, Phel6 e Phel7; Ferreira, 2011).

A PCR para os locos MBI 232 e MBI 254 foi realizada em volume final de 25 µL, contendo 1 µL do DNA extraído, 1 µM de cada primer, 2,5 mM de cloreto de magnésio, 250 µM de cada dNTP, 1X tampão de reação, 1U de Platinum Taq DNA Polimerase (Invitrogen) e água esterilizada. A reação procedeu por 30 a 35 ciclos de 94°C por 30s para desnaturação, 20s à temperatura de hibridação do primer utilizado e 70°C por um minuto para extensão da cadeia.

Para os locos Phel1, Phel2, Phel3, Phel6 e Phel7, as reações de amplificação ocorreram em um volume final de 10µL, nas seguintes condições: água esterilizada, 2,5 mM de cloreto de magnésio, 250 µM de cada dNTP, 1X tampão de reação, 1U de Platinum Taq DNA Polimerase (Invitrogen), 1 µM de cada primer e 1 µL do DNA extraído. A temperatura de hibridização, motivo, tamanho esperado e fluoróforos de cada primer são mostrados na Tabela S1.

Os microssatélites foram agrupados em multiplex, de acordo com a cor da fluorescência, o tamanho do fragmento e a temperatura de anelamento de cada um deles. O produto da PCR foi diluído 10X e uma alíquota de 2 µL foi misturada com 7,75 µL de Tween 20 0,1% e 0,25 µL de ET550-R, sendo o produto então submetido à corrida no sequenciador Mega Bace 1000. Os resultados obtidos foram analisados no aplicativo MegaBACE Genetic Profiler versão 1.2.

Análise dos dados

Inicialmente, utilizamos o programa Micro-checker v. 2.2.3 (van Oosterhout *et al*., 2004) para detecção de possíveis alelos nulos, ausência de amplificação, erros de genotipagem e amplificação preferencial de alelos menores. As análises de diversidade foram implementadas por meio do programa GenAlEx 6.5 (Peakall e Smouse, 2012).

Analisamos a estrutura social de todas as colônias de MVT, verificando se estas são monogínicas e monândricas, como esperado para abelhas sem ferrão (Peters *et al*., 1999; Strassmann, 2001). Para tanto, nós analisamos se os genótipos das operárias de cada colônia são explicados pelo acasalamento entre uma fêmea com um único macho; nesse sentido, as cinco operárias de cada colônia deveriam compartilhar, pelo menos, um alelo para cada loco (alelo paterno), e a colônia deveria apresentar, no máximo, três alelos por loco (caso a fêmea fosse heterozigota para dois outros alelos deste loco). Em seguida, estimamos o parentesco

genético médio entre as cinco operárias de cada uma destas colônias. Estimamos, separadamente, o parentesco genético médio entre operárias de diferentes colônias da mesma localidade e, em seguida, o parentesco entre operárias de colônias de localidades diferentes. Estas duas últimas análises foram realizadas usando apenas uma operária por colônia. Todas as análises de parentesco foram realizadas por meio do índice de Queller e Goodnight (1989), usando o programa Kingroup v2_101202 (Konovalov *et al.*, 2004). Nós removemos os locos monomórficos das análises, uma vez que eles poderiam enviesar os resultados simplesmente pelo fato de não serem informativos. A lista dos locos utilizados para cada bloco de análise é apresentada na Tabela S2.

Resultados

Os alelos nulos foram detectados e ajustados apenas para a população de BVT (Phel-1). Por outro lado, não foram detectados erros de genotipagem ou amplificação preferencial de alelos menores para as populações analisadas. A análise de diversidade genética considerando as três populações mostrou número médio de alelos de 5,14 por loco, variando entre três e nove alelos, e herterozigosidade média de 0,32, variando entre 0,06 a 0,67 (Tabela 1). A análise realizada a partir das cinco operárias de cada colônia de MVT revelou que todos os genótipos observados podem ser explicados por monandria e monoginia (dados não apresentados). A análise de parentesco médio entre operárias destas colônias apresentou r= 0,79 ±0,03 (variando entre 0,42 e 1,00) (Tabela 2). Ademais, foram observados baixos índices de parentesco médio entre as operárias das colônias de MVT e BVT, sendo estes índices ainda menores entre as operárias das colônias de TNO (Tabela 3). Finalmente, a análise de parentesco médio entre operárias de colônias de diferentes localidades mostrou índices ainda menores, exceto para TNO (Tabela 2).

Tabela 1. Número de alelos (*n*) e diversidade genética (H_E) considerando três populações de *P. rustica*, estimados por meio de sete locos microssatélites.

	Locus							
	Phel-1	**Phel-2**	**Phel-3**	**Phel-6**	**Phel-7**	**Mbi254**	**Mbi232**	**Média**
n	8	9	2	3	4	3	7	5,14
H_E	0,60	0,67	0,02	0,45	0,10	0,06	0,35	0,32

Tabela 2. Parentesco genético médio entre operárias de colônias de MVT pelo índice de Queller e Goodnight (1989).

Colônia	**r (±SE)**
MVT1	0,81 ±0,02
MVT2	0,42 ±0,04
MVT3	0,57 ±0,05
MVT4	0,88 ±0,01
MVT5	0,92 ±0,01
MVT6	0,67 ±0,03
MVT7	0,78 ±0,03
MVT8	0,82 ±0,03
MVT9	0,85 ±0,03
MVT10	1,00 ±0,00
MVT11	0,84 ±0,02
MVT12	0,92 ±0,02
MVT13	0,74 ±0,03
MVT14	0,66 ±0,03
MVT15	0,81 ±0,03
MVT16	1,00 ±0,00
Média	**0,79 ±0,03**

Tabela 3. Estimativa de parentesco genético médio (r ± SE) entre indivíduos de colônias de *P. rustica* oriundas da mesma localidade (em negrito) e de colônias de localidades diferentes, estimados pelo índice de Queller e Goodnight (1989).

Localidade	**MVT**	**BVT**	**TNO**
MVT	**0,129 (±0,025)**		
BVT	0,125 (±0,0180)	**0,191(±0,035)**	
TNO	0,101 (±0,018)	0,106 **(**±0,0193)	**0,013(±0,029)**

Discussão

A análise da segregação mendeliana juntamente com o resultado de parentesco médio entre as operárias das colônias de MVT (r= 0,79 ±0,03) mostraram que estas colônias são, possivelmente, resultantes de acasalamentos do tipo monândrico e de colônias monogínicas, padrão que tem sido observado para a maioria dos meliponíneos (Peters *et al.*, 1999; Strassman, 2001; Tóth *et al.*, 2002a, 2003). No entanto, alguns estudos têm revelado poliginia em abelhas sem ferrão, a exemplo de *Plebeia wittmanni* (Witter e Wittmann, 1997), *Melipona scutellaris* (Carvalho-Zilse e Kerr, 2004) e *Melipona quadrifasciata* (Alves *et al.*, 2010). O índice de parentesco genético médio, aqui observado, se mostrou próximo ao esperado (r= 0,75). Reis *et al.* (2010) também encontrou parentesco médio acima do esperado entre operárias de colônias monândricas de *Melipona bicolor*. No entanto, quando estes autores analisaram o parentesco médio, considerando todas as colônias de todas as localidades, observaram índice de parentesco dentro do esperado (r= 0,75 ±0,12).

Os resultados de baixos índices de parentesco médio observados entre as operárias de colônias das localidades que apresentam haplótipos únicos e privados demonstram que apesar destas localidades, possivelmente, terem sido ocupadas por uma ou poucas linhagens maternas (poucas fêmeas) (como mostrado no Capítulo II), o papel dos machos como dispersor e a possível formação de agregações (Engels e Engels, 1984; Paxton, 2000) foram suficientes para reduzir o parentesco entre colônias, tornando o acasalamento panmítico e inserindo variação nova nas populações, evitando, portanto, a endogamia. Além disto, como esperado, o resultado de parentesco genético médio entre as operárias das colônias de TNO (que apresentou maior número de haplótipos) e entre operárias de colônias de diferentes localidades, demonstram índices ainda menores de parentesco médio (Tabela 2). Estes resultados estão em consonância com o estudo de Cameron *et al.* (2004), que também mostrou baixos ou negativos índices de parentesco médio entre colônias de agregações de

Trigona collina devido ao papel dispersor dos machos e à enxameagem, dois mecanismos que evitam o cruzamento entre indivíduos aparentados.

Nossos resultados mostram que, apesar de heterólogos, os locos microssatélites utilizados foram suficientemente variáveis para as estimativas de parentesco genético entre colônias de *P. rustica* da mesma localidade e entre colônias de diferentes localidades, dado os níveis de diversidade observados entre as populações (Tabela 1). No entanto, a quantidade de locos utilizados, a falta de variação para alguns deles em certas populações (Tabela S2) e os muitos genótipos heterozigotos (A/B) observados para as opérarias de MVT, inviabilizaram reconstruir os genótipos parentais destas colônias para cada loco e realizar as estimativas de parentesco genético entre as rainhas das diferentes colônias e entre rainha e zangão de cada colônia. Os genótipos heterozigotos das operárias para um determinado loco impossibilitam inferir com precisão os genótipos parentais das colônias. Diante disto, novas análises envolvendo novos marcadores SSR específicos para *P. rustica* estão em curso e fornecerão um maior número de locos para nova realização destas estimativas.

Nossos resultados corroboram a dispersão sexo-assimétrica nesta abelha sem ferrão, apresentada nos Capítulos II, demonstrando o importante papel dispersor dos machos, promovendo fluxo gênico entre populações, reduzindo o parentesco genético entre colônias e, consequentemente, evitando a homozigose no loco *csd*. Além disto, reforça a necessidade de mudança do foco atual dos estudos da estrutura genética das populações naturais das abelhas que, via de regra, apenas estimam o nível de diferenciação interpopulacional, para estimativas das relações genéticas entre rainhas e machos que fundaram as colônias de uma dada população, como sugerido no Capítulo II e demonstrado neste estudo.

Agradecimentos

Os autores agradecem à Fundação de Amparo à Pesquisa de São Paulo (FAPESP) pelo suporte financeiro (Processos 2012/23342-1 e 2011/21501-2); à Dra. Silvia Regina de Menezes Pedro (FFCLRP - USP), pela identificação taxonômica das abelhas, e a todos os meliponicultores, apicultores e moradores das localidades visitadas que nos auxiliaram nas buscas e coletas das amostras e à Dra. Kátia Ferreira pela ajuda na preparação e genotipagem dos locos microssatélites.

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Material suplementar

Tabela S1. "Motivos" das repetições, fluoróforos, tamanho dos fragmentos e temperaturas de anelamento (Ta) dos sete locos microssatélites utilizados nas análises de estrutura genética das colônias de *P. rustica*.

Loci	Repetição	Fluoróforo	Tamanho	Ta (ºC)
Mbi254	AAG	FAM	208-232	52ºC
Mbi232	CTT	FAM	130-178	48ºC
Phel1	AC	TET	217-247	55ºC
Phel2	TC	TET	260-312	52ºC
Phel3	CT	JOE	99-101	55ºC
Phel6	CA	HEX	174-180	52ºC
Phel7	TG	HEX	282-298	48ºC

Tabela S2. Locos microssatélites utilizados em cada análise de estrutura genética de *P. rustica*. Os traços ("-") representam os locos monomórficos, removidos das análises.

Loci	MVT	BVT	TNO	MVT x BVT	MVT x TNO	BVT x TNO
Mbi254	x	-	x	x	x	x
Mbi232	x	x	x	x	x	x
Phel1	x	x	x	x	x	x
Phel2	x	x	x	x	x	x
Phel3	-	-	x	-	x	x
Phel6	x	x	x	x	x	x
Phel7	-	-	x	-	x	x

Capítulo IV

Phylogeography of *Partamona rustica* (Hymenoptera, Apidae), an endemic stingless bee from the Neotropical dry forest diagonal

Running title: Phylogeography of Neotropical endemic bee

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Phylogeography of *Partamona rustica* (Hymenoptera, Apidae), an endemic stingless bee from the Neotropical dry forest diagonal

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ABSTRACT

South America encompasses the highest levels of biodiversity found anywhere in the world and its rich biota is distributed among many different biogeographical regions. However, many regions of South America are still poorly studied, including its xeric environments, such as the threatened Caatinga and Cerrado biomes. In particular, the effects of Quaternary climatic events on the demography of endemic species from xeric habitats are poorly understood. The present study uses an integrative approach to reconstruct the evolutionary history of *Partamona rustica*, an endemic stingless bee from dry forests in Brazil, in a spatial-temporal framework. In this sense, we sequenced four mitochondrial genes and genotyped eight microsatellite loci. Our results identified two groups: one to the west and the other to the east of the São Francisco River Valley (SFRV). These groups diverged in the late Pleistocene, and the Approximate Bayesian Computation analysis indicates that *P. rustica* originated to the west of the SFRV, subsequently colonising the region to the east. Our tests of migration detected reduced gene flow between these groups. Finally, our results also indicate that the inferences both from the genetic data analyses and from the spatial distribution modelling are compatible with a history of constant size populations.

The Linnean Society of London, Biological Journal of the Linnean Society, 2016, 00, 000–000.

ADDITIONAL KEYWORDS: Approximate Bayesian Computation – bees – dry environments – ecological niche modelling – genetic structure – phylogeographic history.

INTRODUCTION

South America encompasses the highest levels of biodiversity found anywhere in the world (Myers *et al.*, 2000) and its rich biota is distributed among many different biogeographical regions (Morrone, 2014). Studies of the diversification of the Neotropical biota have revealed a complex evolutionary history (Turchetto-Zolet *et al.*, 2013) that appears to have fluctuated continuously throughout the Tertiary and Quaternary (Rull, 2011). This diversification was influenced by tectonic (Hoorn *et al.*, 2010) and paleoclimatic events (Cheng *et al.*, 2013; Carnaval *et al.*, 2014). Many regions of South America are still poorly studied, including its xeric environments (Werneck, 2011; Turchetto-Zolet *et al.*, 2013), such as the threatened Caatinga and Cerrado biomes. In particular, the effects of Quaternary climatic events on the demography of endemic species from xeric habitats are poorly understood.

Phylogeographic studies have indicated that Pleistocene climatic changes may have influenced the species richness, spatial distribution, endemism, genetic diversity, historical demography and biogeographic patterns of the Neotropical biota (Carnaval & Moritz, 2008; Werneck *et al.*, 2015). Present-day Neotropical biodiversity is frequently explained by the Pleistocene "refugia" hypothesis, which relates successive climatic-vegetation cycles during the Pleistocene, in particular glacial events, to vicariant processes and the expansion or retraction of species ranges (Haffer ,1969; Brown & Ab'Saber, 1979). Werneck *et al.* (2011) proposed the occurrence of three principal refuges between the Last Glacial Maximum (~21 ka) and the present day in the Seasonally Dry Tropical Forests (SDTF) of South America – the Caatinga, Misiones/Piemonte and Chiquitano refuges. The Caatinga refuge, which covers most of the present-day distribution of the biome, is the largest stable area of SDTF. However,

some Caatinga ecoregions may have weak climatic stability (Werneck *et al.*, 2011). Werneck *et al.* (2012) investigated the historical distribution of the Cerrado and found evidence of two savanna corridors and predicted the presence of a large refuge in the north-eastern extreme of this biome.

The geological and paleo-climatic history of the Caatinga and Cerrado is complex and controversial (Werneck, 2011), and understanding the evolutionary history of the animal groups adapted to these semiarid regions should provide important insights into the role of Pleistocene climate changes in the diversification of the endemic biota of this unique region and its biogeographic history (Werneck *et al.*, 2012, 2015). In this case, we investigated the role of climatic change on the biota of the Brazilian Caatinga and Cerrado biomes using the stingless bee *Partamona rustica* (Camargo & Pedro, 2003) as a model. *Partamona rustica* is endemic to north-eastern Brazil, where it inhabits the Caatinga of south-western Bahia and ecotone between the Caatinga and the Cerrado in northern Minas Gerais. Its nests are often associated with those of the arboreal termite *Constrictotermes cyphergaster*, where they build its nests (Camargo & Pedro, 2003; Miranda *et al.*, 2015). This bee is a floral visitor of at least 62 plants, and is probably an important pollinator in these biomes. Because of increasing anthropogenic impacts, however, this species is now rare in some areas (Miranda *et al.*, 2015).

In the present study, we used an integrative approach to reconstruct the evolutionary history of *P. rustica* in a spatial-temporal framework. Specifically, we investigated the genetic diversity and structure of its populations, and how climatic changes during the Pleistocene may have influenced the demographic history of these populations. We also inferred species distribution patterns since the late Pleistocene to identify possible species-specific refuges. We then applied a coalescent-based

Approximate Bayesian Computation (ABC) approach to test competing scenarios of the origin and dispersal of *P. rustica* populations across the species current geographic range.

MATERIALS AND METHODS

STUDY AREA AND SAMPLING

We sampled adult *P. rustica* workers from 145 nests at 11 localities in the Caatinga of south-western Bahia (localities 1 to 9 in Table 1) and in the transition zone – the ecotone – between the Caatinga and Cerrado in northern Minas Gerais (localities 10 and 11 in Table 1), in Brazil (Fig. 1A), between May 2012 and January 2014.

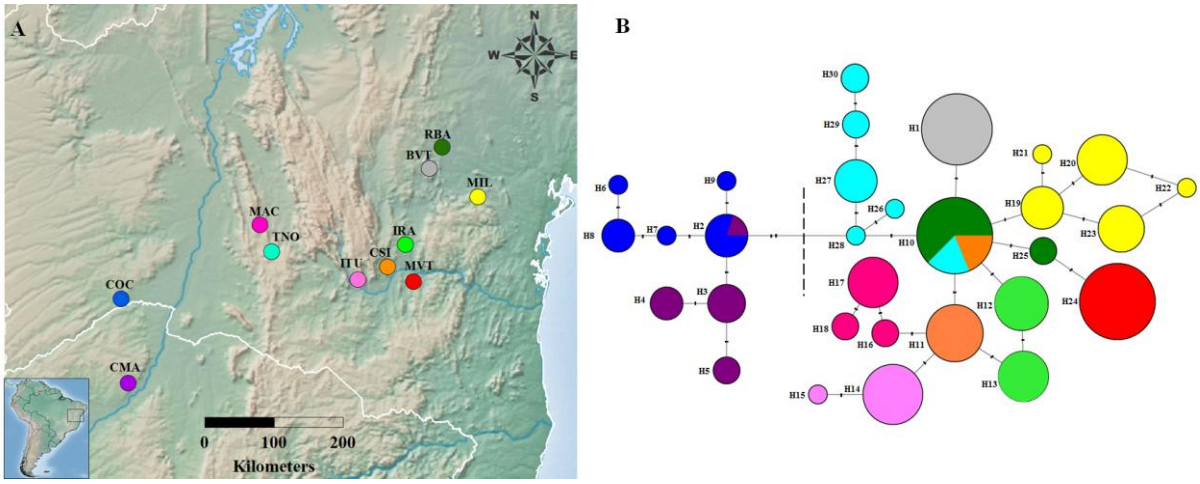

Figure 1. Map of the geographical distribution of populations (A) and median-joining haplotype network of the four mtDNA genes (B) in *P. rustica*. The coloured circles represent the 11 localities sampled in the present study (listed in Table 1). The haplotypes are coloured according to the scheme in "A". The dotted line in the haplotype network separates the eastern and western groups. The black lines represent the positions of the mutations in the haplotypes.

Table 1. Localities sampled in the present study, geographic coordinates, altitude (in meters), number of nests (N) and genetic diversity estimates for the eight microsatellite loci and four concatenated mitochondrial gene regions (12S, 16S, COI and COI-COII) of the *Partamona rustica* populations and for the two population groups (Ar, allelic richness; He, expected heterozygosity; S, number of polymorphic sites; h, number of haplotypes; π, nucleotide diversity; Hd**,** haplotype diversity). All sites are located in the Brazilian state of Bahia (Brazil), except Cônego Marinho, in the state of Minas Gerais.

	Localities						**Microsatellites**		**mtDNA (concatenated)**			
		Code	**S**	**W**	**A**	**N**	**Ar**	**He**	**S**	**h**	**π**	**Hd**
1	B. Vista do Tupim	BVT	-12.502	-40.471	328	14	2.041	0.242	0	1	-	-
2	Contendas do Sincorá	CSI	-13.794	-41.017	300	12	1.785	0.222	1	2	0.00018 (±0.00006)	0.409 (±0.133)
3	Iramaia	IRA	-13.862	-40.081	382	15	2.100	0.251	1	2	0.00024 (±0.00002)	0.533 (±0.052)
4	Ituaçu	ITU	-13.882	-41.325	586	11	2.995	0.383	1	2	0.00008 (±0.00006)	0.182 (±0.144)
5	Macaúbas	MAC	-12.243	-40.250	756	11	2.946	0.379	2	3	0.00029 (±0.00009)	0.582 (±0.142)
6	Milagres	MIL	-12.931	-39.720	365	20	1.961	0.243	3	5	0.00048 (±0.00005)	0.758 (±0.050)
7	Manoel Vitorino	MVT	-13.939	-40.563	263	16	2.475	0.293	0	1	-	-
8	Ruy Barbosa	RBA	-12.228	-40.311	314	12	1.849	0.270	1	2	0.00013 (±0.00007)	0.303 (±0.147)
9	Tanque Novo	TNO	-13.595	-42.521	789	14	3.076	0.354	4	6	0.00070 (±0.00009)	0.835 (±0.070)
10	Cocos	COC	-14.158	-44.403	587	10	3.583	0.505	4	5	0.00066 (±0.00012)	0.800 (±0.100)
11	Cônego Marinho	CMA	-15.314	-44.382	632	10	3.621	0.473	3	4	0.00045 (±0.00009)	0.778 (±0.091)
	Groups											
	Eastern	(localities from 1 to 9)				125	2.359	0.288	15	22	0.00116 (±0.00005)	0.930 (±0.008)
	Western	(localities 10 and 11)				20	3.602	0.489	7	8	0.00085 (±0.00011)	0.879 (±0.040)
	All populations					145	2.585	0.328	22	30	0.00146 (±0.00007)	0.946 (±0.006)

LABORATORY PROCEDURES

The total DNA was extracted from one worker per colony using Sheppard and McPheron's (1991) protocol. The mitochondrial genes 12S, 16S, COI and the terminal region of subunit I and beginning of cytochrome oxidase subunit II (COI-COII) were amplified partially using the techniques described in Appendix S1. Information on the sequence of the primers can be found in Table S1 of Appendix S2. All mitochondrial haplotypes have been deposited in GenBank (accession numbers KT765104-KT765129). Eight microsatellite loci (SSR) were standardized for *P. rustica*. Six of these loci were described for *Partamona helleri* (Phel1, Phel2, Phel3, Phel4, Phel6 and Phel7) (Ferreira 2011) and two for *Melipona bicolor* (Mbi 232 and Mbi 254) (Peters *et al.*, 1998). The PCR cycling conditions followed Ferreira (2011). Information on the sequence of the primers, repeat motifs, annealing temperatures and the respective fluorophores are available in Table S2 of Appendix S2.

GENETIC DIVERSITY

The electropherograms were edited and combined in contigs using the Codon Code Aligner v3.7.1 software (CodonCode, Dedham, Massachusetts, United States). The sequences were aligned using CLUSTAL W in the BioEdit 7.0.9.0 program (Hall, 1999). All alignments were inspected and corrected visually.

To assess genetic diversity in *P. rustica*, we estimated the number of variable sites (S), number of haplotypes (h), haplotype diversity (Hd) and nucleotide diversity (π) for each mitochondrial gene region and for the concatenated gene regions using DnaSP v5.10.01 (Librado & Rozas, 2009).

For the analyses of the SSR dataset, Microchecker v. 2.2.3 (van Oosterhout *et al.*, 2004) was first used to check for null alleles, scoring errors and large allele dropouts.

We estimated the number of alleles, allelic richness (Ar - mean number of alleles, corrected by the smallest sample number) and genetic diversity using Fstat 2.9.3.2 (Goudet, 2001). We also applied Fisher's exact test to test the microsatellite loci for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium, using the Markov chain approach in Genepop v.4.2 (Raymond & Rousset, 1995).

ANALYSIS OF GENETIC STRUCTURE

We obtained a haplotype network for the concatenated gene regions using the median-joining network method (Bandelt *et al.,* 1999) implemented in NETWORK 4.6.1.3 (http://www.fluxus-engineering.com/). In order to verify the occurrence of population groups by the SSR dataset, we used the AMOVA-based K-means clustering method (Meirmans, 2012) in kMeans v.1.1. (http://www.patrickmeirmans.com/software/). We also conducted a spatial analysis of molecular variance (SAMOVA) using the SSR and mtDNA datasets (separately) in SAMOVA 2.0 (Dupanloup *et al.,* 2002). For further information, see Appendix S1.

We also implemented the Mantel test using the Isolation by Distance Web Service 3.23 (IBDWS; Jensen *et al.,* 2005) to investigate the potential correlation between genetic (pairwise Φ_{ST}) and geographic (in kilometres) distances. The significance of this analysis was obtained with 10,000 permutations of both mtDNA and SSR datasets, in analyses run separately.

POPULATION DEMOGRAPHY, DIVERGENCE TIMES AND MIGRATION

To evaluate the dynamics of population size over time, we implemented a Bayesian Skyline Plot (BSP) to reconstruct each population group, with the analysis being run in BEAST 1.8.1 (Drummond *et al.,* 2013), using the concatenated COI and COI-COII

regions. We used the following parameters: a strict clock, 60 million generations for the western group and 200 million generations for the eastern group, sampling parameters at every 1000 generations of the Markov Chain Monte Carlo (MCMC) analysis, with a 10% burn-in period. To obtain the absolute times, we used the COI substitution rate of 1.3–1.9% per lineage per million years (with a uniform distributed prior) calibrated for other hymenopterans (Machado *et al.,* 2001; Moreau *et al.,* 2006). We used the HKY substitution model for the western group and the GTR+I model for the eastern group, as selected by jModeltest v.2.1.5 (Darriba *et al.,* 2012), based on the Akaike information criterion (AIC). We checked for convergence between runs and the performance of the analysis using Tracer 1.5 (Rambaut *et al.,* 2013), and accepted the results if effective sample size (ESS) values were greater than 200.

Divergence times, effective population sizes and migration rates between western and eastern *P. rustica* groups were calculated based on the isolation with migration (IM) model (Nielsen & Wakeley, 2001; Hey & Nielsen, 2004) using IMa2 (Hey, 2010), for the COI and COI-COII sequences. This program estimated six demographic parameters for pairs of populations: the splitting time (t = mutation-scaled time since divergence), migration rates between groups ($m_{west>east}$ and $m_{east>west}$ = mutation-scaled migration rate), and the effective population sizes of the eastern, western and ancestral populations ($\theta_{eastern}$, $\theta_{western}$ and $\theta_{ancestral}$).

We first conducted a series of relatively short preliminary runs in the 'MCMC mode'. These short runs were used to adjust the run duration and prior distribution for each parameter and the heating scheme for the subsequent, longer runs. For the final run, we used 40 MCMC chains with a geometric heating scheme (g1 = 0.975 and g2 = 0.75), with the maximum prior population sizes set at 30, migration rates at 20 and

divergence time at 20. We used the HKY substitution model and an inheritance scalar of 0.25. We assumed the same mutation rate for COI (1.3–1.9% per lineage per million years) as in BEAST and a generation time of one year. The run had eight million steps. The first two million steps were discarded as burn-in. We also checked convergence by analysing the posterior distributions, and the ESS values that were higher than 200. We also tested the SSR dataset (on its own or together with the mtDNA data). We removed the ribosomal regions from the IMa2 and BSP analyses because no substitution rates have yet been estimated for these regions in the Hymenoptera.

Finally, we tested for evidence of recent population bottleneck events in the SSR dataset in BOTTLENECK v. 1.2.0.2 (Cornuet & Luikart, 1996). The run for each population (eastern and western groups) was based on the stepwise mutation model (SMM) and the significance was evaluated by Wilcoxon's test with 10,000 replications. A significant number of loci with excess heterozygosity is expected in bottlenecked populations, while a deficiency of heterozygosity is expected in expanding populations (Cornuet & Luikart, 1996).

TESTING ALTERNATIVE SCENARIOS BY ABC MODELLING

To evaluate the fit of the different historical scenarios to the data obtained for the *P. rustica* populations, we implemented the Approximate Bayesian Computation procedure (ABC; Beaumont *et al.,* 2002) available in DIYABC v2.1 (Cornuet *et al.*, 2014). We assumed the existence of two groups of populations (eastern and western), based on the K-means and the results of the SAMOVA (see Table 2). We compared competing hypotheses on the origin and dispersal of the *P. rustica* populations by investigating five potential historical scenarios: scenario 1 - at t1, the eastern population

group gives rise to the western group; scenario 2 - at t1, the western group gives rise to the eastern group; scenario 3 - at t1, an ancestral population splits and gives rise to both groups; scenario 4 - at t2, an ancestral population gives rise to the eastern group, and at t1, the eastern group gives rise to the western group; scenario 5 - at t2, an ancestral population gives rise to the western group, and at t1, the western group gives rise to the eastern group (see Fig. 2). We estimated the posterior probability of these five putative scenarios for *P. rustica* using the mtDNA and SSR datasets both separately and together. For both datasets, the priors were set at a uniform distribution, with a generation time of one year. For the mtDNA dataset, the simulations were run for four million iterations, with a different set of summary statistics being generated for each population (the number of haplotypes and segregating sites) and between populations (the number of haplotypes, FST and the mean pairwise differences, w), with the mutation rate being left on the default setting. For the SSR dataset, the simulations were also run for four million iterations with summary statistics being generated for: (1) the number of alleles, (2) mean genetic diversity, (3) mean size variance, (4) FST, (5) the classification index and (6) the $(d\mu)^2$ distance. Once again, the mutation rate was left on the default setting. We assumed a generation time of one year for all analyses. All other settings were configured as suggested by the DIYABC recommendations for the SSR and mtDNA datasets. The posterior probability of each scenario was calculated by logistic regression, considering between 500 and 50,000 datasets that were closest to the observed values. The scenario that best explained the data was used to estimate the origin and dispersal of *P. rustica.*

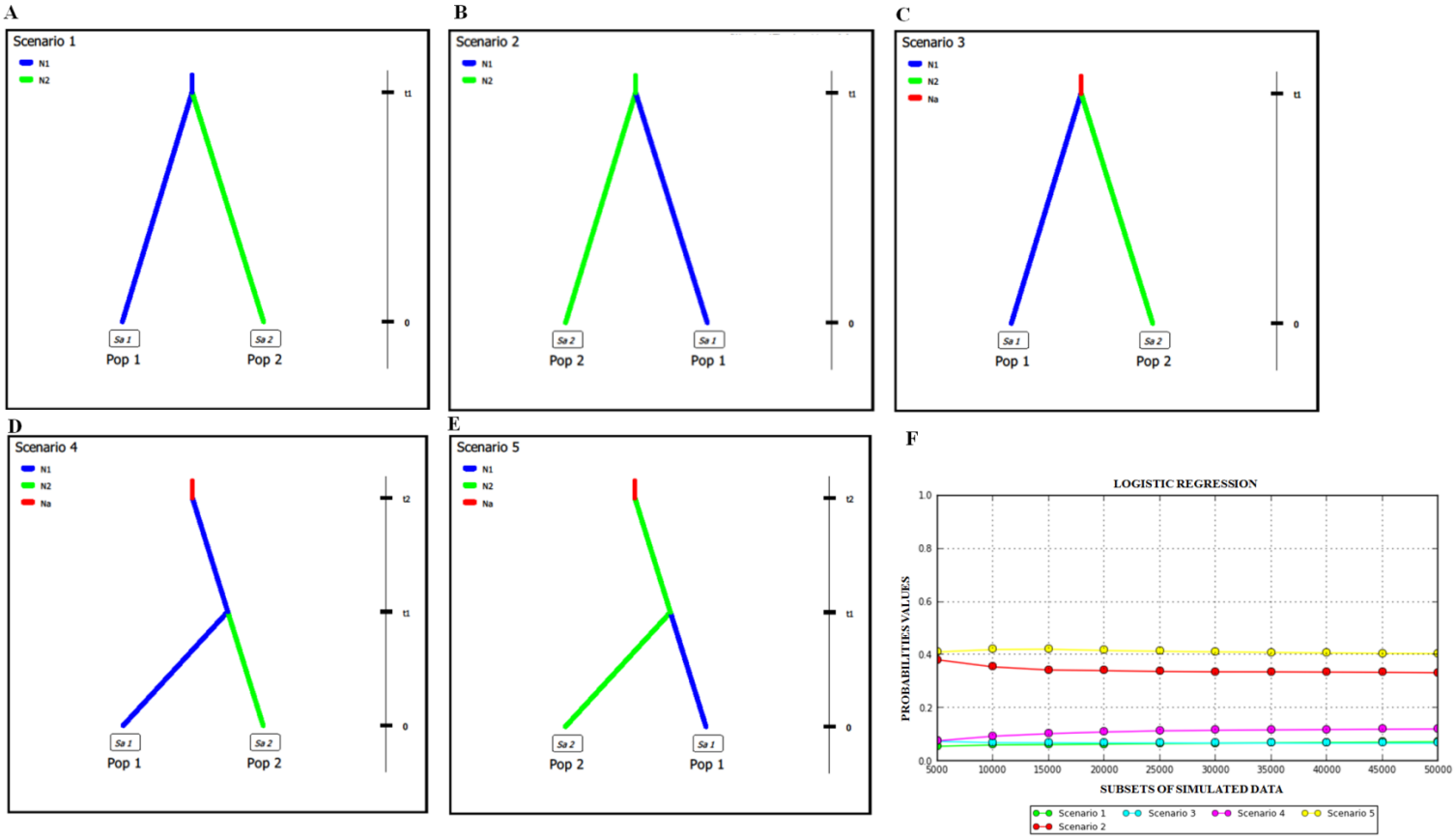

Figure 2. The scenarios tested in the DIYABC analysis and logistic regression. Graphic representation of the five scenarios tested for the eastern (pop1) and western groups (pop2) in DIYABC. In scenario 1 (A) - at t1, the eastern population group gives rise to the western group; scenario 2 (B) - at t1, the western group gives rise to the eastern group; scenario 3 (C) - at t1, an ancestral population splits and gives rise to both groups; scenario 4 (D) - at t2, an ancestral population gives rise to the eastern group, and at t1, the eastern group gives rise to the western group; scenario 5 (E) - at t2, an ancestral population gives rise to the western group, and at t1, the western group gives rise to the eastern group. The logistic regressions of the posterior probabilities for each scenario are shown in "F".

HISTORICAL CLIMATE MODELLING

Once a contemporary model (i.e., 1950–2000) of the potential distribution of *P. rustica* was generated, based on Miranda *et al.* (2015), we projected the model onto past climatic conditions to predict the potential distribution of the species during three distinct periods of the late Quaternary: the Last Interglacial period (LIG, 120 ka), the Last Glacial Maximum (LGM, 21 ka), and the Mid-Holocene (M-H, 6 ka). We modelled potential distributions using MaxEnt 3.3.3k (Phillips *et al.,* 2006; Phillips & Dudík, 2008) based on the combined data for 20 localities, including the 11 localities sampled in the present study (Table 1) and a further nine sites recorded in the literature (Table S3 in Appendix S2), with 19 bioclimatic environmental descriptors available in the WorldClim database (www.worldclim.org). Because the collinearity of variables can result in the overfitting of the model (Carvalho & Del Lama, 2015), we omitted correlated variables through a multivariate analysis (Appendix S1).

We modelled the potential paleo-distribution of the species during the LGM and M-H periods using downscaled data derived from two Global Climate Models (GCMs) commonly used in recent analyses (e.g., Raes *et al.,* 2014; Lim *et al.,* 2015), the CCSM4 and MIROC-ESM models, with a spatial resolution of 2.5 minutes. To estimate the areas that putatively remained suitable for the species throughout the Late Quaternary, we applied a threshold based on the minimum training presence (i.e., the value of the lowest contemporary logistic prediction) to transform continuous suitability outputs into presence/absence raster files. The areas of stability were then estimated from the intersection of all the projections (CCSM4 + MIROC-ESM) and separately, on a downscaled GCM. For further information, see Appendix S1.

RESULTS

GENETIC DIVERSITY

We obtained a total of 2260 bps for the four mtDNA gene regions (Table 1). No indels were found in any of these regions. Nucleotide (π) diversity for the concatenated dataset was 0.00146 (±0.00007) and haplotype (h) diversity was 0.946 (±0.006) (Table 1).

The microsatellite loci had between two and 17 alleles. The allelic richness (standardised to a minimum sample size of eight) of the polymorphic loci ranged from 1.785 to 3.621, and the mean gene diversity (He - expected heterozygosity) was 0.328, ranging from 0.222 to 0.505 (Table 1). The genetic diversity of the western group was higher overall than that of the eastern group (see Table 1). Significant departures from HWE were found for the Mbi232 locus at ITU and Phel-1 at CMA, although no pairwise linkage disequilibrium was detected in any case. Finally, null alleles were detected only at BVT (Phel-1) and ITU (Phel-2 and Mbi232), and no evidence was detected of large allele dropout or scoring errors due to stuttering.

POPULATION STRUCTURE

We adopted different approaches to estimate the degree of population differentiation. Our haplotype network, based on the four mitochondrial gene regions, revealed the presence of two groups separated by two mutations, the first being restricted to the west of the São Francisco River Valley (SFRV), i.e., eight haplotypes at COC and CMA, and the second, to the east of the SFRV, represented by all the other nine populations and 22 haplotypes (Fig. 1B).

The AMOVA-based K-means cluster analysis of the SSR dataset indicated these two groups as the best clusters (see Table S4 in Appendix 2). The SAMOVA also confirmed the existence of the two groups (Fig. 3 and Table 2) with moderate differentiation between the

groups, based on both the mtDNA (Φ_{CT} = 0.468, P<0.0001; Φ_{ST} = 0.774; P<0.0001) and SSR datasets (Φ_{CT} = 0.218, P < 0.0001; Φ_{ST} = 0.247; P < 0.0001).

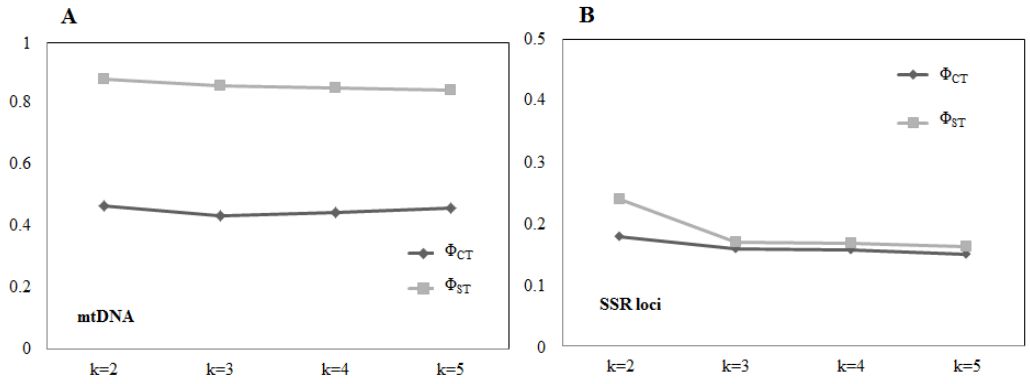

Figure 3. Spatial Analysis of Molecular Variance (SAMOVA) assessed for grouping schemes 2–5 of the *P. rustica* population from the 11 study localities based on the mitochondrial (A) and microsatellites (B) datasets, using estimates of fixation indices (Φ_{CT} and Φ_{ST}). All P-values were significant (P < 0.005).

Table 2. Results of the Analysis of Molecular Variance (AMOVA) with three hierarchical levels, testing the differentiation between the *P. rustica* groups. The analysis was run using the four concatenated mtDNA genes and eight SSR loci.

Region	Variation source	d.f	Variation (%)	Φ	P-Value
mtDNA	Between groups	1	46.88	Φ_{CT} = 0.468	<0.0001
	Among localities within groups	9	41.13	Φ_{ST} = 0.774	
	Within localities	134	11.99		
	Total	144			
SSR	Between groups	1	21.87	Φ_{CT} = 0.218	<0.0001
	Among localities within groups	9	2.85	Φ_{ST} = 0.247	
	Within localities	279	75.27		
	Total	289			

The Mantel test did not detect any significant correlation between genetic and geographic distances in the mtDNA data (r_{total} = -0.087, P = 0.627; $r_{eastern}$ = r = -0.402; P = 0.9820). This indicates that there is no isolation by distance between the populations analysed.

On the other hand, the results of the Mantel test for the SSR dataset indicated a significant positive correlation between genetic and geographic distances (r_{total} = 0.882, P < 0.0010; $r_{eastern}$ = 0.646; P = 0.0010), indicating isolation by distance among the populations (Fig. S1 in Appendix S2).

DIVERGENCE TIMES AND MIGRATION

In all tests using only SSR dataset and combined dataset with SSR and mtDNA we obtained poor values of ESS (ESS <11). Therefore, it was not possible to use the SSR loci (alone or with mtDNA dataset) in IMa2 analysis because the runs did not converge and became stationary. Thus, it was just possible to implement this analysis using mtDNA dataset. The estimate of the divergence time between the two groups of *P. rustica* by the IMa2 method indicated that the split occurred in the Late Pleistocene at *ca.* 102 ka (HPD 95%: 45,588 – 979,902 ka). The isolation with migration model also revealed low migration rates between the groups (Table 3 and Fig. 4). The IMa2 estimates of the effective population size (Ne) showed that the peak of the probability distribution is found in the eastern population, followed by the western and ancestral populations.

Table 3. Estimates of demographic parameters by the IMa2 model for the *P. rustica* mtDNA dataset.

	t	$\mathbf{m_{west > east}}$	$\mathbf{m_{east > west}}$	$\mathbf{\theta_{eastern}}$	$\mathbf{\theta_{western}}$	$\mathbf{\theta_{ancestral}}$
Highest point	102,451	0.01087	0.02573	122,610	71,875	22,610
Lower HPD of 95%	45,588*	0	0	59,007	18,934	0*
Upper HPD of 95%	979,902*	1.847	3.284	237,684	220,404	345,772*

HPD: High posterior density

t: Splitting time in years

θ: Effective population sizes of west, east and the ancestral population.

m: Migration rate in coalescent time.

*: HPD values with high precision and influenced by priors, because the likelihood distribution was flat at the end and did not touch zero.

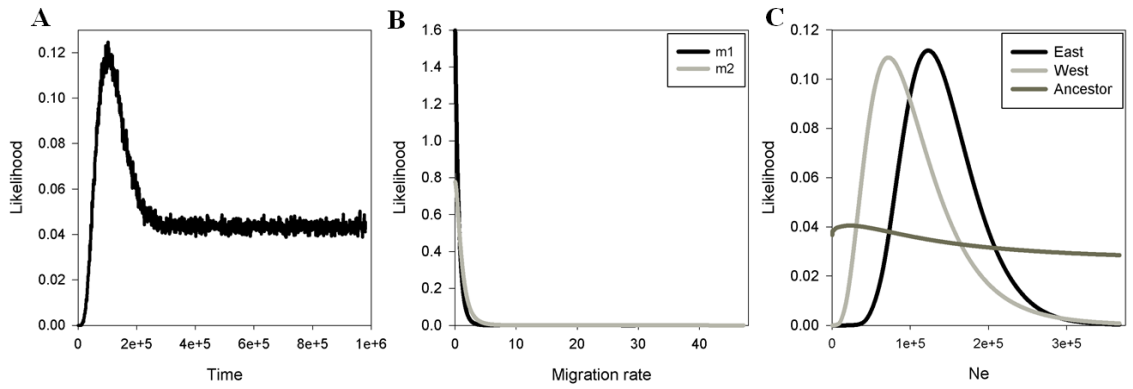

Figure 4. The IMa2 analysis of the *P. rustica* population groups from the west and east of the SFRV using the COI and the COI-COII gene fragment. In "A" Posterior probability distributions of the divergence time (t) between the two population groups is shown in millions of years (Mya); in "B" Posterior probability distribution of migration (m) from west to east ($m_{west > east}$) and east to west ($m_{east > west}$); in "C" Posterior probability distribution of the Ne (effective population size), given as the number of individuals

POPULATION HISTORY BY ABC

The SSR dataset was omitted from the ABC analyses because it did not adjust to any of the models tested, alone or together mtDNA dataset. The ABC analysis for the mtDNA dataset identified scenario 5 as the origin and dispersal scenario with the highest posterior probability, followed by the scenario 2 (Fig. 2). Both scenarios comprehend a western origin with a recent origin of eastern populations. In scenario 5, the ancestral originating western group at *ca*. 258 ka (HPD 95%: 7.0– 476 ka) and western group then diverged to form the eastern group at *ca.* 13 ka (HPD 95%: 10 – 26 ka). In scenario 2, western group gave rise to the eastern group at *ca.* 14 Ka (HPD 95%: 10,500 - 34,300) (Fig. 2). There is overlapping of confidence intervals between the posterior probabilities of the scenario 5 (P_{S5} = 0.4114, 0.3383 - 0.4845) and scenario 2 (P_{S2} = 0.3819, 0.2986 -0.4651), therefore, we can consider both scenarios as probable.

HISTORICAL DEMOGRAPHY

The BSP did not reveal any marked fluctuations in the effective size (Ne) of the population in either group, although there was a slight reduction in the median Ne values at 5 Ka (Fig. 5), although this was accompanied by an increase in confidence intervals. Similarly, no evidence was found of any recent population bottlenecks or demographic expansion in the SSR dataset of the western group (Wilcoxon's test - He deficiency, P = 0.1250; He excess, P = 0.9023). On the other hand, we found a non-significant excess in He (Wilcoxon: P = 1.0000) and a significant deficiency (P = 0.0019) in the eastern group, which suggests a population expansion.

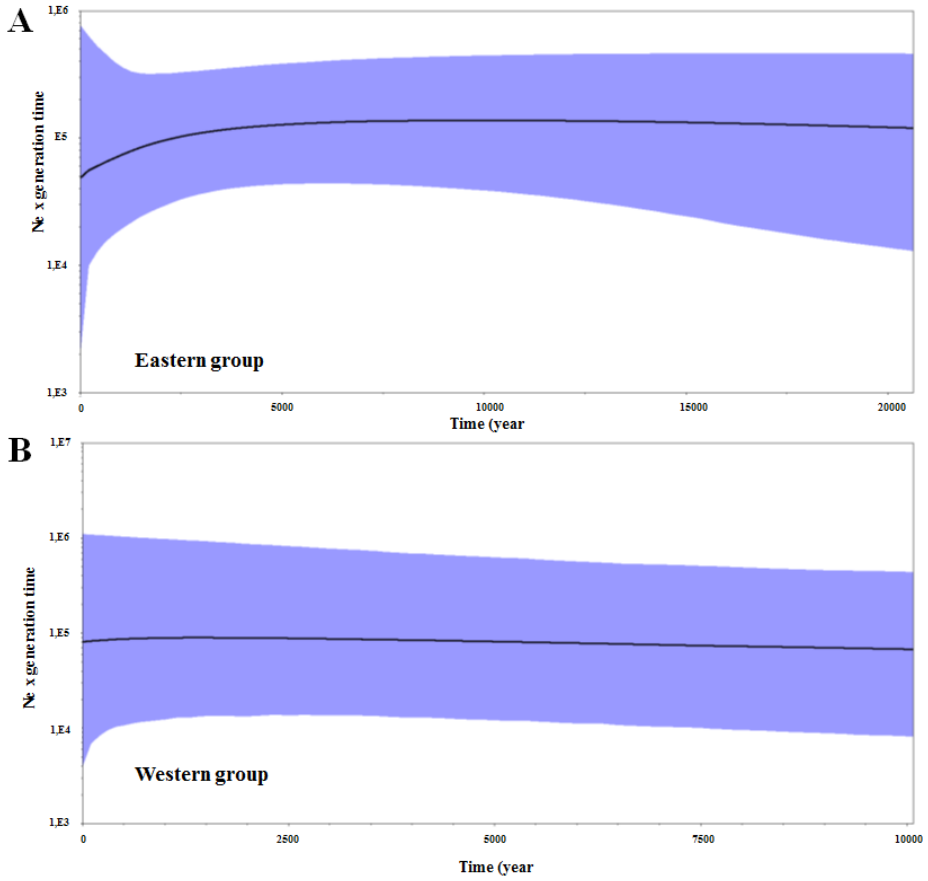

Figure 5. Coalescent Bayesian Skyline Plot (BSP) used to infer the demographic history of *P. rustica* populations to the east and west of the SFRV. The dotted horizontal line shows the median estimate of the BSP and the blue area shows the upper and lower 95% highest posterior density limits.

HISTORICAL DISTRIBUTION PATTERNS

Our ENM analysis accurately predicted the current distribution of *P. rustica*, with an AUC of 0.96. During the LIG, few suitable areas were observed within the current range of *P. rustica* (Fig. 6F), whereas a higher degree of suitability was observed during the M-H (Fig. 6B and 6C), and a slightly lower one during the LGM (Fig. 6D and 6E). All the models indicated climate stability to the east of the SFRV, although alternative outcomes were observed in the simulations for the western group (Fig. 6). Considering that the downscaled Global Climate Models (GCM), i.e. CCSM4 and MIROC-ESM, are generated by different processes that naturally produce different outcomes, we present the results separately (Fig. 6G and 6H), before combining the simulations in a consensus model (Fig. 6I). Zones of stability were defined here as areas in which at least three models indicated the potential local occurrence of *P. rustica* during the historical period analysed (Fig. 6G, 6H and 6I).

Despite some differences among the models, a continuum of putatively stable areas for *P. rustica* was identified to the east of the SFRV in all models. This zone covers central Bahia, including the Chapada Diamantina hills, and northern Minas Gerais (Fig. 6I). Almost all the eastern populations are found in this potentially stable area, which is characterised by a high degree of historical suitability, whereas the western group is located within an area of much lower stability. This analysis also pointed to climatic instability in the SFRV and north-eastern Bahia (Fig. 6I).

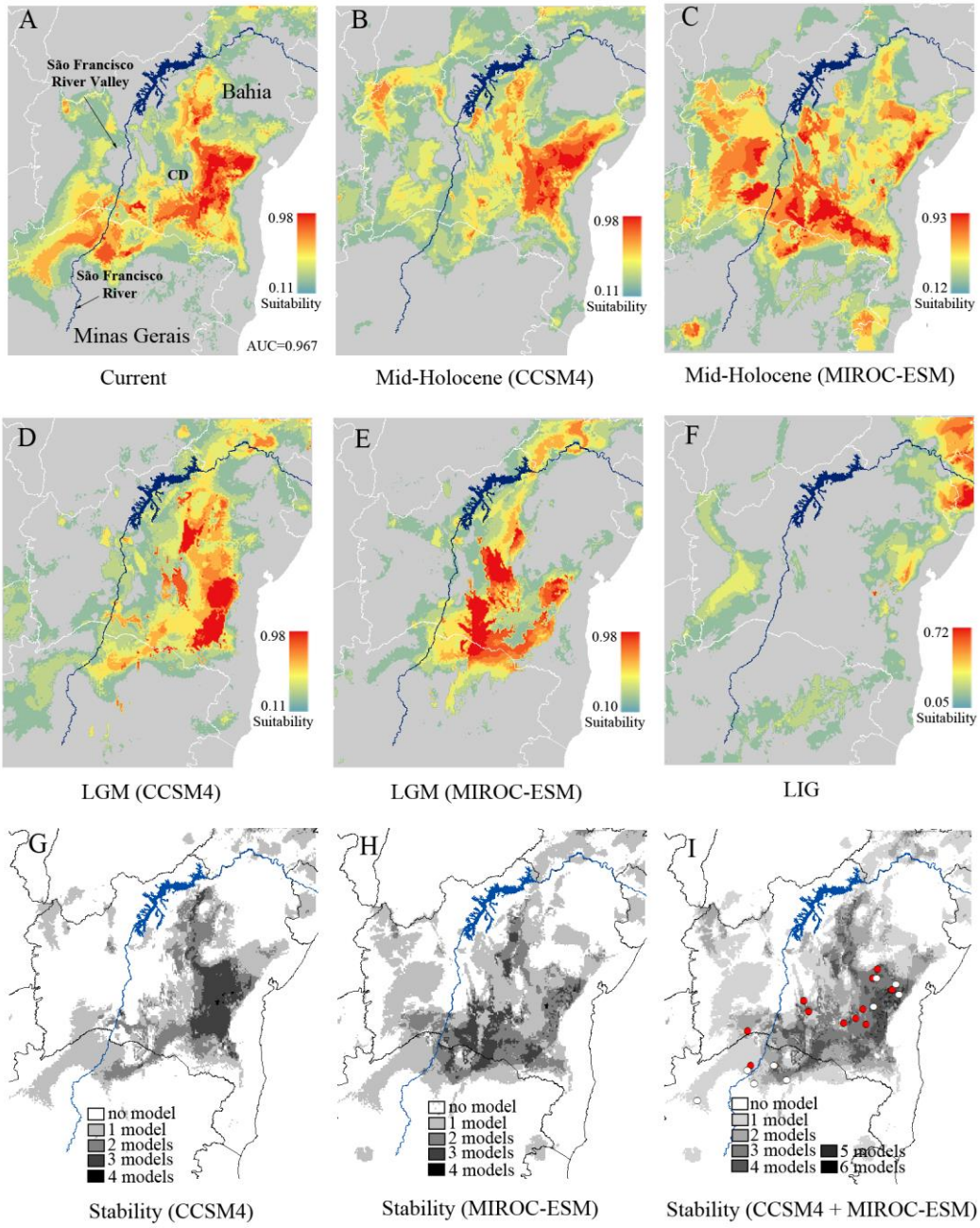

Figure 6. Ecological niche modelling showing the potential geographical distribution of *P. rustica* in different periods and stability models. In "A" Current, (B and C) Mid-Holocene, (D and E) Last Glacial Maximum (LGM) and "F" Last Interglacial period (LIG). The stability models (G to I) refer to the overlap of the potential distribution maps using two Global Climate Models combined (I) and each GCM separately (G and H). In "I", the red points represent the localities sampled in the present study and white points represent other localities obtained from collections and museums. The legends indicate the probability of suitable conditions for the species. Abbreviations: CD = Chapada Diamantina; AUC = area under the curve.

DISCUSSION

PHYLOGEOGRAPHIC STRUCTURING AND EVOLUTIONARY HISTORY

Overall, our population structure analyses (haplotype network, K-means and SAMOVA) of both mtDNA and SSR datasets were consistent in their allocation of *P. rustica* populations to two groups, one distributed to the east of the SFRV, and the other to the west. The IMa2 approach indicated that the two groups diverged in the Late Pleistocene. The ABC analysis proposed a historic process in which the *P. rustica* populations diverged from an ancestral population that initially gave rise to the western group (found in north-western Minas Gerais), which then dispersed to the east of the SFRV, where the species probably colonised central and south-western Bahia (scenario 5). Another probable hypothesis is that *P. rustica* populations diverged from western group, which then dispersed to the east of the SFRV, as showed by the scenario 2. Therefore, both scenarios have shown the western group as ancestral of the eastern group. This probably accounts for the greater genetic diversity found in the SSR data for the western group in comparison with the eastern group, despite the much smaller sample available for the former group. This result agrees closely with Pedro & Camargo, (2003), who confirmed that *P. rustica* is endemic to the region that stretches from northern Minas Gerais, through the Espinhaço hills to south-western Bahia.

While the two *P. rustica* groups are well-defined, estimates of migration indicate reduced levels of bidirectional gene flow between these groups. This reflects the moderate degree of differentiation between groups in both the mtDNA and the SSR dataset, which contrasts with the within-group variation especially in the SSR markers, and may be accounted for by the relatively recent evolutionary history of *P. rustica*. However, the divergence time indicated by the ABC approach was shorter than that suggested by the IMa2, which may be due to the migration between groups, which is not contemplated by the

DIYABC, which assumes an absence of migration among populations after they have diverged (Cornuet *et al.*, 2014).

While our results have revealed a moderate degree of differentiation between *P. rustica* population groups, our reduced sampling of the western region, and the probable gap of some 200 km between the groups hampers the analysis of the potential role of the SFRV as a barrier to gene flow, ecological differences (ecotone habitats in the west *vs*. Caatinga proper in the east) or isolation by distance, as found in the SSR data. This is despite our considerable sampling efforts within the known ranges of both groups and at others sites in the western region, which were unsuccessful.

ISOLATION BY DISTANCE AND SEX-BIASED DISPERSAL

The Mantel test found no evidence of isolation by distance in the mtDNA data, indicating that the differences found among populations cannot be accounted for by the physical distances among them. By contrast, strong evidence of isolation by distance was found in the SSR dataset. Similarly, while the mitochondrial genes indicated a high degree of differentiation between populations, only moderate differentiation was found in the SSR dataset (Table 2). These apparent disagreements are common in recently-diverged lineages (Gómez-Zurita & Vogler, 2003) and may be the result of long-term male-biased dispersal, which results in genetic structuring in the mtDNA, but panmixia in the nuDNA (SSR). Wille & Orozco (1975) confirmed that new *Partamona* queens are phylopatric, remaining in their place of origin. These new queens cannot migrate more than 300 meters from the maternal nest in the swarming process, given their limited dispersal capacity (Wille & Orozco, 1975; Araújo *et al.*, 2004). Even so, due to the peculiar system of sex determination in Hymenoptera, it is necessary for these bees to have mechanisms that prevent endogamy in order to avoid the generation of diploid males due to homozygosity in the sex locus (Hasselmann *et al.*, 2008). According to Fernandes *et al.* (2012), only a few founder phylopatric female *Partamona* will

colonise new areas, while the males disperse and promote gene flow among populations. This pattern was can also be observed in our mtDNA haplotype network, which showed that each locality may have been colonised by only one or few maternal lineages. Therefore, this disagreement between the isolation by distance tests, together with the observed population differentiation, can be explained by the phylopatric females and dispersing males in *P. rustica*, featuring a sex-biased dispersal pattern. Alternatively, these results may reflect true differences in the history of genetic markers within organisms, i.e., modes of inheritance and degree of ploidy (Gómez-Zurita & Vogler, 2003). However, studies of the colonisation processes resulting from female-mediated and male-biased dispersal patterns are currently in progress in our laboratory, to test whether these findings are widely applicable to *Partamona* populations in general.

HISTORICAL DEMOGRAPHY AND PALEOMODELLING

The BSP yielded consistent results indicating that there were no marked changes in effective population size in either the western or eastern groups of *P. rustica* (Fig. 5). While we found a significant deficiency of heterozygosity in the eastern group, which might indicate a process of expansion, this signal may actually correspond to a false signal of recent demographic expansion induced by isolation by distance, asymmetric gene flow and the recent emergence of rare alleles through migration (Leblois *et al.,* 2006; Paz-Vinas *et al.,* 2013), all scenarios that were observed during the present study. These results were confirmed by the paleomodelling, which showed a marked increase in the potential range of *P. rustica* from LIG to the present day, as well as areas of stability that harbour most of the known range of *P. rustica* (Fig. 6I), including most of the localities sampled in this study. These areas may have acted as refuges for the species, in particular to the east of the SFRV. On the other hand, the results of our ENM also indicated few areas of stability in the western region, which may have been at least partly influenced by the relatively small number of records obtained from

the western region in comparison with the eastern portion of the study area. Therefore, our results indicate that the inferences both from the genetic data analyses and from the spatial distribution modelling are compatible with a history of constant size populations.

The refuge areas proposed here for *P. rustica* (Fig. 6I) overlap the SDTF refuge proposed by Werneck *et al.* (2011) for the southern portion of the Southern Backlands Depression (Depressão Sul-Sertaneja) of Bahia. Our results also point to the southern Chapada Diamantina and the SFRV as areas of instability for the species, as well as north-eastern Bahia (Fig. 6G, 6H and 6I), as observed by Werneck *et al.* (2011). Our findings also confirm the potential current distribution of *P. rustica* proposed by Miranda *et al.* (2015).

The use of a single molecular marker, such as mtDNA, to infer demographic history, divergence time, the origin of a species, and its history of dispersal has certain limitations and potential biases (Ballard & Whitlock, 2004), although the validity of a molecular marker depends primarily on the structure of populations being analysed. In this context, some characteristics of the stingless bees, such as limited long-distance migration (Araújo *et al.*, 2004), female phylopatry (Wille & Orozco, 1975), and colonies composed primarily of females make the analysis of maternally-inherited mtDNA markers a highly suitable approach for studying the evolutionary history of these animals. In this case, the population history of the females is what probably determines the long-term reproductive success and survival of the population. Overall, then, while the exclusive analysis of mtDNA markers does not cover the entire demographic and evolutionary history of a species, it has proven to be a very useful tool for the study of closely-related populations (Gilbert *et al.*, 2008; Alberdi *et al.*, 2015).

Quaternary oscillations in climate influenced a number of aspects of the evolution of species (Hewitt, 2004), including extinctions and changes in geographic ranges. In many cases, these climatic effects have yielded population genetic signatures (Avise, 2009), and the detection of these signatures is essential for the reliable evaluation of a species distribution,

genetic diversity, demographic history, and genetic endemism (Graham *et al.*, 2006; Carnaval *et al.*, 2009, 2014).

Understanding the history of the distribution of a species in essential for the development of effective conservation strategies, especially for species such as *P. rustica*, which have a very restricted distribution. Miranda *et al.* (2015) concluded that bee hunters are the most serious threat to *P. rustica*, which has been overexploited for the collection of honey, pollen, and wax, with the result that nests are becoming rare or even absent in many areas. Deforestation and livestock farming have further contributed to the extinction of the species in some areas.

While studies of the biogeography and phylogeography of the taxa found in dry forest environments are increasing, the role of Pleistocene glaciations and vicariant events in the diversification of this biota are still poorly understood. These ecosystems have high levels of endemism, which are threatened increasingly by ongoing anthropogenic impacts. For this reason, it is essential to understand the evolutionary processes that have shaped the genetic diversity of the dry forest biota in order to develop appropriate conservation strategies. Our results contribute to the interpretation of the biogeographic scenarios that arose in the Caatinga and Cerrado biomes during the Pleistocene and reinforce the need for further, more detailed investigation of the dry environments of the Neotropical region.

ACKNOWLEDGMENTS

We are grateful to Fundação de Amparo à Pesquisa de São Paulo - (FAPESP, processes numbers 2012/23342-1, 2011/21501-2, 2011/13391-2 and 2013/04317-9) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, PNPD 1462228) for financial support, to Dra. Silvia Regina de Menezes Pedro (FFCLRP - USP) for the identification of the bees and to all beekeepers and locals that helped us during field surveying. We also thank to Dr. Charles Goodnight for comments on an earlier version of this manuscript and to Carolina Machado and Manolo Perez for help with ABC analyses.

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SUPPORTING INFORMATION

Appendix S1 Details of the methodologies used.

Appendix S2 Supporting tables (Tables S1–S5) and supporting Figs (Fig. S1).

SUPPORTING INFORMATION

Appendix S1 - Details of the methods used

AMPLIFICATION OF THE MITOCHONDRIAL DNA

The PCR (25 μL) contained template DNA (50 ng), 1X of Taq buffer (Invitrogen), 250 μM of each dNTP, l.0 μM of each primer, 2.5 mM of $MgCl_2$ and 1 U of Taq polymerase (Invitrogen). The PCR conditions were as follow: an initial denaturation step at 94ºC for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C (COI, COI-COII and 12S) or 48ºC (16S) for 20 s, and extension at 70°C for 1 min, with final extension at 70°C for 10 min. The PCR products were electrophoresed in agarose gel stained with Gel Red™.

The amplified DNA was purified using illustra ExoProStar 1-Step kit (GE). The purified products were used as templates for sequencing in both forward and reverse directions. The DNA sequencing was carried out using a BigDye v 3.0 Dye Terminator Cycle Sequencing kit (Applied Biosystems, Inc., Carlsbad, CA, USA) following the manufacturer's protocol, with the same primers used for the amplification. The sequences were analysed in an ABI 3730 XL automated sequencer (Applied Biosystems).

GENETIC STRUCTURE ANALYSIS

We used the AMOVA-based K-means clustering method (Meirmans, 2012) for the analysis of the populations groups. This procedure was implemented in kMeans v.1.1 (http://www.patrickmeirmans.com/software/) using the SSR dataset, following the settings recommended by the author of the program: a maximum number of clusters of 11; AMOVA, to calculate the distances; Pseudo-F to determine the optimal number of clusters; simulated annealing, as a clustering algorithm; 50,000 steps for the simulated annealing chain; and 10

repeats of the algorithm. We also used the Spatial Analysis of Molecular Variance (SAMOVA) to test for geographical groupings that are genetically homogeneous and maximally differentiated from each other, without making *a priori* assumptions on group assignments, using both mtDNA (all concatenated gene regions) and SSR datasets separately; this approach was implemented in SAMOVA 2.0 (Dupanloup *et al.*, 2002). In this analysis, we tested for the existence of between two and five groups (k2 – k5), based on 1000 simulations of the annealing process in each case. We then compared the fixation indices (Φ_{ST} – structure among localities among groups; Φ_{CT}–structure among groups) and percentage of variation between ks and chose the k with the highest levels of differentiation.

HISTORIC CLIMATE MODELLING

As redundant variables may cause overfitting of the outputs of the modelling, we identified variables that were highly correlated with one another using the multivariate Principal Component Analysis (PCA), run in PAST (Hammer *et al*. 2001). We excluded those variables with the lowest percentage contribution to the final model of *P. rustica* distribution under current conditions. This is the only step that differs from Miranda *et al*. (2015), and it was applied after the assignment of the values of each bioclimatic variable to the *P. rustica* occurrence records using the "extract multi values to points" tool in ArcMap 10.1. Following this step, we constructed a correlation matrix in PAST to produce the scatter diagram resulting from the PCA. The redundant variables were identified from their respective contribution to the model, and the correlated variables with the lowest percentage contribution to the model were eliminated (Carvalho & Del Lama, 2015). Based this approach, we removed two variables, BIO1 and BIO19. The final model was built after the exclusion of these variables, and the projections of past scenario were implemented using the same

MaxEnt parameters described by Miranda *et al*. (2015) during the construction of the definitive model of climatic stability for *P. rustica*.

Ecological niche modelling was conducted based on the minimum area for the known range of occurrence of the species in order to minimise the influence of background noise on the model output (Anderson & Raza, 2010). The potential distribution map was edited in Arc-Gis 10.1 and we used DIVA-GIS 7.5.0 (http://www.diva-gis.org/) to define the overlap of the potential distribution maps generated for different periods and identify the putative areas of stability within the range of *P. rustica.*

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SUPPORTING INFORMATION

Appendix S2 - Supporting tables and supporting figures

Table S1. Primers used for PCR of mitochondrial genes amplifications.

Region	Primers	Reference
16S	F: 5'-TATAGATAGAAACCAATCTG-3' R: 5'-CACCTGTTTATCAAAAACAT-3'	Hall & Smith (1991)
12S	F: 5'-TACTATGTTACGACTTAT-3' R: 5'-AAACTAGGATTAGATACCC -3'	Simon *et al*. (1994)
COI	F: 5'-GGAGATCCAATTCTTTATCAAC-3' R: 5'-GATATTAATCCTAAAAAATGTTGAGG-3'	F: Afonso (2012) R: Dick *et al*. (2004)
COI-COII	F: 5'-TCTATACCACGACGTTATTC-3' R: 5'-GATCAATATCATTGATGACC-3'	Hall & Smith (1991)

Table S2. Repeat motifs, annealing temperatures (Ta) and each respective fluorophores of the microsatellite loci used for analysis of *P. rustica*.

Locus	Motif	Size	Ta (ºC)	Fluorophore	Primer
Phel-1	AC	217-247	55ºC	TET	F: 5'- TCGGCCGCTCATGGATAAGT- 3' R: 5' - TCAACGCCAGTCGAGAAGAGGATG - 3'
Phel-2	TC	260-312	52ºC	TET	F: 5'- CGTTCAATTTACCGCACAA - 3' R: 5' - CCACGTATCCAGGCTTTTTA - 3'
Phel-3	CT	99-101	55ºC	JOE	F: 5' - GTCGCAATAGCAATAGG - 3' R: 5' - TGGTCGTCATCTGTTTT - 3'
Phel-4	GT	246-254	62ºC	FAM	F: 5' - AATAACACGCGCACCATCA - 3' R: 5' - ACACATACAGAAGAACGAAGAAAA - 3
Phel-6	CA	174-180	52ºC	HEX	F: 5' - TTGGCACGAAAAGAACA - 3' R: 5' - TTGAAAGCTGAAAAATCCA - 3'
Phel-7	TG	282-298	48ºC	HEX	F: 5' - TTACATAAGAGCAAAACT - 3' R: 5' - TCGAAAATGAAATAAATA - 3'
Mbi-254	AAG	208-232	52ºC	FAM	F: 5' - CAATCGTTGGAAGGGAAC - 3' R: 5' - GGACCTATACCCAAGTCCAT - 3'
Mbi-232	CTT	130-178	48ºC	FAM	F: 5' - TTTTTCTCTTAAATTTTCTTCT - 3' R: 5' - CTTACTCGACGACTTTATTT - 3'

Table S3. Records of occurrence of *P. rustica* obtained in Camargo and Moure's collection used in ecological niche modeling.

Site	S	W
Bonfinópolis de Minas-MG	-16.441	-46.110
Lontra-MG	-15.897	-44.294
Nova Porteirinha-MG	-15.776	-43.252
Januária-MG	-15.470	-44.512
Jaíba-MG	-15.317	-43.650
Maracás-BA	-13.424	-40.447
Amargosa-BA	-13.052	-39.631
Itatim-BA	-12.723	-39.728
Itaberaba-BA	-12.512	-40.351

Table S4. AMOVA-based K-means clustering by pseudo-f statistics (Calinski-Harabasz, 1974). The test indicate two groups (eastern and western – see Table 1)

numK	SS-total	SS-among	SS-within	r-squared	pseudo-f	Phi_ct
1	472.63	0.00	116.91	0.000	0.000	0.000
2	472.63	66.53	406.10	0.569	**11.887**	0.383
3	472.63	82.83	389.80	0.709	9.725	0.281
4	472.63	90.81	381.83	0.777	8.118	0.221
5	472.63	97.72	374.91	0.836	7.641	0.228

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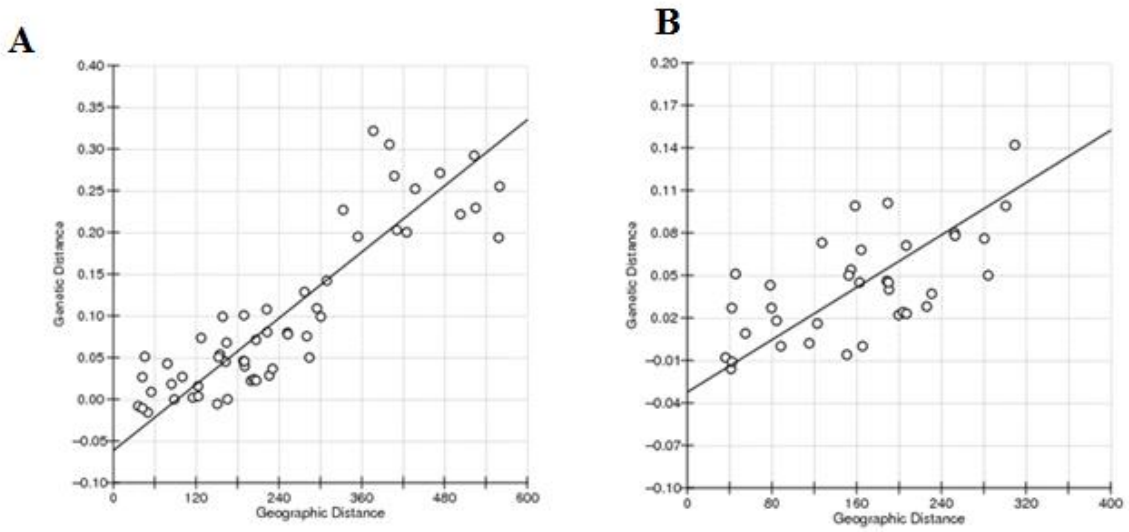

Figure S1. Isolation by distance test conducted for 11 populations of *P. rustica* using SSR markers (a - r_{total} = 0.882; P < 0.0010) and to the eastern group (b - $r_{eastern}$ = 0.6467; P = 0.0010).

CONSIDERAÇÕES FINAIS

CONSIDERAÇÕES FINAIS

Nesta tese foram apresentados aspectos da história natural, biogeográficos e ecológicos de *P. rustica* (Capítulo I); foi testada a hipótese de dispersão sexo-assimétrica por meio da análise genética de suas populações (Capítulos II e III) e foi contada a possível história evolutiva desta abelha, apontando os possíveis efeitos das mudanças climáticas ocorridas nos biomas Caatinga e Cerrado durante o Pleistoceno tardio (Capítulo IV).

Considerando o conjunto dos trabalhos produzidos, nota-se que o primeiro possibilitou conhecer melhor a distribuição geográfica (clima, elevação, biomas) de *P. rustica*, o hábito de nidificação desta abelha e as plantas visitadas por ela. Este último dado demonstrou o possível papel desta abelha como polinizador da flora da Caatinga e do Cerrado. Ademais, foi apontado o *status* de conservação de *P. rustica* frente às iminentes ameaças de meleiros e do desmatamento, em especial no bioma Caatinga.

Os trabalhos apresentados nos Capítulos II e III são complementares, uma vez que o primeiro apresentou resultados que suportaram a hipótese de ocupação de áreas por poucas fêmeas das abelhas sem ferrão, apontando a necessidade de mudança de foco nos estudos envolvendo a genética de populações das abelhas sem ferrão e sugerindo que os novos estudos foquem na verificação do fluxo gênico para evitar acasalamentos entre indivíduos aparentados, que é danoso para estas abelhas, principalmente, devido ao sistema de determinação do sexo. De forma complementar e a fim de testar o possível modelo de dispersão sexo-assimétrica apontada no segundo trabalho, no terceiro capítulo foi discutido o papel dispersor dos machos na promoção do fluxo gênico entre colônias e entre populações, demonstrando, como esperado, baixos índices de parentesco médio entre operárias de colônias da mesma localidade e de localidades diferentes. Além disto, o terceiro trabalho mostrou a estrutura social de *P. rustica* que, como esperado na maioria das abelhas sem

ferrão, é monogínica e monândrica. Em suma, estes dois trabalhos suportam a dispersão sexo-assimétrica em *P. rustica*, em que as fêmeas, filopátricas, ocupam as novas áreas e, por enxameagem, fundam as "populações locais"; por outro lado, os machos por seu comportamento dispersor e de formação de agregações (Engels e Engels, 1984; Paxton, 2000), promovem o fluxo gênico e favorecem o acasalamento panmítico, respectivamente, entre colônias e populações, reduzindo as chances de endogamia. A hipótese aqui testada e suportada pelos dados obtidos nos dois trabalhos deve ser testada em outros meliponíneos, a fim de checar se este padrão é comum às demais espécies do grupo.

O Capítulo III ainda apresenta a necessidade de novos estudos envolvendo estimativas de parentesco genético entre as rainhas das diferentes colônias e entre rainha e zangão de cada colônia de uma dada localidade, estimados de forma indireta pela recontrução dos genótipos parentais a partir das operárias de cada colônia, como nas análises realizadas por Cameron *et al.* (2004). Neste estudo, não foi possível reconstruir os genótipos parentais devido aos locos em que todas as operárias das colônias analisadas eram heterozigotas, dificultando a atribuição dos alelos parentais, à falta de variação de alguns locos para certas colônias, e ao baixo número de locos empregados nas análises. Diante disto, novas análises envolvendo novos marcadores SSR específicos para *P. rustica* estão em curso e fornecerão um maior número de locos para realização destas estimativas.

Os dados apresentados no Capítulo IV permitiram elucidar a possível história evolutiva de *P. rustica* numa pespectiva espaço temporal, complementando a história natural, apresentada no Capítulo I. Dentre outros resultados, este último trabalho demonstrou que as populações de *P. rustica* se apresentam diferenciadas em dois grupos, separados pelo Vale do Rio São Francisco (SFRV). Esta informação corrobora com os dados apresentados no primeiro capítulo, que mostrou que apesar dos esforços de coleta, não foram encontrados registros de ocorrência para a espécie no SFRV (ver Figura 1 do Capítulo I). Este trabalho

também demonstrou a necessidade da utilização de diversas ferramentas como marcadores mitocondriais, nucleares e rebuscadas análises para elucidar o padrão filogeográfico de *P. rustica*, além de lançar mão de informações bioclimáticas e geológicas para contar a possível história da espécie e dos biomas ocupados por ela. Ademais, este constitui um dos primeiros estudos relacionando os efeitos das mudanças Pleistocênicas sobre as populações de abelhas de áreas secas da região Neotropical e pode contribuir com a interpretação dos cenários históricos e biogeográficos da Caatinga e do Cerrado durante o Pleistoceno. Por exemplo, nosso estudo mostrou potenciais áreas de refúgio e áreas de menor estabilidade para *P. rustica* durante o Pleistoceno tardio que são comuns a alguns refúgios e áreas de instabilidade para Florestas Tropicais Sazonalmente Secas propostas por Werneck *et al.* (2011, 2012). Além disto, este trabalho também apontou para uma possível dispersão sexo-assimétrica em *P. rustica*, resultados apresentados nos Capítulos II e III.

Diante do endemismo e da importância de *P. rustica* como potencial polinizador e frente às pressões antrópicas que têm levado ao desaparecimento das suas populações (como mostrado no Capítulo I), os dados sobre a história natural, ecologia, biogeografia e a genética das suas populações poderão ser utilizados para nortear estratégias de conservação e manejo da espécie, bem como dos biomas Caatinga e Cerrado. Além disto, os dados apresentados nos Capítulos II e III ampliam o conhecimento acerca da biologia e da genética das abelhas sem ferrão e podem ser úteis na criação racional destas abelhas. Diante disto, novos estudos envolvendo abelhas sem ferrão, principalmente aquelas que vivem em agregações e que, teoricamente, correriam maiores riscos de endogamia, poderão reforçar os resultados aqui apresentados, além de corroborar os resultados obtidos por Cameron *et al*. (2004), que mostraram o papel dispersor dos machos em espécie de abelha que forma agregações, reduzindo os riscos de endogamia. Finalmente, reforça-se a necessidade de novas e mais detalhadas investigações sobre as mudanças climáticas Pleistocênicas e seus efeitos na

diversificação da biota endêmica das florestas secas da região Neotropical, principalmente, novos estudos envolvendo a grande diversidade de abelhas existente nesta região.

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