UNIVERSIDADE FEDERAL DE SÃO CARLOS
CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E EVOLUÇÃO

"DINÂMICA E ESTRUTURA GENÉTICA POPULACIONAL DE ABELHAS EUGLOSSINI (HYMENOPTERA: APIDAE) VISITANTES FLORAIS DE *Thevetia peruviana* (APOCYNACEAE) EM ÁREAS URBANAS"

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1.1 As abelhas como polinizadores

As abelhas oferecem serviços de polinização a um grande número de espécies de plantas e, por esta razão, são consideradas espécies chave nos ecossistemas naturais e agroecossistemas (KLEIN et al., 2003; ROUBIK, 1989). As abelhas possuem várias características biológicas que as fazem um grupo de polinizadores particular. A primeira é que todas as 20.000 espécies de abelhas, com exceção das cleptoparasitas, são visitantes florais obrigatórios devido a que o pólen é a principal fonte de proteínas, gorduras, minerais e vitaminas das suas larvas. Isto cria uma forte interação mutualista entra as plantas e as abelhas na qual, as plantas oferecem alimento às abelhas e estas últimas oferecem os serviços de polinização às plantas. Outra característica é que as abelhas são forrageadores do tipo "central", já que as fêmeas saem do ninho em busca de alimento e voltam a este para aprovisionar as células onde ovipositarão (ROUBIK, 1989). Isto faz com que, diferentemente de outros insetos polinizadores, as abelhas utilizem áreas de forrageamento limitadas às proximidades do ninho.

Um ambiente é propício para uma abelha quando, dentro da distância de forrageio da espécie, este proporciona: (1) sítios de nidificação, (2) materiais para a construção de ninhos, (3) fontes de néctar para os adultos e (4) fontes de pólen para as larvas (GATHMANN & TSCHARNTKE, 2002). Este conjunto de características descreve necessidades específicas das abelhas para sua sobrevivência em um lugar. No entanto, alterações como degradação do

habitat, fragmentação, agricultura, pesticidas e herbicidas (KEARNS et al., 1998) podem modificar drasticamente o habitat e fazer com que estas necessidades não sejam satisfeitas levando à extinção de populações locais.

Nos últimos anos tem sido descrito um declínio das populações de abelhas, o que tem levado à formação de várias iniciativas regionais e mundiais para a conservação de polinizadores (e.g. São Paulo Declaration of Pollinators: http://www.biodiv.org/doc/case-studies/agr/cs-agr-pollinator-rpt.pdf). Uma das maiores dificuldades para a conservação de abelhas é o desconhecimento da biologia da maioria das espécies. Este cenário é ainda pior para as abelhas não-eusocias, um grupo menos conhecido devido aos seus ninhos cobertos, hábitos solitários e menor abundância quando comparadas ao das abelhas eusociais. Estudos sobre a interação destes insetos com o ambiente são necessários para saber como conservá-los (ELLIS et al., 2006).

1.2 As abelhas Euglossini: um grupo interessante de abelhas nativas

O Brasil possui aproximadamente 1600 espécies de abelhas pertencentes às famílias Colletidae, Halictidae, Andrenidae, Megachilidae e Apidae (SILVEIRA et al., 2002). Dentro desta diversidade de abelhas encontram-se as abelhas da tribo Euglossini (Apidae: Apinae) que fazem parte das abelhas corbiculadas junto com as mamangavas, abelhas de mel e abelhas sem ferrão. As abelhas Euglossini distribuem-se do México ao centro da Argentina e podem chegar a constituir até 25% da diversidade em algumas matas (ROUBIK & HANSON, 2004). Estas abelhas possuem uma grande capacidade de vôo, sendo que cada indivíduo pode cobrir até 1000 km em um dia, de 10 a 20 km de raio aos sítios de nidificação (ROUBIK &

HANSON, 2004). A tribo Euglossini contém cerca de 200 espécies distribuídas em cinco gêneros: *Euglossa* (114 spp.), *Eufriesea* (62 spp.), *Eulaema* (18 spp.), *Exaerete* (7 spp.) e *Aglae* (1 sp.) (Fig. 1.1), sendo as dois últimos gêneros cleptoparasitas de outras espécies de Euglossini.

Os ninhos de *Euglossa, Eulaema* e *Eufriesea* estão geralmente localizados nas partes altas das árvores (RAMÍREZ-ARRIAGA et al., 1996; YOUNG, 1985), no solo (Dressler, 1982) ou em cavidades pré-existentes (EBERHARD, 1989; SANTOS & GARÓFALO, 1994). Todas as fêmeas destas espécies, exceto as cleptoparasitas, utilizam resinas e outros materiais como fezes, lama, cortiça, talo e folhas para a construção de células e ninhos (DRESSLER, 1982) (Fig. 1.2). No entanto, a biologia de nidificação é desconhecida para 80% das espécies devido à dificuldade de se achar estes ninhos na natureza (CAMERON, 2004).

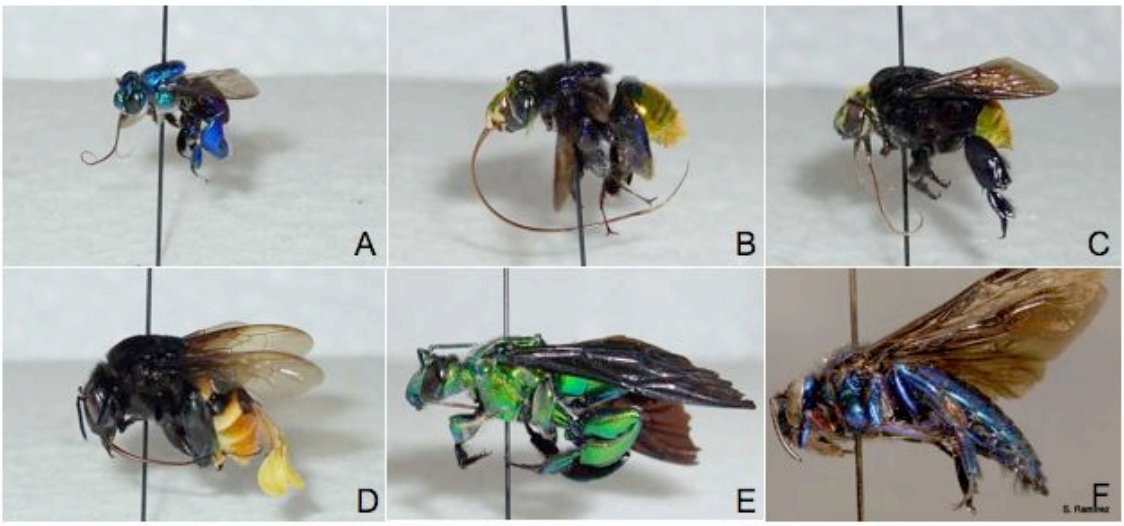

Figura 1.1. Gêneros da tribo Euglossini. (A) *Euglossa cognata*. (B) *Euglossa intersecta* (C) *Eufriesea pulchra* espécie mimética de *Euglossa intersecta*. (D) *Eulaema cingulata* carregando polinários de orquídea. (E) *Exaerete frontalis* e (F) *Aglae caerulea*, espécies cleptoparasitas.

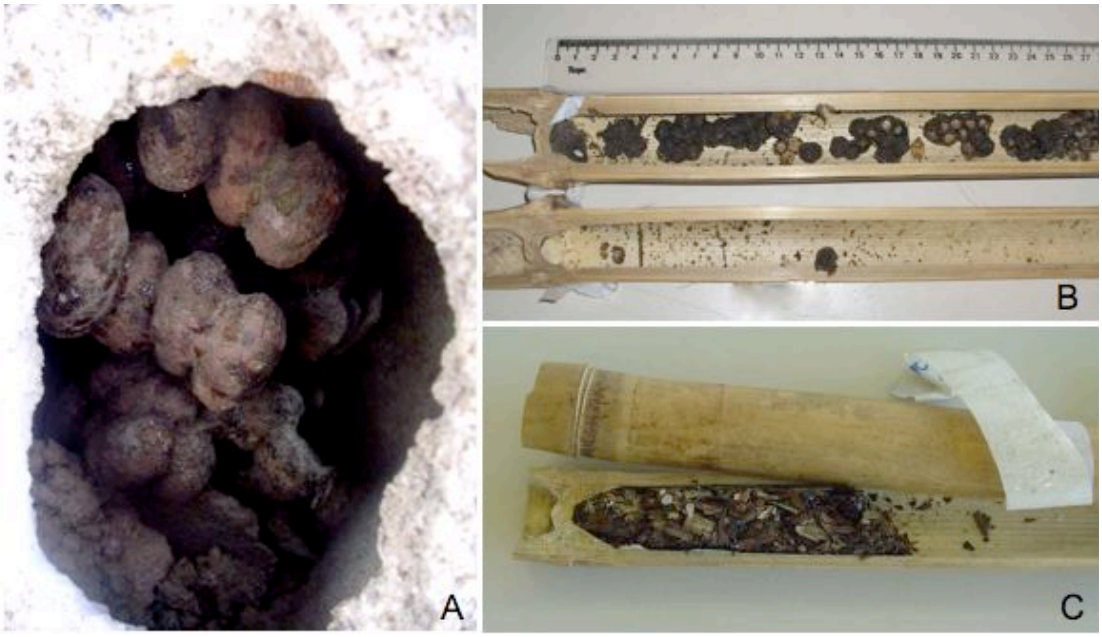

Figura 1.2. Ninhos de abelhas Euglossini. (A) Ninho de *Eulaema nigrita* construído dentro de um tijolo. As células são feitas com resina, barro e fezes. (B) Ninho de *Euglossa townsendi* construído dentro de bambu. As células são feitas a partir de resinas. (C) Ninho de *Eufriesea sp.* em bambu-armadilha com células construídas de cortiça e folhas.

Este grupo de abelhas nativas tem sido considerado "espécie-chave" de muitos ecossistemas devido ao seu papel como polinizadores de espécies de Orchidaceae (HILLS et al., 1972; SINGER & SAZIMA, 1999), Lecythidaceae (KNUDSEN & MORI, 1996; PRANCE, 1976), Euphorbiaceae (ARMBRUSTER et al., 1989), Araceae (MONTALVO & ACKERMAN, 1986) e Solanaceae (SOARES et al., 1989), entre outras famílias. Em particular, os machos desta tribo são polinizadores importantes de muitas espécies de plantas. Os euglossíneos possuem a característica particular de coletar ativamente fragrâncias de flores e outras fontes como fungos e madeira em decomposição (DRESSLER, 1982). Acredita-se que estas fragrâncias estejam associadas à seleção sexual, já que as fêmeas se acasalam com os machos que possuem a "melhor" qualidade de fragrâncias (CAMERON, 2004). Algumas espécies de plantas, particularmente orquídeas da família Catasetinae (ROMERO &

NELSON, 1986), têm se especializado na produção destes compostos, oferecendo exclusivamente fragrâncias como recurso floral. Este tipo de flor é associada à denominada 'síndrome da polinização euglossínea', pois são polinizadas exclusivamente pelos machos da tribo Euglossini.

1.3 Estudos sobre abelhas Euglossini

A atratividade dos machos euglossíneos por compostos similares às fragrâncias que eles coletam nas flores tem permitido estudar a riqueza e diversidade de espécies de Euglossini em diferentes habitats. Entre os ecossistemas estudados, encontram-se os contínuos, fragmentos de mata (floresta amazônica, mata atlântica) e agroecossistemas, os quais têm demonstrado que a degradação do habitat afeta negativamente a riqueza de espécies de Euglossini (TONHASCA et al., 2003). No entanto, trabalhos realizados em áreas com forte ação antrópica indicam que algumas espécies são mais abundantes nestes ambientes altamente perturbados (OTERO & SANDINO, 2003), contradizendo o que estudos preliminares haviam relatado (MORATO, 1994).

Contrariamente aos trabalhos ecológicos, os estudos que avaliam a diversidade genética destas espécies são escassos, porém mostram resultados interessantes. Análises genéticas de populações destas abelhas mediante o uso de alozimas têm detectado uma grande proporção de machos diplóides em populações do Panamá e Colômbia (LÓPEZ-URIBE et al., *in press;* ROUBIK et al., 1996; ZAYED et al., 2004). Esta alta proporção de machos diplóides tem sido associada a efeitos antrópicos relacionados à perda de variabilidade genética, o que tem alertado sobre o possível declínio de algumas destas populações. No

entanto, outros trabalhos genéticos em populações brasileiras apontam resultados diferentes (TAKAHASHI et al., 2001), exigindo estudos adicionais sobre os efeitos de habitats perturbados sobre a comunidade de abelhas Euglossini.

Como os métodos de amostragem para o estudo destas abelhas consistem principalmente no uso de iscas-armadilha, o conhecimento sobre a sua sistemática, ecologia e genética está baseado em informações obtidas a partir de machos. As fêmeas são desconhecidas para cerca de 30% das espécies da tribo e pouco se conhece sobre a sua biologia (RAMÍREZ et al., 2002). O uso desta metodologia pode gerar um viés nas inferências sobre a distribuição destas espécies, já que nem todas elas são atraídas igualmente a estes compostos. Assim, amostragens baseadas em iscas podem não refletir diretamente a abundância e riqueza de espécies em um lugar. De igual forma, a estimativa de parâmetros populacionais, como tamanho efetivo populacional, a partir de dados genéticos de machos, podem não refletir as características populacionais se a razão sexual for diferente de 1:1 ou se as populações se encontram fora de equilíbrio.

Do ponto de vista da conservação destas espécies, a metodologia de iscas pode levar também a conclusões equivocadas. Por exemplo, se os machos diplóides são tão freqüentes, como tem sido proposto para algumas populações, a metodologia de iscas pode mascarar o declínio destas. Devido ao fato de que as fêmeas das espécies de Hymenoptera têm controle sobre a razão sexual da sua progênie, a razão sexual de uma população é o resultado da escolha das fêmeas. Porém, os machos diplóides ao serem ovos fertilizados, são indivíduos que deveriam ser fêmeas e desviam a razão sexual. Portanto, a partir de amostragens baseadas unicamente em machos pode-se superestimar a abundância de uma espécie em um lugar. Por

esta razão, dados sobre a biologia de fêmeas são de vital importância para a conservação destas espécies.

1.4 As abelhas em áreas urbanas

Os ecossistemas urbanos são áreas caracterizadas pela constante atividade humana devido à presença de uma alta densidade de população humana e centros industriais e comerciais (MCINTYRE et al., 2001). O processo de urbanização leva à transformação do habitat nativo em outras formas de uso da terra. Fatores climáticos como radiação solar, temperatura, umidade relativa, nebulosidade e precipitação mudam drasticamente nas cidades, o que torna estes ambientes não aptos para muitos animais e plantas. No entanto, algumas espécies parecem se adaptar bem às mudanças que o processo de urbanização induz, o que tem levado a estudos sobre o componente biótico nas cidades.

Alguns estudos sobre a diversidade de insetos nas cidades (MCINTYRE, 2000; ZANETTE et al., 2005) demonstram que as abelhas são parte importante do componente biótico urbano. Podem ser vistas abundantemente em parques e jardins que oferecem a elas recursos florais. Nas cidades, as construções de madeira, pedra e tijolo provêm sítios ótimos para nidificação de muitas espécies de abelhas (CANE, 2005). Os recursos tróficos geralmente são abundantes, já que a presença de flores é constante durante todo o ano, embora possam estar dispersos (ZANETTE et al., 2005). Portanto, os jardins, parques e fragmentos de mata junto às construções humanas podem proporcionar um habitat adequado para algumas espécies de abelhas.

Uma abelha deve maximizar a relação entre o ganho de energia oferecida em uma fonte de alimento e a perda de energia na procura deste (HEINRICH, 1975). Assim, em um habitat onde os recursos tróficos estão em baixas densidades, a capacidade de dispersão das abelhas é essencial para sua sobrevivência (STEFFAN-DEWENTER, 2003). Da mesma forma, a amplitude do nicho trófico é outro fator importante já que generalistas, que exploram vários tipos de recursos, têm maior chance do que especialistas de se estabelecerem em ambientes onde os recursos se encontram dispersos (STEFFAN-DEWENTER, 2003). Desta forma, a presença ou ausência de espécies de abelhas em áreas urbanas pode ser influenciada pela distância de forrageio e amplitude do nicho trófico destas.

As abelhas Euglossini são insetos com grande capacidade de dispersão e potencialmente generalistas já que o comprimento de suas línguas permite explorar muitos recursos diferentes (BORRELL, 2005). Estas características fazem delas espécies com potencial adaptabilidade a ambientes urbanos. No entanto, a comunidade de abelhas Euglossini ainda não foi estudada em áreas urbanas e se desconhece a interação que as euglossíneas têm com estes ambientes.

2. Objetivos

O objetivo deste trabalho foi estudar a dinâmica e estrutura genética populacional das abelhas Euglossini em áreas urbanas, com o propósito de contribuir para o conhecimento da interação destas abelhas com ambientes com forte intervenção antrópica. Como lugares de coleta foram utilizadas árvores de *Thevetia peruviana* (Apocynaceae) (Fig. 2.1), também conhecida como "chapéu-de-napoleão", a qual é uma planta exótica, nativa do Peru, que possui flores tubulares e muito ricas em néctar. Esta planta é muito comum na paisagem arbórea das cidades do estado de São Paulo, o que possibilitou amostragens nas cidades de São Carlos, Rifaina, Pedregulho, Jaboticabal e Araras.

Figure 2.1. (A) Árvore de *Thevetia peruviana* em uma calçada na cidade São Carlos mostrando o ambiente urbano do entorno. (B) Flor de *Thevetia peruviana* sendo visitada por um macho de *Eulaema nigrita*.

No presente trabalho, duas hipóteses foram testadas:

1. Algumas espécies de Euglossini, devido aos seus vôos rápidos e amplos nichos tróficos, são abundantes em áreas urbanas onde a estrutura da vegetação e as características climáticas foram drasticamente modificadas pela ação antrópica.

2. As fêmeas de Euglossini têm uma área ampla de forrageio e se movimentam muito dentro das cidades devido ao fato dos recursos florais estarem dispersos.

Com esta finalidade, foram realizados experimentos de marcação e recaptura juntamente com análises moleculares (alozimas e DNAmt) das espécies de abelhas coletadas nas cinco cidades amostradas. Foi detectada a presença de seis espécies de abelhas Euglossini: *Eulaema nigrita, Eufriesea violacens* e *Exaerete smaragdina* (Fig. 2.2), além de três espécies do gênero *Euglossa* (*Eg. cordata, Eg. securigera* e *Eg. townsendi*) que foram identificadas mediante o uso de marcadores moleculares (alozimas e DNAmt).

Figura 2.2. Espécies de abelhas Euglossini coletadas em flores de *Thevetia peruviana* na cidade de São Carlos. (A) *Euglossa cordata*, (B) *Eulaema nigrita*, (C) *Eufriesea violacens* e (D) *Exaerete smaragdina.*

Os resultados deste trabalho mostram que *T. perviana*, embora introduzida, é uma importante fonte de recursos para as abelhas Euglossini e muitos outros insetos. Além das seis

espécies de Euglossini, foram encontradas outras abelhas (solitárias e sociais), vespas e borboletas visitando a planta (Fig. 2.3).

Figura 2.3. Outras espécies de insetos vistos em *Thevetia peruviana*. (A) Vespa procurando uma larva de Piralidae, a qual forma o casulo enrolando as folhas da árvore. (B) Adulto de Piralidae sobre o fruto de *Thevetia peruviana*. (C) *Polistes* sp. saindo da flor de *Thevetia peruviana.*

Das seis espécies encontradas, *Eg. cordata* e *El. nigrita* mostraram ser muito abundantes nas áreas amostradas. No entanto, os experimentos de marcação-recaptura na população de São Carlos evidenciaram diferenças nas abundâncias relativas, horário de atividade e sazonalidade entre estas espécies.

Finalmente, as análises genéticas realizadas nas cinco populações de *Eg. cordata* evidenciaram estruturação populacional com os marcadores mitocondriais e a sua ausência com os marcadores nucleares. Estes resultados sugerem que para as abelhas Euglossini, os machos são provavelmente o sexo dispersor, enquanto as fêmeas apresentam um comportamento mais filopátrico.

3. Molecular Identification of Females of *Euglossa spp.* Latreille (Hymenoptera: Apidae: Euglossini) Floral Visitors of *Thevetia peruviana* (Apocynaceae) in Urban Areas

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3.1 Resumo

Identificação Molecular de Fêmeas de *Euglossa spp.* Latreille (Hymenoptera: Apidae: Euglossini) Visitantes Florais de *Thevetia peruviana* (Apocynaceae) em Áreas Urbanas

RESUMO - As abelhas euglossíneas estão entre os polinizadores nativos mais importantes da região neotropical. A riqueza e a abundância destas abelhas têm sido intensamente estudadas em diferentes ecossistemas baseando-se na captura de machos em iscas-armadilhas. As fêmeas são pouco conhecidas para a maioria das espécies e, portanto, caracteres morfológicos que permitam sua identificação taxonômica não têm sido descritos. O propósito deste trabalho foi identificar fêmeas das espécies de *Euglossa* Latreille que visitam as flores de *Thevetia peruviana* (Apocynaceae) em cinco cidades do estado de São Paulo (Brasil) usando alozimas e padrões de restrição das regiões mitocondriais 16S e Cit b. Foram identificadas três espécies de *Euglossa* entre os 305 indivíduos coletados durante as visitas às flores de *T. peruviana* nas cinco cidades amostradas. *Euglossa cordata* (Linnaeus) foi a única espécie presente em todas as localidades, enquanto *E. securigera* Dressler e *E. townsendi* Cockerell foram encontradas em duas e uma cidade, respectivamente. *EST-3* mostrou-se ser loco diagnóstico, enquanto *ICD*, *MDH* , *ME* e *PGM* foram locos informativos para a identificação de espécies quando fenotipados em conjunto. Os mitótipos da região 16S digeridos com *Vsp*I, além de diferenciar as três espécies, apresentaram polimorfismo intra-específico para *E. cordata* e *E. securigera.* A região Cit b apresentou um padrão característico para *E. townsendi,* mas não permitiu diferenciar as outras duas espécies. Nossos resultados descrevem marcadores genéticos potencialmente úteis para a sistemática de fêmeas de *Euglossa ssp.* ao nível de (1) espécie e (2) subgênero-grupo.

PALAVRAS-CHAVE: abelhas das orquídeas, cidades, Estado de São Paulo, alozimas, PCR-RFLP

3.2 Abstract

Euglossine bees are among the most important native pollinators of lowland forest in the Neotropical region. The richness and abundance of these bees have been intensively studied in different ecosystems mainly based on information obtained from males captured using chemical baits. Females are poorly known for most of the species and, therefore, morphological characters for their taxonomic classification have not yet been described. The purpose of this study was to identify the species of *Euglossa* Latreille that visit *Thevetia peruviana* (Apocynaceae) flowers in five cities of the state of São Paulo (Brazil) using allozymes and restriction patterns of the 16S and Cyt b mitochondrial regions. Three *Euglossa* species were identified among the 305 individuals collected during their visits to *T. peruviana* flowers in the cities sampled. *Euglossa cordata* (Linnaeus) was the only species found in all cities, while the distribution of *E. securigera* Dressler and *E. townsendi* Cockerell was restricted to two and one cities respectively. *EST-3* was a diagnostic locus, whereas *ICD*, *MDH*, *ME* and *PGM* data were informative for species identification when used together. Mitotypes of the 16S region digested with *Vsp*I differentiated the three species and showed intraspecific polymorphism for *E. cordata* and *E. securigera.* The Cyt b region showed a distinctive pattern for *E. townsendi* but it was not possible to distinguish the other two species. Our results describe potentially useful genetic markers for the systematics of *Euglossa spp.* females at the (1) species level and (2) subgenus-group level.

KEY WORDS: orchid bees, cities, state of São Paulo, allozymes, PCR-RFLP

3.3 Introduction

Euglossine bees (Apidae: Apinae) pollinate about 180 plant species in Neotropical forests and comprise almost 25% of the bee community in some ecosystems (Roubik & Hanson 2004). These bees, also known as orchid bees, are hard to detect in nature because of their fast flying, solitary habits and the difficulty of locating their nests (Cameron 2004). Knowledge on the biology of this group of bees increased only after the isolation of compounds that mimic the fragrances males look for in flowers. As females are not attracted to these chemical baits, literature on systematics, ecology and genetics of orchid bees is mostly based on males (Roubik & Ackerman 1987; Ramírez *et al.* 2002; Zayed *et al.* 2004).

As most euglossine bee species have been described based on male holotypes, the presence of males is usually necessary for taxonomic identification of females, especially of the genus *Euglossa* Latreille, which encompasses most species of the Euglossini tribe. Females are unknown for over 40% of the *Euglossa* species and even taxonomic identification of males is problematic in some cases due to the morphological similarity among species (Ramírez *et al.* 2002).

Genetic markers have been intensively used as a tool for species identification when morphological characters are unknown or of difficult interpretation (Brunner *et al.* 2002; Naeole & Haymer 2003; Schama *et al.* 2005). Among the molecular markers, allozymes may be used as diagnostic markers for identification of cryptic species, as they have low within-species polymorphism levels and usually show fixed alleles within species (Schama *et al.* 2005). However, the need of fresh material in allozyme analysis restricts its large-scale use for systematic purposes. Since the development of the PCR (Polymerase Chain Reaction), DNA markers as RAPD, AFLP, RFLP and DNA sequencing are commonly used for species identification once a small amount of dried material is sufficient for the analysis.

As female identification is a problematic issue for the genus *Euglossa,* the aim of this study was to taxonomically identify female individuals of *Euglossa spp.* that visit *Thevetia peruviana* (Apocynaceae) flowers in five urban areas of the state of São Paulo (Brazil) using allozymes and mtDNA PCR-RFLP of the 16S and Cytochrome b (Cytb b) regions as genetic markers. For this purpose, we estimated (1) between-species variation to identify diagnostic markers and (2) within-species variation to evaluate the accuracy of the markers proposed. Here, a molecular methodology for females identification of *Euglossa* bees is proposed. This method provided a reliable inexpensive tool for identification of female euglossine specimens that visit of *T. peruviana* flowers in the five urban areas sampled.

3.4 Materials and Methods

3.4.1 Sample collection. Euglossine bees were collected in urban areas of the cities of Rifaina (20º04'50"S 47º25'17"W), São Carlos (22º00'16"S 47º53'18"W), Pedregulho (20º15'25"S 47º28'36"W), Jaboticabal (21º15'17"S 48º19'20"W) and Araras (22º21'25"S 47º23'03"W) (state of São Paulo - Brazil) (Fig. 3.1) during the hot rainy season from 2003 to 2005. Specimens were collected in plastic bags when they were exiting *T. peruviana* flowers, placed in plastic vial and stored at -20ºC until analysis. As *T. peruviana* is an ornamental exotic plant, the number of sites sampled in each city varied among localities (Fig. 3.1). A total of 305 individuals of *Euglossa,* 277 females and 28 males, were captured (Table 3.1). Males captured were identified following the taxonomic key of Rebêlo and Moure (1995) and later used as controls for the allozyme and mitochondrial restriction patterns of females.

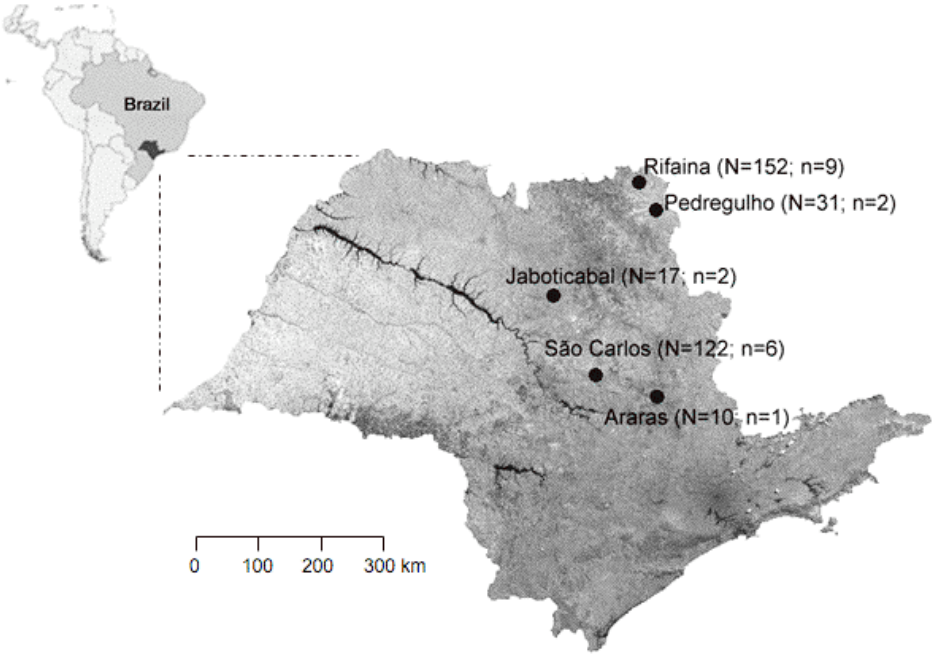

Figure 3.1. Map of the state of São Paulo (Brazil) showing the location of the five cities sampled. N= number of individuals sampled; n=number of sites sampled.

3.4.2 Allozyme analysis. Allozymes were used for identifying the number of species present in our samples. The "filed for recombination" criterion was used which identifies species based on the non-overlapping sets of heterozygous individuals (Sites & Marshall 2003). Proteins were extracted from the head in a 0.2% 2-mercaptoethanol solution and later analyzed through horizontal electrophoresis in 14% starch gels (Penetrose 30^{TM}, Corn Brazil S/A) using (1) Tris Citric Acid pH 7.5, (2) Tris Citric Acid pH 8.0 and (3) Tris Citric Acid pH 8.0 – Borate pH 8.3 as buffers. Twenty-two enzymes corresponding to 24 loci were tested: acid phosphatase (*ACP*, TC 7.5); aconitase (*ACO*, TC 7.5); adenylate kinase (*AK*, TC 8.0); aldolase (*ALD*, TC 7.5); arginine kinase (*ARGK*, TC 8.0 and TCB 8.0-8.3); creatine kinase (*CK*, TC 8.0); esterase (*EST* TC 7.5 and TCB 8.0-8.3); fumarase (*FUM*, TC 7.5); glucose-6-phosphate dehydrogenase (*G6PDH*, TC 7.5); α-glycerophosphate dehydrogenase (*GPDH*, TC 8.0); glucosephosphate isomerase (*GPI*, TC 8.0); β-hidroxybutirate dehydrogenase (*HBDH*,

TC 7.5); hexokinase (*HK,* TC 8.0); isocitric dehydrogenase (*ICD*, TC 7.5); leucine aminopeptidase (*LAP*, TC 8.0 and TCB 8.0-8.3); malate dehydrogenase (*MDH* , TC 8.0); malic enzyme (*ME*, TC 8.0); mannose-6-phosphate isomerase (*MPI*, TC 7.5); peptidase A (leu-ala) (*PEP-A*, TCB 8.0-8.3); phosphoglucomutase (*PGM*, TC 8.0); 6-phosphogluconate dehydrogenase (*6-PGD*, TC 7.5) and superoxide dismutase (*SOD*, TCB 8.0-8.3). All enzymatic reactions were prepared according to Harris and Hopkinson (1976).

3.4.3 PCR-RFLP analysis. Total genomic DNA was extracted from thoracic tissues grounded in a 1.5mL microtube with extraction buffer and incubated with proteinase K at 60ºC for 2h. Incubation was followed by a standard phenol-chloroform and ethanol precipitation protocol (Sheppard & McPheron 1991). The resultant DNA pellet was resuspended in 50µL of TE buffer (10mM Tris-HCl, pH 8.0; 1mM EDTA) and later stored at -20ºC.

PCR products were amplified in a Bio-Rad Gene thermocycler. For amplification of the Cyt b fragment, we used the primers described by Crozier *et al*. (1991) with 30 amplification cycles (94ºC for 30s; 56ºC for 15s; 72ºC for 1min). The 16S fragment was amplified using the primers 16SWb (Dowton & Austin 1994) and 874-16SlR (Cameron *et al.* 1992). PCR conditions consisted of 35 amplification cycles (94ºC for 45s; 5 cycles at 46ºC, 5 cycles at 48ºC and 25 cycles at 50ºC for 45s; 72ºC for 45s) followed by a final elongation step for 5min at 72ºC. Each reaction was performed in a 25µL final volume containing 12.5µM of each dNTP, 2.5 (Cyt b) or 5.0 (16S) mM of $MgCl_2$, 2.5µL Biotools Buffer 10x, 0.5µM of each Primer, 0.8U of Taq Polymerase Biotools and 1µL of DNA solution.

Digestion of the amplified DNA products was performed in a 10µL final volume solution containing 1µL of the PCR product, 1µL of One-for-All (Amershan) buffer and 1U of the restriction enzyme. Restriction patterns were visualized in 12% silver stained

polyacrylamide gels. The endonucleases *Bgl*II, *Dra*I, *EcoR*I, *Mbo*I, *Taq*I and *Vsp*I were initially tested for the Cyt b and 16S fragments since they show polymorphism when used in *Apis mellifera* (Linnaeus) for these regions.

After detecting different restriction mitotypes with these endonucleases, fragments of each prior identified species were sequenced to check the restriction patterns visualized on the polyacrylamide gels. The amplified products of both regions were purified using 1U of SAP (Shrimp Alkaline Phosphates, Amersham Pharmacia Biotech) and 10U of *Exo*I (Exonuclease I, Amersham Pharmacia Biotech) for 8µL of DNA and later incubated at 37ºC for 1h and at 80ºC for 15min. The sequencing reaction was performed in a 10µl final-volume reaction containing 3.5µl of Save Money Buffer 2.5x, 0.5µL of Big Dye (Applied Biosystems), 1µM of Primer and 1µl of DNA. Direct sequencing of forward and reverse strands were obtained in an ABI3700 automatic sequencer.

3.4.4 Data analysis. Sequences of the 16S and Cyt b regions were edited using CodonCode Aligner v 1.5.2 software (CodonCode, Dedham, Massachusetts, United States) and later on aligned using the CLUSTAL X multiple sequences editor (Thomson *et al.* 1997). Within-species polymorphism was estimated as the average number of pairwise differences within species (d_W) and as nucleotide diversity (π, Nei 1987) using the ARLEQUIN 2.0 software (Schneider *et al.* 2000). Between-species variation was estimated as the average number of pairwise differences between populations (d_B) and as F_{ST} statistics (Weir and Cockerham 1984). A UPGMA genetic distance tree was constructed by the PAUP* program (Swofford 1999) to estimate genetic similarity among sequences. Data were resampled 10000 times to obtain bootstrap support values for each cluster. To assess restriction patterns with restriction enzymes, sequences were analyzed with the Sequence Analysis 1.6.0 software (http://informagen.com/SA/).

3.5 Results

3.5.1 Species identification. The males captured were taxonomically identified as *E. cordata* (Linnaeus)*, E. securigera* Dressler and *E. townsendi* Cockerell. Even though males were intended to be collected at all cities, 90% of the flower visitors were females, consequently males were not sampled at all localities (Table 3.1). This preliminary classification could have underestimated the number of species, however, the allozymic analysis confirmed the presence of just three species of *Euglossa. E. cordata* was the only species found at all localities and was also the most common species. Samples from São Carlos, Jaboticabal and Araras were exclusively composed of *E. cordata. E. securigera* occurred in Rifaina and Pedregulho, while *E. townsendi* was found exclusively in Rifaina.

Table 3.1. Sampling localities showing number of sampled sites; total number of females (N_f) and males (N_M) analyzed; and total number of individuals of each of the three *Euglossa* species found in each locality. Numbers in parenthesis correspond to sampled males.

Locality	Sites	N_f	N_m	*E. cordata*	*E. securigera*	*E. townsendi*
Rifaina	9	145	7	89(3)	12(3)	44(1)
São Carlos	6	102	20	122(20)	-	-
Pedregulho	2	30	1	29(1)	2	-
Jaboticabal	2	17	0	17	-	-
Araras	1	13	0	12	-	-
Total		277	28		14	44

3.5.2 Allozyme analysis. Five of the 24 allozyme loci analyzed were informative for species identification. *EST-3* was a diagnostic locus since it showed exclusive fixed alleles for each of the three species (Table 3.2). *MDH* , *ME, ICD* and *PGM* were also informative for species identification because they presented fixed alleles that, in association, were also diagnostic.

Loci *ACP, EST-1, HK-2, G6PDH* and *FUM* showed intra-specific variation uninformative for species identification but useful for population genetic studies.

Table 3.2. Electromorphs of the five enzyme loci useful for identification of the three *Euglossa* species. The most common electromorph is called 100 and the others variants are named according to their relative mobility to 100.

Marker	*E. cordata*	*E. securigera*	*E. townsendi*
EST-3	100	141	143
ICD	100	96	100
MDH	92/100	100	90
ME	100	100	94
PGM	100	100/110	102

3.5.3 PCR-RFLP analysis. Only three of the six endonucleases tested revealed variation: *Dra*I, *Mbo*I and *Vsp*I. The restriction analysis of the 16S sequences with *Vsp*I showed diagnostic restriction patterns, allowing the identification of each of the three species. The region amplified varied in the number of restriction sites for *Vsp*I from 7 to 10. However, some fragments were not visualized in the polyacrylamide gel because of their small size (Fig. 3.2). The largest fragment was characteristic of *E. securigera* (174) while the smallest fragments differentiated *E. townsendi* (34) from *E. cordata* (30). Besides being diagnostic for the three species, *Vsp*I digestion of the 16S fragment revealed two different mitotypes for *E. cordata* (C_A; C_B) and *E. securigera* (S_M; S_N) (Fig. 3.2). Digestion with *Dra*I also revealed intra-specific polymorphism, but was not useful for species identification, as *E. cordata* and *E. securigera* shared one mitotype.

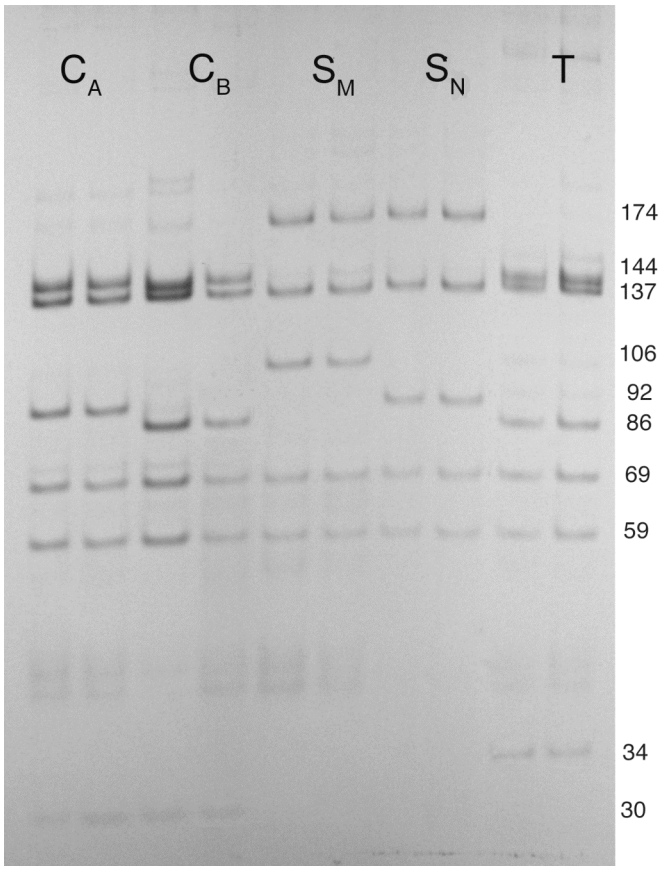

Figure 3.2. Polyacrylamide gel showing the restriction patterns for the 16S region digested with *Vsp*I. (C= *E. cordata*; S= *E. securigera*; T= *E. townsendi*)

Restriction analyses of the Cyt b fragment with *Mbo*I and *Vsp*I revealed common mitotypes for *E. cordata* and *E. securigera.* However, *E. townsendi* was easily identified when digested with these restriction enzymes. The amplified Cyt b fragment showed the occurrence of three *Mbo*I restriction sites for *E. townsendi*, whereas *E. cordata* and *E. securigera* presented four. Digestion of Cyt b with *Vsp*I detected one restriction site for *E. townsendi*, whereas the other two species had at least two. *Vsp*I also revealed intra-specific polymorphism for *E. cordata* (C_E; C_F) but the C_F mitotype was shared with *E. securigera*..

3.5.4 Nucleotide sequences. The restriction patterns were confirmed by sequencing the 16S and Cyt b mitotypes identified by the RFLP analysis. The 16S region showed a high number of polymorphic sites and was variable in size among and within species ranging from 579 bp to 582 bp (Table 3.3). The Cyt b fragment showed equal size of 485 bp among all individuals analyzed and the variable sites were mostly synonymous substitutions at the third codon position (Table 3.4).

Table 3.3. Consensus of the variable sites of the 16S fragment from the three *Euglossa* bee species analyzed. Within-species polymorphic sites are presented in bold using the IUB code for degenerate bases (M = A/C; R = G/A; S = C/G; W = A/T; Y = C/T). Asterisks (*) indicate polymorphic sites where some individuals showed a deletion.

	56	57	76	85	86	101	103	190	193	194	206	218	239	241	242	244	246	247	260	283	289
E. cordata	T	**W**	A	C	T	T	A	T	A	T	**W**	**Y**	T	A	T	T	A	A	A	T	T
E. securigera	T	T	A	T	**M**	T	**W**	T	**W**	T	T	T	T	A	T	**Y**	A	A	A	C	**Y**
E. townsendi	A	T	T	T	T	A	A	C	T	A	A	T	A	T	A	T	-	-	T	T	T

	301	315	317	320	322	323	324	325	326	331	411	420	441	446	454	484	488	499	501	552	553
E. cordata	A	**W**	**W**	T	**W**	T	**A***	**A***	T	T	A	A	A	**Y**	T	C	C	**Y**	A	-	**Y**
E. securigera	A	**R**	T	T	A	T	-	-	-	**Y**	A	A	G	T	T	**S**	**S**	C	A	**A***	T
E. townsendi	T	T	T	A	T	A	A	A	T	T	T	T	A	T	C	C	C	C	G	-	T

E. townsendi was very different from the other two species having on average over 20 pairwise differences with *E. cordata* and *E. securigera* for both mitochondrial regions, whereas the latter two showed on average less than 11 pairwise differences (Table 3.4). F_{ST} values for the 16S region were significant ($P<0.05$) for all pairs of species, demonstrating high between-species differentiation and therefore supporting the quality of this assessment for species identification through the 16S locus. The UPGMA distance tree clustered the mitotypes of each species (C_A, C_B for *E. cordata,* S_M, S_N for *E. securigera* and T for *E. townsendi*) with high bootstrap values (Fig. 3.3) indicating low probability of wrong assignment of the species. For the Cyt b fragment, the average number of between-species

differences (d_W) and F_{ST} values showed great differences between *E. townsendi* and the other two species (Table 5). In contrast, this locus had low between-species differentiation for *E. cordata* and *E. securigera*, as shown by the non-significant F_{ST} value (F_{ST}=0.097; p=0.17). The UPGMA tree evidences the strong differences between *E. townsendi* and the other two species, while *E. cordata* and *E. securigera* form a single group with reticulated mitotypes (Fig. 3.4).

Table 3.4. Consensus of the variable sites and respective codon positions of the cytochrome *b* fragment from the three *Euglossa* bee species analyzed. Within-species polymorphic sites are indicated in bold using the IUB code for degenerate bases (R = G/A; W = A/T; Y = C/T).

	39	58	69	72	85	100	102	114	120	129	168	189	225	228	234	235
E. cordata	T	A	**Y**	T	**Y**	**R**	**W**	**Y**	T	A	**W**	**W**	A	**W**	T	**R**
E. securigera	T	A	T	A	A	A	T	**Y**	T	**W**	A	**W**	T	**W**	T	**R**
E. townsendi	A	T	T	A	A	A	T	T	C	G	T	A	A	T	A	A
Codon Position	3	1	3	3	1	1	3	3	3	3	3	3	3	3	3	1

	255	256	267	270	276	316	320	369	375	402	406	411	441	447	456
E. cordata	T	**Y**	A	T	T	**Y**	C	**W**	T	A	A	T	T	**W**	A
E. securigera	T	T	A	**Y**	T	**Y**	T	T	T	A	A	T	T	A	A
E. townsendi	C	T	T	T	A	T	C	A	C	T	C	C	A	A	T
Codon Position	3	3	3	3	3	1	2	3	3	3	1	3	3	3	3

Table 3.5. Between-species parameters of variation for the 16S and Cyt b mitochondrial regions. Above diagonal; average number of pairwise differences between species. Below diagonal; F_{ST} values for pairwise comparisons of the euglossine bee species. Values in bold are significant (p<0.05) estimated after 10000 permutations.

	16S			Cyt b		
	E. c.	*E. s.*	*E. t.*	*E. c.*	*E. s.*	*E. t.*
E. cordata	*	9.56	20.34	*	6.76	21.4
E. securigera	**0.516**	*	20.67	0.097	*	21.8
E. townsendi	**0.738**	**0.806**	*	**0.799**	**0.871**	*

Table 3.6. Values for within-species variation of the 16S and Cyt b mitochondrial regions. Number of individuals analyzed per species (n), number of haplotypes found (N), average number of pairwise differences within populations (d_W) and nucleotide diversity (π).

	16S				Cyt b			
	n	N	d_W	π	n	N	d_W	π
E. cordata	9	4	5.3	0.006 ± 0.0044	10	6	6.5	0.013 ± 0.0078
E. securigera	5	3	4	0.006 ± 0.0044	5	3	6.8	0.012 ± 0.0078
E. townsendi	2	1	0	0	5	1	0	0

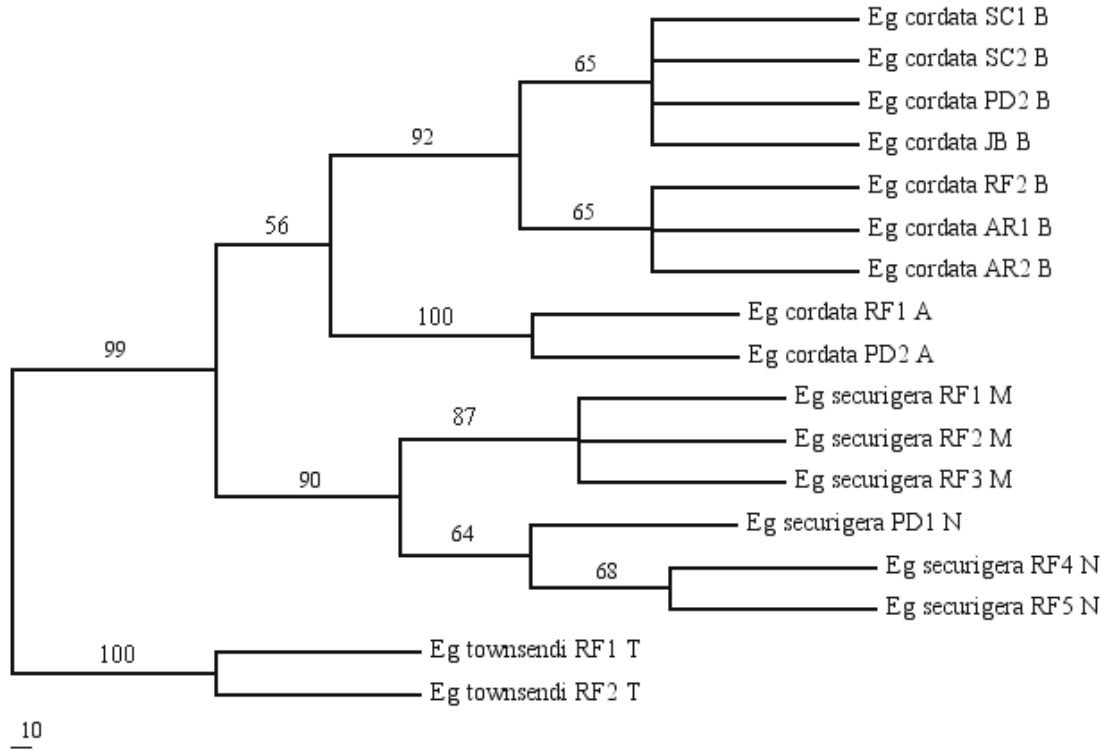

Figure 3.3. UPGMA dendrogram showing the genetic distances among the individuals sequenced for the 16S region. Numbers on branches are bootstrap values over 10000 replicates.

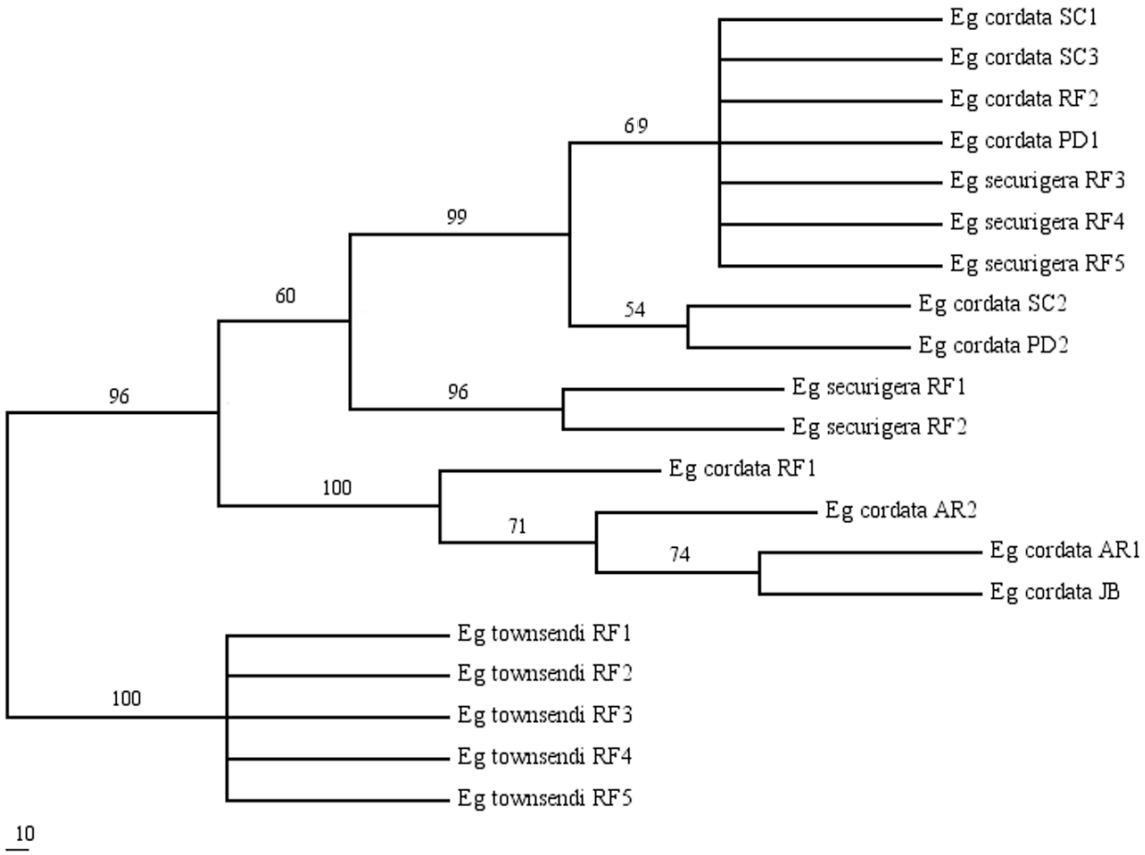

Figure 3.4. UPGMA dendrogram showing the genetic distances among the individuals sequenced for the cytochrome *b* region. Numbers on branches are bootstrap values over 10000 replicates.

3.6 Discussion

Allozymes proved to be useful markers for identifying the number of species in our samples, as the absence of heterozygotes in a three groups of individuals was an indubitable evidence of the presence of three distinct species. *EST-3* was an important diagnostic marker since each species showed a private fixed allele. Furthermore, *ICD* 96 allele was diagnostic for *E. securigera* and *MDH* 90, *ME* 94 and *PGM* 102 alleles were diagnostic for *E. townsendi.*

After, comparing with the male patterns used as controls, these markers allowed the simple identification of each of the three species of *Euglossa*.

The 16S gene is an untranslated region of the mtDNA that is highly variable in insects showing length variation absent in sequences of protein-coding genes as Cyt b (Whitfield & Cameron 1998). As it is a very variable region, this gene has been widely used for phylogenetic purposes at the species level in Hymenoptera (Cameron *et al.* 1992; Whitfield & Cameron 1998). For the *Euglossa* species analyzed in this paper, this gene showed diagnostic patterns for the three species when digested with *Vsp*I besides showing within-species polymorphism for *E. cordata* and *E. securigera*. Therefore, it was a very informative locus for species identification.

The Cyt b fragment did not perform as well as the 16S for species identification since *E. cordata* and *E. securigera* shared most of the restriction sites. However, restriction patterns of *E. townsendi* for all the tested endonucleases proved to be very different. Although a complete phylogeny of the *Euglossa* genus is yet to be done, a morphological classification made by Dressler (1978; 1982c; 1982a; 1982b) and Moure (1967) clusters *Euglossa* species in six subgenus and 12 species groups. The three species analyzed in this study belong to the subgenus *Euglossa s. str.* due to the presence of short tongues and the form of the mid-tibial tuff. At the species group, *E. townsendi* belongs to the purpurea group – XI, whereas *E. cordata* and *E. securigera* belong to the cordata group – XII (Dressler 1982c). This morphological classification may reflect the phylogenetic relationships among the species of the genus *Euglossa*, explaining the similarity found in the sequences of *E. cordata* and *E. securigera.* Similar results were also found by Dick *et al.* (2004) after analyzing mitotypes of *E. mixta* Friese and *E. cognata* Moure (analis group – VIII) for the COI region.

We found diagnostic RFLP markers of good quality for the identification of the three species. However, it should be taken into consideration that these DNA markers have two

limitations for this purpose: (1) presence of high intra-specific polymorphisms or (2) ancestral polymorphisms. These problems are seen in the 16S and Cyt b fragments, respectively. The high variation found in the 16S region makes it an optimal marker for identification of phylogenetically related species. However, before considering a mitotype as particular of one species, intra-specific variation should be assessed due to the high polymorphism of this region. In contrast, the reticulated mitotypes of *E. cordata* and *E. securigera* for the cytochrome *b* region probably resulted from the presence of ancestral polymorphism between two recently formed species. Our results suggest that for the *Euglossa* genus, the cytochrome *b* gene is useful for identification at the species group level or subgenus whereas the 16S region is informative at the species level.

T. peruviana showed to be actively visited by euglosine bees, and therefore was very useful for collecting *Euglossa* females. Besides the *Euglossa* species sampled, *Eulaema nigrita* Lepeletier, *Exaerete smaragdina* (Guérin-Méneville) and *Eufriesea violacens* (Mocsáry) females were also seen visiting the *T. peruviana* flowers. As euglosine females are rarely seen in nature, this plant may be an easy way of sampling females in urban areas or in areas where its native distribution. In addition, information about plants visited by euglossine bees is necessary to detect presence of some species that are not attracted to chemical baits.

Samples collected in the five cities evaluated here show differences in the number and abundance of euglossine bee species visiting *T. peruviana.* Nevertheless, this result could be an artifact of the different sampling efforts in each city. Nests of *E. townsendi* have been found in São Carlos and Araras (Del Lama, pers. obs.) suggesting that other species may occur in the sampled cities but were not collected in *T. peruviana* flowers. Moreover, as distribution of bees depends on the availability of resources for food and nest construction, even if present, other *Euglossa* species may have not been sampled because (1) they prefer

other nectar sources or (2) *T. peruviana* trees were not near enough to other food sources they need to survive.

The urbanization process seems to have an impact on euglossine species richness once fewer species are found in urban areas than in their surroundings. The euglossine bee community of a semideciduous forest of northeast São Paulo state, near Pedregulho, was described as having 10 species of *Euglossa*, for which *E. pleosticta* Dressler was the most common species, with a frequency of 64% among all the *Euglossa* species sampled (Rebêlo & Garófalo 1997). In our urban surveys of *T. peruviana* plants, we found only three species, of which *E. cordata* was the most common species. These differences could be related to (1) capacity of adaptation to disturbed areas or (2) habitat preferences of the euglossine bees. Milet-Pinheiro & Schlindwein (2005) found that *E. cordata* males left the forest to search for chemical baits in sugarcane monocultures, suggesting that this species flies through open areas. In contrast, *E. townsendi* seems to be more restricted to forested areas, as shown by a study comparing euglossine bee abundances across a human intervention gradient (Otero & Sandino 2003). *E. townsendi* is rarely captured using chemical baits (Rebêlo & Garófalo 1997), but was quite common in the Rifaina samples showing the relevance of plant surveys for species richness evaluation.

The results here presented show a useful genetic tool for the identification of *Euglossa* female individuals at the species level. Although our analyses describe an exclusive tool for the species that visit *T. peruviana* flowers, it can be expanded and applied to other *Euglossa* species for which morphological identification of females is not currently possible. Moreover, as their biology is practically unknown, *T. peruviana* flowers offer an easy and efficient way to assess *Euglossa* females for research.

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4. Mark-recapture experiments in urban euglossine bees using flowers of *Thevetia peruviana* (Apocynaceae) as a sampling method

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4.1 Abstract

1. Urbanization has been predicted to decrease species richness since it drastically alters vegetation structure and climate conditions of environments.

2. Euglossine bees are important native pollinators of the Neotropical region and have been proposed as bioindicators of habitat quality due to their susceptibility to habitat perturbation. Therefore, these species are not expected to exist in highly disturbed environments as urban areas.

3. Four euglossine species were found inside the urban area of São Carlos city (SP-Brazil). The small euglossine bee *Euglossa cordata* was the most abundant species during the nine-month mark-recapture survey.

4. These results suggest that although urbanization decreases richness of euglossine bees, species like *Eg. cordata* may be preserved in this highly perturbed areas if food and nest resources are offered to these bees.

Key words. orchid bees, seasonality, abundance, *Euglossa cordata*

4.2 Introduction

Euglossine bees (Hymenoptera: Apidae), commonly known as orchid bees, are important native pollinators in the Neotropics since they interact with more than 50 families of plants (Ramírez *et al.*, 2002). These bees are found from south of the United States to Central Argentina, from altitudes of 0 to 2700m (Roubik & Hanson, 2004). Orchid bees are characterized by their long tongues, fast flights and solitary habits. They are mostly cavity nesters and although typically solitary, some species present facultative primitively social behavior (Augusto & Garófalo, 2004). Since euglossine bees are difficult to detect in nature, the most widely used sampling method is traps with chemical baits that effectively attract males. This methodology has allowed a wide research on their biogeographical patterns, abundances, richness and diversity in different habitat (Roubik, 2001; Tonhasca *et al.*, 2003).

It has been proposed that euglossine bees could be used as bioindicators of disturbed Neotropical forests since they are widely distributed native pollinators. However, there is no agreement on that point. Some studies have found that euglossine bee community is susceptible to fragmentation since species richness decreases with fragment size (Powell & Powell, 1987). Nonetheless, other studies showed that species richness does not change with fragment size (Tonhasca *et al.*, 2002) and some species even show preference to disturbed areas (Otero & Sandino, 2003).

Urban areas are environments characterized for an intense human influence: a matrix dominated by buildings, roads and other paved surfaces (McIntyre, 2000). This heterogeneous environment provides different habitat types to animal and plant species. Among these, bees are frequently found visiting flowers of native and exotic plants at parks and gardens in urban areas (Zanette *et al.*, 2005; McFrederick & LeBuhn, 2006). In spite of this, urbanization may

have severe effects on bee assemblage since it changes plant community composition, landscape structure and abiotic conditions (Cane, 2005).

Rich communities of native bees have been found in gardens and parks of populous cities like Rome, São Paulo and New York (Cane, 2005). This unusual pattern may be due to the fulfillment of bees' needs in urban areas. In general, a patch fulfills bees' needs if, within the bee flight range, it offers (1) nesting places, (2) floral sources for larval feeding and (3) nest construction materials. Studies on floral visitors of the exotic plant *Tecoma stans* (Bignoniaceae) in three Brazilian cities reported the presence of euglossine bees (Silva submitted) but at very low frequencies compared to eusocial (honey and stingless bees) and other solitary bee species (*Xylocopa spp, Centris spp., Epicharis spp.*). López-Uribe and Del Lama (submitted) reported six euglossine bee species floral visitors of *Thevetia peruviana* (Apocynaceae) after a survey in five cities of the São Paulo state in Brazil. However, it is not clear if urban areas may be suitable environments for euglossine bee populations.

We studied urban populations of euglossine bees through mark-recapture experiments using flowers of the exotic plant *T. peruviana* as sampling sites. The aims of this paper are (1) to describe aspects of the interaction of euglossine bees species with *T. peruviana* in urban areas and (2) to estimate the abundance and survival of populations of *Euglossa cordata* visiting *T. peruviana* trees at three sites in the city of São Carlos (SP - Brazil). Our results show that euglossine bees are a notable component of the urban bee community and that *T. peruviana* may be an important nectar source for these and other pollinators in urban areas. We call to the urban landscape planning to allow the conservation of these native bees through city arborization.

4.3 Material and Methods

4.3.1 Sampling area

The study was conducted inside the urban area of the city of São Carlos (SP - Brazil) located between 21º30'S 47º30'S and 22º30'S 48º30'W. The climate of the region is characterized by dry winters (April – September) and wet summers (October – May) with an average rainfall of 1400mm. The native vegetation of the area was mainly composed of savannas, arboreal savannas and riparian forests, but it is currently very fragmented, remaining only 7% of its original coverage (Soares *et al.*, 2003). The city boundary shelters 1.132 km^2 but only 67 km^2 (6%) are actually urbanized while the rest is mainly composed of sugar-cane plantations. Green areas inside the city represent only 2% of the territory and the Green Area Index (GAI) is about 2.6 m^2/habitant (Henke-Oliveira, 1996).

Trees of *T. peruviana* were found at 20 sites in the urban area of São Carlos (Fig. 4.1). From those 20 sites, twelve were sampled at least once to establish if euglossine bees were found in the area (Table 4.1). All trees were located on street sidewalks. These sampling sites were classified as having high, medium or low transit interferences (4.7 Appendix).

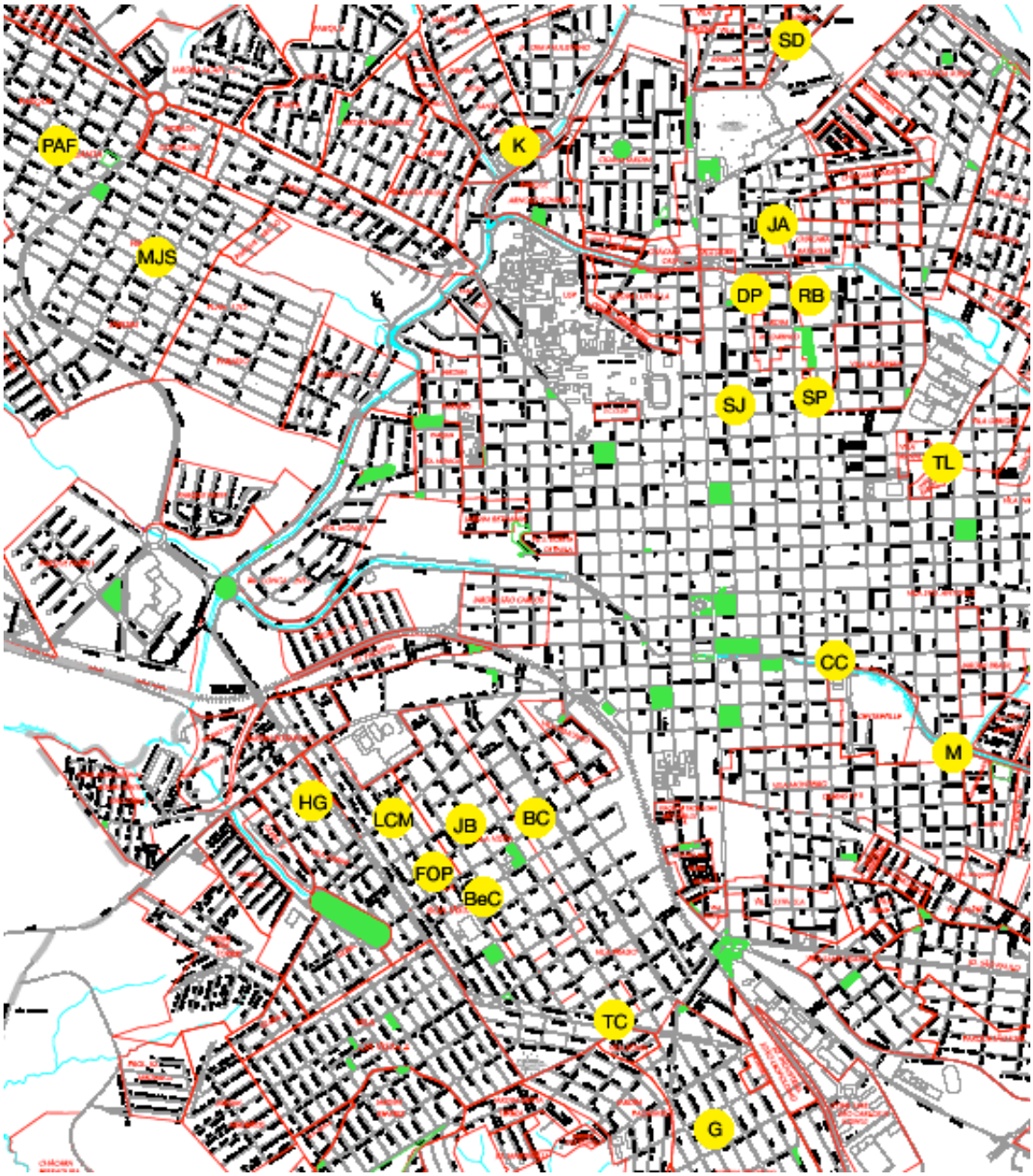

Figure 4.1. Map of the city of São Carlos. The green areas represent parks and squares inside the city. The yellow points are the sites where *Thevetia peruviana* trees were found. (Benjamin Constan (BeC); Bernardino de Campos (BC); Casa da Cultura (CC); Dom Pedro II (DP); Francisco Oliveira Penteado (FOP); Guadalajara (G); Henrique Gregori (HG); José Benetti (JB); José de Alencar (JA); Kartódromo (K); Luiz Carlos Arruda Mendes (LCM); Manoel José Serpa (MJS); Marginal (M); Propócio de Araújo Ferraz (PAF); Rui Barbosa (RB); Santos Dumont (SD); São Joaquim (SJ); São Paulo (SP); Theodureto de Camargo (TC); Totó Leite (TL)).

Table 4.1. Linear pair distance (km) between sites sampled for presence of euglossine bees in the São Carlos city.

Sites[1]	BC	DP	G	JB	K	M	MJS	PAF	RB	SJ	SP	TL
BC	*											
DP	2.51	*										
G	1.83	3.97	*									
JB	0.36	2.68	1.93	*								
K	2.88	1.18	4.61	2.98	*							
M	1.87	2.29	2.14	2.07	3.29	*						
MJS	3.21	2.67	5.04	3.21	1.60	4.35	*					
PAF	3.64	3.17	5.45	3.61	2.07	4.84	0.51	*				
RB	2.49	0.24	3.86	2.66	1.42	2.10	2.9	3.40	*			
SJ	1.99	0.59	3.38	2.16	1.56	1.76	2.84	3.34	0.51	*		
SP	2.24	0.76	3.52	2.54	1.79	1.62	3.32	3.82	0.44	0.54	*	
TL	2.42	1.26	3.33	2.62	2.42	1.28	3.80	4.30	1.02	0.96	0.63	*

[1] Bernardino de Campos (BC), Dom Pedro II (DP), Guadalajara (G), José Benetti (JB), Kartódromo (K), Marginal (M), Manoel José Serpa (MJS), Procópio de Araújo Ferraz (PAF), Rui Barbosa (RB), São Joaquim (SJ), São Paulo (SP) e Totó Leite (TL).

4.3.2 Mark-Recapture

Three of the sampling sites with *T. peruviana* trees were used for the mark-recapture experiments: Marginal (M), José Benetti (JB) and Kartódromo (K). A pilot experiment was done from February to March 2005 to test persistence of the marks used. Experiments for population size estimates were performed from November 2005 to May 2006. Sampling periods were done from 6h to 17h at least once every two weeks at each sampled site if weather conditions were favorable (still and sunny days).

Bees were collected in plastic bags when they were exiting the tubular *T. peruviana* flowers. Immediately after, they were chilled in ice for three to five minutes until they were in torpor. Then, a numbered colored label (Opalithplättchen mit Nr. 1-99) was glued on their

thorax. Bees were kept in vials until they were awake to ensure the glue was dry and the label was not coming off. Afterward, vials were opened and, one or two minutes later, bees found their way out and flew away.

4.3.3 Data record

After each specimen was individually captured, it was recorded (1) species, (2) sex, (3) date, time and place of collection, (4) presence/absence of pollen or resins and (5) wing wear classified into three categories (class I= no tears; class II= tears, class III= notches). Every time an individual was captured in the flowers, it was photographed to record the gradual damage of wings. History of each individual was recorded as an absence/presence matrix for the sampling days.

4.3.4 Data analysis

To estimate parameters for abundance (N), survival (ϕ) and birth+immigration (b), it was used the Jolly-Seber model (POPAN) for open populations (Jolly, 1965; Seber, 1965) which includes birth and immigration as parameters to be estimated. Parameters were calculated using software MARK (White & Burnham, 1999) and the "Akaike Information Criterion" (AIC) was used to select the most parsimonious model. The AIC is calculated as:

$$\mathrm{AIC} = -2\ln(\mathcal{L}) + 2K \tag{4.1}$$

where $\mathcal{L}$ is the likelihood of the model and K is the number of parameters estimated. However, for models with many parameters in relation to the size of the sample and

overdispersed count data, like mark-recapture data, a modification of the information criterion is used:

$$\mathrm{QAIC}_c = \frac{-2\ln(\mathcal{L})}{c} + 2K + \frac{2K(K+1)}{M-K-1} \tag{4.2}$$

where c is the estimated overdispersion quasi-likelihood parameter and M is the effective sample size (Burnham & Anderson, 2002). The AIC is a model selection criterion that balances fit and precision since as indicated in formula (4.1). The smaller the log likelihood, the better the fit but the greater the number of parameters, the lower the precision.

The "goodness-of-fit test" (GOF) of RELEASE software available in MARK was estimated to measure how well the more general model fit to the data, this means testing the assumptions underlying the models. Precision of each parameter was estimated calculating the standard error (SE).

Each of the three sampled sites was treated as an independent experiment and different models were tested at each site. These sites were chosen because an species was particularly abundant at that place and a high number of marked and recaptured individuals was recorded. *Eg. cordata* populations were modeled at M and JB, estimating abundance (N), birth+immigrants (b) and probability of survival (ϕ). For *El. nigrita*, the same parameters were estimated for males and females at K site because sexual ratio was 1:1.

4.4 Results

4.4.1 Floral visitors species

After nine months of survey and 210 hours of sampling, there were marked 799 individuals belonging to the species: *Euglossa cordata* (575), *Eulaema nigrita* (216),

Eufriesea violacens (6) and *Exaerete smaragdina* (3). Sex ratios of the visitors of *T. peruviana* were female biased for *Eg. cordata* (1:12), *Ef. violacens* (1:5) and *Ex. smaragdina* (1:2), while for *El. nigrita* sex ratio was not significantly different from 1:1.

Other insect species were frequently seen at *T. peruviana* trees as flowers visitors. *Bombus atratus, B. morio, Centris spp., Apis mellifera, Auglochlora sp1.* and *sp2.* were seen inside the flowers drinking nectar. In contrast, *Xylocopa suspecta, Trigona sp1.* and *sp.2* were nectar robbers since they did a little cut at the base of the flower and sucked the nectar from there. A few times, butterflies of the families Hesperidae and Pieridae were also seen sipping nectar. Some wasp species were found at the plants mostly looking for larvae of Piralidae moths that encapsulated using the leaves of the plant. In addition, it was seen a species of *Polistes sp.* inside the flower probably looking for nectar.

Distribution of floral visitors varied among sites (Table 4.2). The social species *Trigona spp.* and *Apis mellifera* were seen visiting almost at all sites. *Xylocopa suspecta* was also very common in several sites. In contrast, *Augochlora spp., Centris sp.* and *Bombus spp.* were restricted to few sites. Euglossine bees were not found at all sites either (absent at G, SD and PAF), however where they were found, their abundances were high compared to the other species. As well, relative abundance of euglossine bees was different among sites. At M and JB, *Eg. cordata* was more abundant while *El. nigrita* was more common at K. Other sites like BC, MJS, SP, and TL showed in general intermediate to low abundances of euglossine bees. RB and SP exhibited high abundances of both species during few months.

Table 4.2. Distribution of floral visitor species[1] in the twelve sample sites. ✓ indicates the species was seen at least once at the site

	AM	AU1	AU2	BA	BM	CE	EC	EV	EN	ES	TR1	TR2	XS
BC	✓						✓		✓		✓	✓	✓
DP	✓			✓			✓		✓		✓	✓	✓
G	✓										✓	✓	✓
JB	✓						✓		✓		✓	✓	✓
K	✓				✓		✓	✓	✓		✓	✓	
ME		✓	✓				✓		✓	✓	✓	✓	
MJS							✓		✓	✓	✓		✓
PAF	✓	✓			✓						✓	✓	✓
RB	✓			✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
SJ	✓					✓	✓		✓				✓
SP							✓	✓	✓		✓		✓
TL	✓						✓		✓		✓	✓	✓

[1] *Apis mellifera* (AM); *Augochlora sp.1*, *sp2*. (AU1, AU2); *Bombus atratus* (BA); *B. morio* (BM); *Centris sp.* (CE); *Eg cordata* (EC); *Ef. violacens* (EV), *El. nigrita* (EN); *Ex. smaragdina* (ES); *Trigona sp1.*, *sp2*. (TR1, TR2) and *Xylocopa suspecta* (XS).

4.4.2 Seasonality

Species showed a tendency to seasonality throughout the nine sampled months (Fig. 4.2). *Eg. cordata* was more abundant from December to April but individuals were also captured during the other months. In addition, this species was clearly more abundant in 2006 than in 2005. *El. nigrita* was more abundant from November to January and drastically decreased in February. Even though few individuals of *Ef. violacens* and *Ex. smaragdina* were captured, individuals of *Ef. violacens* were more abundant in February while *Ex. smaragdina* was captured only in November and February.

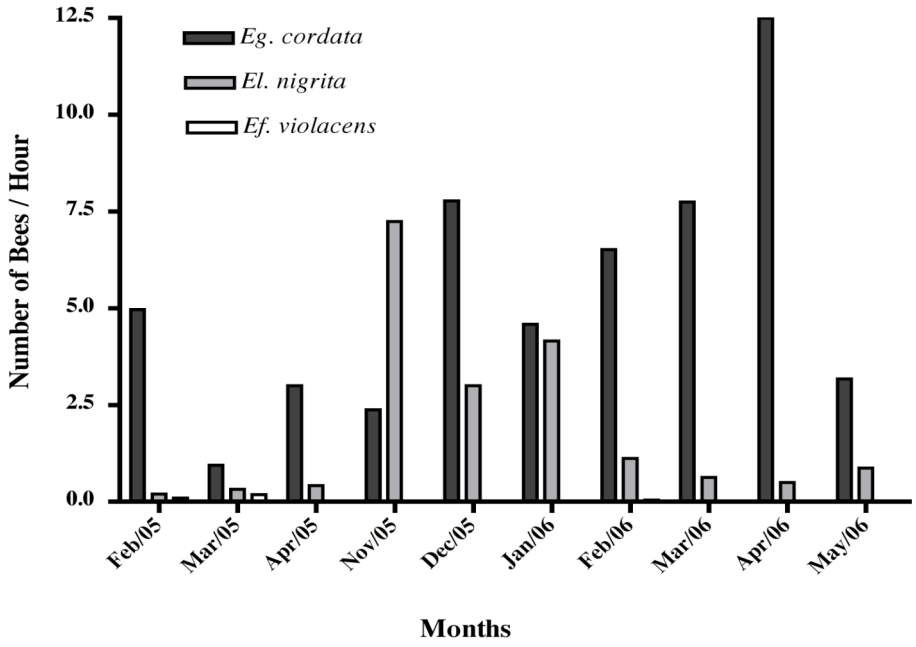

Figure 4.2. Abundance over time of the year measure as number of bees per hour of *Euglossa. cordata*, *Eulaema nigrita* and *Eufriesea violacens* along the nine sampled months.

4.4.3 Foraging activities

Previous reports show that euglossine bees are usually more active during the morning period. Even though, our data support this statement, the two species showed different patterns of activity during the day. *Eg. cordata* showed a peak of activity at 11h (Fig. 4.3) and was present at the flowers all day long. Differently, *El. nigrita* showed two peaks, one at 7h, time of major activity, and at 11h (Fig. 4.4) having a drastic decrease afterwards.

Only seven females were marked at a place and recaptured somewhere else (Table 4.3). One individual of *El nigrita* was marked and recaptured at two different sites, 360m apart, within a period of 40 min. The other individuals belong to the species *Eg. cordata*. Four of them probably used RB and DP, 240m apart, as common sites of nectar source since they

were recaptured at these two sites several times. The other two individuals were recaptured at a distance considerably higher (1 and 2.10 km) but were not seen at their original sites again. Individuals recaptured in a place different from the one where they were marked represents only 2% of total recaptures.

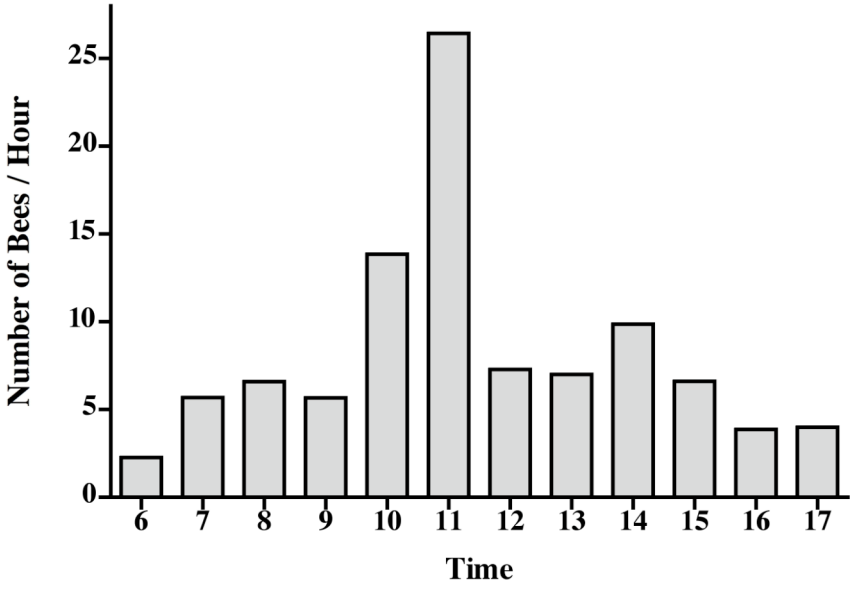

Figure 4.3. Abundance of *Euglossa cordata* at *Thevetia peruviana* flowers along the day. Abundance is measured as number of bees per hour from 6h to 17h.

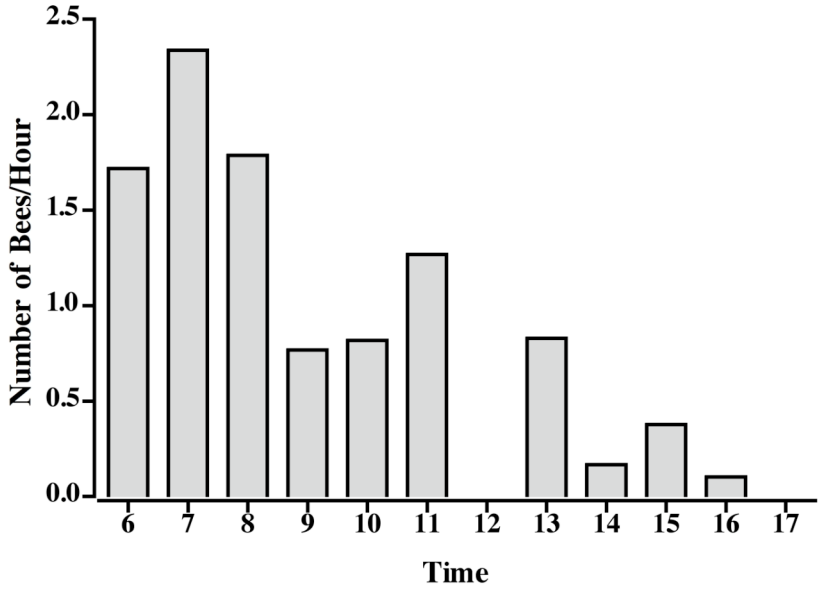

Figure 4.4. Abundance of *Eulaema nigrita* at *Thevetia peruviana* flowers along the day. Abundance is measured as number of bees per hour from 6h to 17h.

Table 4.3. Dispersal events registered. Sites that presented individuals marked and recaptured somewhere else, number of individuals (N), species and time (days) between the events.

Mark	Recapture	N	Species	Days
Rui Barbosa	Dom Pedro II	2	*Eg. cordata*	1, 12
Dom Pedro II	Rui Barbosa	2	*Eg. cordata*	2
Totó Leite	Rui Barbosa	1	*Eg. cordata*	3
Rui Barbosa	Marginal	1	*Eg. cordata*	1
José Benetti	Bernardino de Campos	1	*El. nigrita*	0

Our data show that foraging activities for pollen differ between *Eg. cordata* and *El. nigrita.* A high proportion of females of *Eg. cordata* collect pollen early in the day (7h) and later the percentage of bees carrying pollen is constant (Fig. 4.5). In contrast, *El. nigrita* showed no peak and it was constant during the morning for these activities (Fig. 4.6). Resin collection was registered only for eight individuals, five of them during the afternoon.

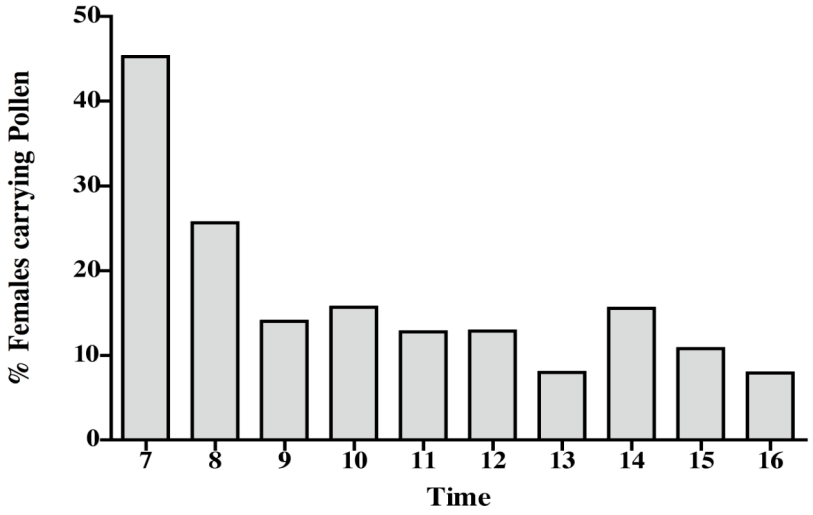

Figure 4.5. Proportion of *Euglossa cordata* females carrying pollen along the day.

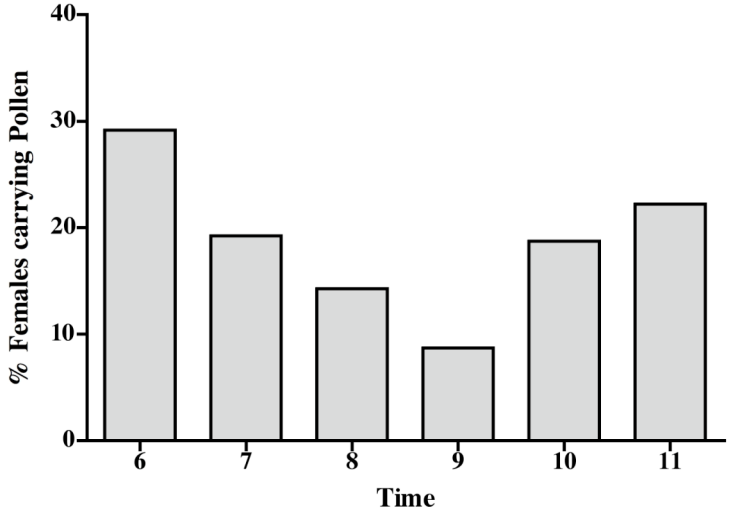

Figure 4.6. Proportion of females of *Eulaema nigrita* bees carrying pollen along the day.

4.4.5 Wing wear

Wing wear data showed that most *Eg. cordata* bees were classified as I and very few as class III (Fig. 4.7). The three classes did not vary much among the different months sampled. Our data suggest wing wear is not a good estimators of the age for *Eg. cordata* (Oi, in prep.). One individual was marked the first time as wing class III and recaptured four and a half months later. Moreover, some individuals marked as class I the first time, were recapture as class III one month later. Individuals that kept wing class I over two months were also observed. Data for *El. nigrita* are not shown because sample sizes were too small and did not show a significant pattern.

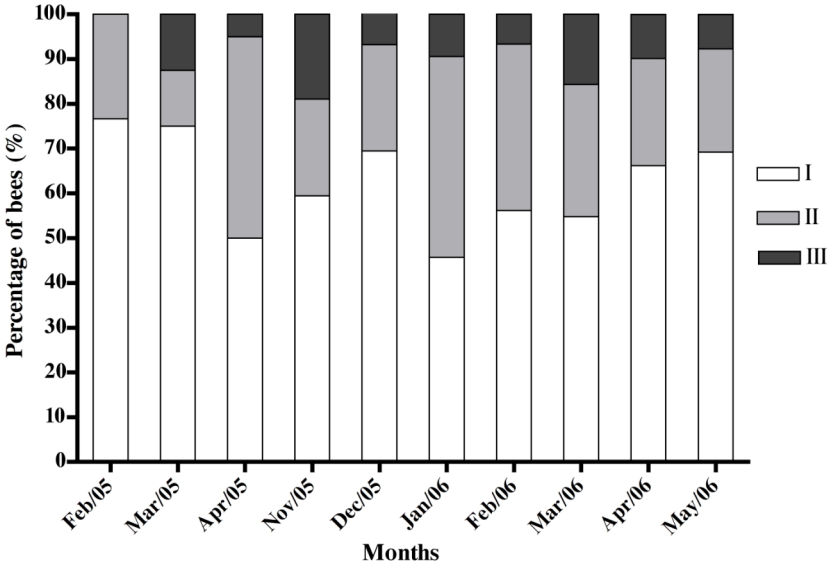

Figure 4.7. Wing wear of *Euglossa cordata* during the sampled months. White indicates class I, gray class II and black class III.

4.4.6 Population abundance

Data used for the analysis of M included nine recapture occasions (Table 4.4). The most parsimonious model chosen by the AICc had survival probability (ϕ) independent of time while probabilities of recapture (p) and entry (b) to the population were dependent of time (Table 4.5).

Table 4.4. Resume of the data for *Euglossa cordata* at M. Time between recaptures occasions (t_i); number of marked (m_i) and unmarked (u_i) individuals; total of individuals released at occasion i (R_i); and number of animals of R_i subsequently captured after occasion i (r_i).

Occasion	t_i	m_i	u_i	R_i	r_i
1	1	0	31	31	7
2	24	21	3	24	8
3	31	10	2	12	5
4	54	22	9	31	22
5	56	14	14	28	24
6	60	13	30	43	29
7	64	14	24	38	21
8	68	17	24	41	15

Table 4.5. Model selection for the most parsimonious model for the M experiment. It shows models tested, number of parameters of the model, AICc values, delta AICc between models, (ΔAICc) and the AICc weight.

Model	No. Parameters	AICc	ΔAICc	AICc Weight
ϕ(.), p(t), b(t)	17	562.161	0	0.99714
ϕ(t), p(t), b(t)	24	573.992	11.83	0.00269
ϕ(t), p(.), b(.)	17	579.482	17.32	0.00017
ϕ(t), p(t), b(.)	15	28443.493	27881	0
ϕ(.), p(.), b(.)	2	28566.266	28004	0

The gross estimate of the abundance of the *Eg. cordata* population visiting the *T. peruviana* trees at M was of 578 (SE 40.78) individuals and the survival probability for the two-month experiment was 0.96 (SE 0.006). Net abundance estimates per sample occasion increased for almost all sampling occasion. This pattern suggests the population of *Eg. cordata* at the M sites was increasing during the nine sampling occasion which agrees with the birth+immigration (B+I) estimates that were also high for all occasions (Fig. 4.8). B+I estimates were almost constant for the last capture occasions indicating a more stable stage of the population. These constant values are probably the result of the equal time intervals of last occasions indicating that the number of individuals entering the population is quite constant.

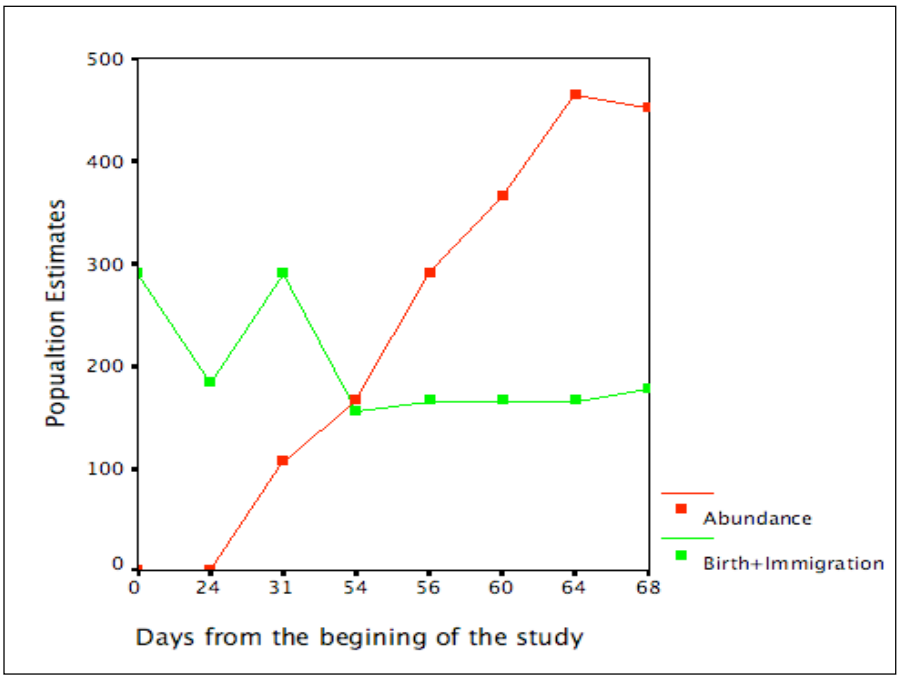

Figure 4.8. Maximum likelihood estimates for the abundance and birth+immigration of the population of *Euglossa cordata* at M using the ϕ(.), p(t), b(t) model.

Experiment at JB was composed of 11 occasions as shown in Table 4.6. *Eg. cordata* was less abundant at this site that at ME, as indicated by the low R_i values. The most parsimonious model following the AICc was time dependent for survival and recapture

probabilities (Table 4.7). However, data for this sample site did not fit well to the Jolly-Seber model ($\chi^2 = 0$) and therefore estimates of population size and survival are meaningless (data not shown).

Table 4.6. Resume of data for *Euglossa. cordata* at JB. Time between recaptures occasions (t_i); number of marked (m_i) and unmarked (u_i) individuals; total of individuals released at occasion i (R_i); and number of animals of R_i subsequently captured after occasion i (r_i)

Occasion	t_i	m_i	u_i	R_i	r_i
1	6	0	27	27	10
2	14	6	3	9	0
3	29	0	2	2	0
4	41	2	6	8	3
5	44	2	6	8	3
6	48	7	6	13	2
7	50	4	5	9	2
8	55	5	1	6	0
9	62	1	2	3	0
10	64	3	4	7	4

Table 4.7. Model selection for the most parsimonious model for the JB experiment. It shows models tested, number of parameters of the model, AICc values, delta AICc between models, (ΔAICc) and the AICc weight.

Model	No. Parameters	AICc	ΔAICc	AICc Weight
ϕ(.), p(t), b(t)	17	562.161	0	0
ϕ(t), p(t), b(t)	24	573.992	11.83	0
ϕ(t), p(.), b(.)	17	579.482	17.32	0
ϕ(t), p(t), b(.)	15	28443.493	27881	27450.687
ϕ(.), p(.), b(.)	2	28566.266	28004	27601.262

Mark-recapture experiments for abundance and survival estimates of *El. nigrita* at K were composed of nine recapture occasions (Table 4.8). The most parsimonious model was time and sex dependent for survival and probability of entry to the population (Table 4.9).

However, model with survival probabilities depending on sex also fit well the data. Gross estimates of population size were 75 (SE 10) for males and 110 (SE 13) for females.

Table 4.8. Resume of data for *Eulaema nigrita* at K. Time between recaptures occasions (t_i); number of marked (m_i) and unmarked (u_i) individuals; total of individuals released at occasion i (R_i); and number of animals of R_i subsequently captured after occasion i (r_i)

Occasion	t_i	m_i	u_i	R_i	r_i
1	4	0	33	33	16
2	13	15	21	36	2
3	30	3	7	10	1
4	37	4	20	24	2
5	56	3	7	10	1
6	58	2	25	27	12
7	63	13	5	18	0
8	67	0	5	5	0

Table 4.9. Model selection for the most parsimonious model for the *Eulaema nigrita* experiment at K. It shows models tested, number of parameters of the model, AICc values, delta AICc between models, (ΔAICc) and the AICc weight (t= time and s=sex).

Model	No. Par.	AICc	ΔAICc	AICc Weight
ϕ(t*s), p(.), b(t *s)	22	231.043	0	0.73954
ϕ(t*s), p(. s), b(t*s)	23	233.425	2.38	0.22482
ϕ(t*s), p(t*s), b(t*s)	30	237.108	6.06	0.03564
ϕ(. s), p(t*s), b(t*s)	23	258.627	27.58	0
ϕ(.), p(.), b(.)	7	18384.446	18153	0

Estimates of abundance and birth+immigration to the population show that both, males and females, decline in abundance during the eight occasions sampled (Fig. 4.9). Males and females were at similar abundances at the beginning of the experiment, and later there is a population decline more drastically in males than females. Distinctly of *Eg. cordata, El.*

nigrita did not show a constant number of individuals entering the population which is probably an indication of a demographical pattern at this species for this time of the year, which severely declines in February after a peak of abundance from September to January (Rebêlo & Garófalo, 1991).

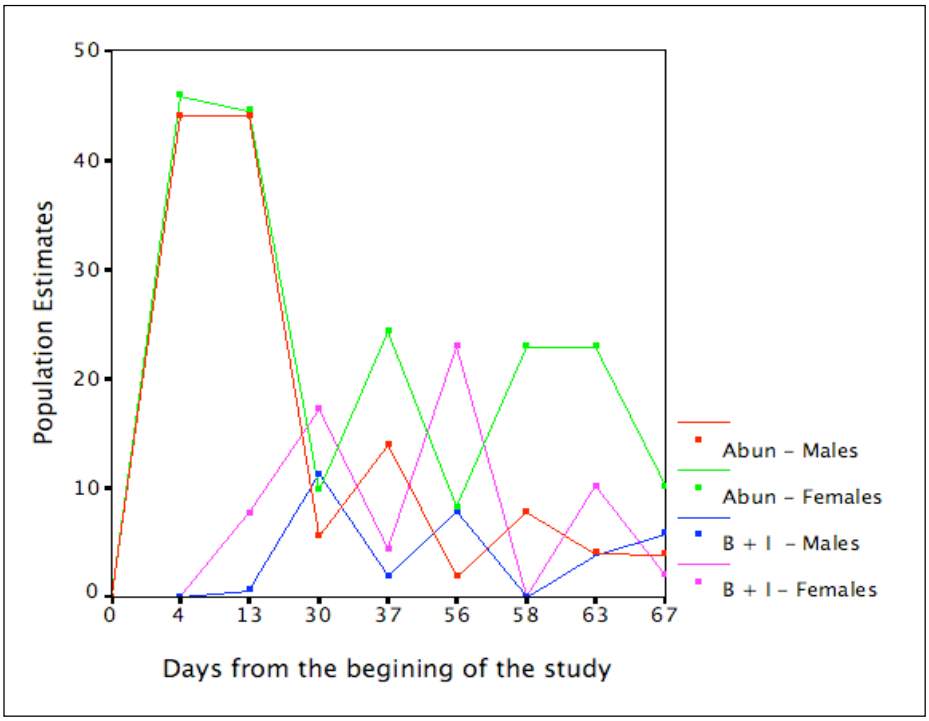

Figure 4.9. Maximum likelihood estimates for the abundance (Abun) and birth+immigration (B+I) for males and females of *Eulaema nigrita* at K using the ϕ(t s), p(.), b(t s) model.

4.5 Discussion

4.5.1 Interaction with Thevetia peruviana

Sex ratios were found to be female-biased for all euglossine species except for *El. nigrita* that showed both sexes at the same proportion. This bias is expected since females are active all day long foraging for pollen, resins and other materials for nest construction, which

demands a huge energy input that nectar supplies. In contrast, euglossine males only forage for (1) fragrances, probably for female attraction, and (2) nectar as an energy source. We hypothesize the equal sex ratio for *El. nigrita* is due to the presence of a male aggregation at or very near site K. Sleeping male aggregations have been previously described for other euglossine species (Silva & Garófalo, 2004).

Eg. cordata and *El. nigrita* showed different peak activities during the day. *Eg. cordata* showed a clear activity peak at 11h probably correlated to the high temperatures during this time of the day. *El. nigrita* showed two peaks of activity, the higher at 7h and a smaller at 11h. This pattern indicates that *El. nigrita* is more active early in the day, which may be due to their large body size. The fact that both species showed an activity peak at 11h could also be due to a variation in the quantity of nectar *T. peruviana* flowers offer during the day. Foraging activity of bees may be affected by their physiology as well as the quality of floral resources. However, data about variation of nectar quantity of *T. peruviana* along the day is necessary to confirm the latter hypothesis.

4.5.2 Bee diversity in urban areas

Four euglossine bee species were detected visiting *T. peruviana* flower in the urban areas surveyed. This number of species is low when compared to forest fragments in the state of São Paulo where there have been registered 14 species when using chemical baits (Rebêlo & Garófalo, 1997). Differences in the number of species found in urban and forestry environments could be due to negative effects of urbanization to species richness (Niemelä et al., 2002). Studies on urban areas suggest that in general urbanization decreases species richness, although some species actually become highly abundant in these habitats (McFrederick & LeBuhn, 2006). In fact, this seems to be the case of *Eg. cordata* and *El.*

nigrita, which appear to be well-adapted species to open areas, like agroecosystems (Milet-Pinheiro & Schlindwein, 2005) and cities. In the same manner, *Ef. violacens* has also been reported in other urban areas (Silva *et al.*, submitted). In our surveys, this species showed a strong seasonality, as it was present exclusively during three months of the year and in low abundance. Seasonal abundances are common for species of the genus *Eufriesea spp.* as shown by Roubik (2001) after a 21-year survey in Panama.

Presence of *Ex. smaragdina* is perhaps associated to the availability of their host, *El. nigrita,* in urban areas. However, the abundance of *Ex. smaragdina* was extremely low when compared to *El. nigrita* (1:72). This could be due to one or a combination of the following. First, it is possible that our sampling method using *T. peruviana* flowers underestimated the abundance of *Ex. smaragdina* since, as a cleptoparasitic species, may not be as foraging active as other euglossine species and therefore it differs in its nectar needs. It could also be due to the less abundant populations of *Ex. smaragdina* at low latitudes indicating that this species is not strictly associated to their potential host *El. nigrita* (Nemésio & Silveira, 2006). As a final point, the low abundance of *Ex. smaragdina* may be just the result of its susceptibility to open environments.

Besides the four euglossine species, other nine bee species were frequently seen visiting *T. peruviana* flowers (Table 4.2) indicating that this exotic tree is an important nectar source for native bees in urban areas. Short-tongued species usually prefer flowers with shallower corollas, which explains the preference of *X. suspecta* and *Centris spp.* for other nectar sources like *Tecoma stans* (Bignoniaceae) (Del Lama, pers. obs.). Contrasting, we observed that even when *T. peruviana* trees were next to *T. stans* trees, bumblebees and euglossine bees preferred *T. peruviana* flowers. The fact that both, euglossine and bumblebees, have long tongs suggests that *T. peruviana* flowers are preferred by long-tongued bees because of the deep corollas these tubular flowers have.

The nectar rich flowers of *T. peruviana* may be the most important factor for the attraction of several bee species to its flowers (Thakar et al., 2003). However, it is possible that none of the bee species here reported are actual effective pollinators of the plant. A preliminary genetic analyses of 5 urban populations of *T. peruviana* in the state of São Paulo showed that this shrub is monomorphic for 20 allozyme loci suggesting that autofecundation is the commonest reproductive mechanism of this species, at least outside its native distribution (Del Lama, unpubl. data).

4.5.3 Seasonality

Abundances of the euglossine bees found at *T. peruviana* flowers showed different seasonal patterns. *Eg. cordata* was present during all the sampled month, which agrees with previous bait samplings showing that *Eg. cordata* males are present during the whole year but are more abundant from November to April (Rebêlo & Garófalo, 1991). *El. nigrita* was very abundant from November to January but their visits to *T. peruviana* flowers drastically decrease afterwards. Even though, this species is present all year long, it is more abundant from September to January. *Ef. violacens* and *Ex. smaragdina* also showed a strong seasonality, as they were captured only during three of the months surveyed. Since bee populations dynamics may be very variable, these results on the seasonality of these euglossine bee species are restricted to this short-term study.

Contrary to the activity peak that *Eg. cordata* presented at 11h, our data show that a greater proportion of females carried pollen from 7h to 8h. On the contrary, data for *El. nigrita* did not show a peak for pollen collection activity. This different pattern in *Eg. cordata* could be a response to competition for pollen resources. However, our data cannot confirm

this hypothesis since there is no information about the overlap of trophic niche between these species in urban areas.

4.5.4 Abundance and survival

Estimates of abundance suggest that the population of *Eg. cordata* visiting the flowers of *T. peruviana* at M was larger than the population of *El. nigrita* at K. Even though, this result could be indicating just a preference of *El. nigrita* to other floral resources during different times of the year, abundance estimates strongly suggest that *El. nigrita* drastically decreased in February (Fig. 4.9). Population size estimates indicate that the population of *Eg. cordata* was growing during our sampling, whereas population of *El. nigrita* was decreasing. This is also evident looking at the models selected for both species, *Eg. cordata* survival was independent of time while model of *El. nigrita* was strongly dependent on this parameter.

Estimates of population size and survival of *Eg. cordata* indicate that this population are large and "healthy" in the areas surveyed. Because dispersal was not a common behavior in this species, population size estimated for the different sites are representative of each particular area. Therefore, if all sites where *T. peruviana* trees were found (4.7 Appendix) hold a different population of *Eg. cordata*, population size of *Eg. cordata* in São Carlos is considerably larger. Our results show that populations of *Eg. cordata* and *El. nigrita* are important components of the bee community in the city of São Carlos. Even though previous evidence suggested that *Eg. cordata* and *El. nigrita* prefer open areas, this is the first study reporting data on their abundance and seasonality in urban environments.

Surveys in forest fragments using chemical baits usually detect *Eg. cordata,* but this species is not the most abundant in these environments. Although baits data are not comparable to *T. peruviana* surveys, our results suggest that *Eg. cordata* is the most abundant

species in urban areas, and most likely it is more abundant in urban areas than in forestry environment. However, data using the same sampling method is necessary for comparing abundances in these habitats to confirm this hypothesis.

We found that euglossine species richness is lower in urban areas than in forest fragments. However, some species are found in great abundances which is a pattern found in other urban surveys already reported (Niemelä *et al.*, 2002; McFrederick & LeBuhn, 2006). These results also evidence that some species could be preserved in urban areas, if food and nest resources are offered to bees in urban environments.

It is important to evaluate if arborization in the cities is in favor of the maintenance of these important pollinators. We hope the data here presented will be considered for future landscape planning in the cities to have in mind the importance of trees, like *T. peruviana,* as food sources for the preservation of euglossine and other species of native bees.

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4.7 Appendix

Description of the sites where *T. peruviana* trees where found in the city of São Carlos (SP). Number of trees per site, geographical position given as latitude and longitude, altitude (m) and index of transit interference (TI).

Sample Sites	Simbol	No. Trees	Latitude	Longitude	Altitude	TI
Bejamin Constan	BeC	1	22º01'59.7"S	47º53'57.5"W	835	L
Bernardino de Campos	BC	2	22º01'31.9"S	47º53'53.9"W	852	M
Casa da Cultura	CC	2	22º01'14.8"S	47º53'06.6"W	813	H
Dom Pedro II	DP	1	22º00'16.8"S	47º53'18.9"W		H
Francisco Oliveira Penteado	FOP	2	22º01'37.9"S	47º54'15.1"W	833	M
Guadalajara	G	2	22º02'26.2"S	47º53'27.1"W	838	L
Henrique Gregrori	HG	1	22º01'54.2"S	47º54'11.9"W	845	H
José Benetti	JB	2	22º01'35.4"S	47º54'05.7"W	861	L
José de Alencar	JA	1	22º00'20.9"S	47º53'11.7"W	836	L
Kartódromo	K	16	21º59'58.4"S	47º53'55.2"W	858	M
Luiz Carlos Arruda Mendes	LAM	2	22º02'05.6"S	47º53'43.3"W	842	L
Manoel José Serpa	MJS	2	22º00'15.2"S	47º54'51.4"W		L
Marginal	M	3	22º01'26.0"S	47º52'49.0"W	816	M
Procópio de Arújo Ferraz	PAF	8	21º59'55.0"S	47º55'07.2"W	860	M
Rui Barbosa	RB	8	22º00'20.9"S	47º53'11.8"W	812	H
Santos Dumont	SD	4	21º59'44.2"S	47º53'11.2"W	835	M
São Paulo	SP	1	22º00'34.3"S	47º53'08.1"W	868	L
Theodureto de Camargo	TC	3	22º02'05.5"S	47º53'43.4"W	824	H
Totó Leite	TL	3	22º00'44.1"S	47º52'46.7"W	839	L

5. Population structure of *Euglossa cordata* (Linnaeus 1758) in urban areas as revealed by allozymes and mtDNA

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Keywords: Euglossini, genetic diversity, population structure, urban areas

5.1 Abstract

Urban areas harbor fewer species of animals and plants compared to surroundings because urbanization severely changes ecological conditions of habitats. However, some species seem to be well-adapted to urban ecosystems. *Euglossa cordata* is an euglossine bee abundant in urban areas of the São Paulo State (Brazil). In this study, it was estimated the genetic variability of five urban populations of *E. cordata* in the state of São Paulo using one mitochondrial locus (16S) and three allozyme loci (*MDH, ACP, EST-1*). Heterogeneous genetic variability was found in the five cities surveyed. F_{ST} values showed genetic structure for the mitochondrial locus but not for the nuclear markers. Our results suggest that (1) urban areas are reservoirs of *E. cordata* and (2) females have a philopatric behavior whereas males are the dispersal sex.

5.2 Introduction

Urbanization drastically alters vegetation structure, climatic conditions of areas and it has a direct impact on species diversity (Niemelä et al. 2002). The international GLOBE-NET, which is a project for the appraisal of human activities effects on the diversity of the community of carabid beetles, has found that specialist and non-flying species of beetles tend to decline in urban environments (Niemelä et al. 2000). In contrast to these ecological results, a study on the genetic diversity of small isolated urban populations of the declining beetle *Abax ater* showed no signs of genetic erosion and, on the contrary, showed great adaptability to the heterogeneous urban environments (Desender et al. 2005). These differences on ecological and genetic results evidence the importance of genetic studies to asses the impact urbanization has on animal and plant populations.

Studies on the bee assemblage of urban areas have shown that urbanization decreases bee diversity but some species are usually adapted to this habitats and become very abundant (McIntyre and Hostetler 2001; Zanette et al. 2005; McFrederick and LeBuhn 2006). In general, species of bees that are abundant in urban areas are generalists that explore the floral resources offered by the exotic ornamental species present in the cities (McFrederick and LeBuhn 2006). However, there are very few studies evaluating the genetic composition of urban populations of bees to compare the ecological patterns with genetic data (Chapman et al. 2003).

Euglossine bees are potential generalist pollinators since they possess long tongues and a great flight capacity (Janzen 1971; Borrell 2005). In particular, the large bodied

euglossines of the *Eulaema* genus are strong fliers and have been associated to open disturbed areas. A morphometric trend in euglossine bees predicts that large-sized long-tongued bees tend to forage more in open areas like farm sites (Otero and Sandino 2003). This trend has been associated to two features: (1) long proboscis that enables these bees to explore a great variety of floral resources and (2) the large body size that allows bees to tolerate high temperatures and low humidity.

Euglossa cordata is a small-bodied and short-tongued bee of the Euglossini tribe distributed at low altitudes in Brazil, the Guiana's, Surinam, Trinidad and Tobago and Venezuela (Ramírez et al. 2002). Because of its small size, the euglossine morphometric trend does not predict the ability this species has to fly long distances across open areas (Raw 1989; Tonhasca et al. 2003). In addition, recent studies have revealed that among the euglossine bees, *E. cordata* is the most common species in urban areas of the São Paulo state (Brazil) (López-Uribe & Del Lama *submitted*).

Census data on bees are not always a good indicator of healthy populations (Zayed *et al.* 2004) and therefore genetic data are important to detect possible population declines. For this reason, estimation of the genetic variability of populations is a central issue to be measured. With this purpose, the genetic diversity of urban population of *Euglossa cordata* was measured to establish if urban population of *E. cordata* are as healthy as census data indicate. Genetic variability of five urban areas was measured using three allozyme loci (*MDH, ACP* and *EST-1*) and one mitochondrial locus (16S) through PCR-RFLPs. Our results showed different levels of genetic diversity for the five urban populations sampled. F_{ST} values of the mitochondrial locus evidenced strong population structure. However, nuclear markers

did not show this pattern suggesting that females present a more philopatric behavior whereas males are the dispersal sex in *E. cordata.*

5.3 Methods

Samples were collected in five cities of the São Paulo state (Brazil) from 2003 to 2005 (Table 5.1). Specimens of *Euglossa cordata* were collected when they were visiting flowers of the exotic plant *Thevetia peruviana* (Apocynaceae) located on sidewalks inside the cities. Each individual was placed in a plastic vial on ice and later kept at -20ºC until analysis.

Table 5.1. Geographical information of the five sampled cities of the São Paulo state (Brazil). Population size (thousands); population density (hab/Km^2); number of females (N_f) and males (N_m) analyzed.

Locality	Latitude	Longitude	Pop. Size	Pop. Density	N_f	N_m
São Carlos	22º00'16"S	47º53'18"W	210.8	184.79	102	20
Rifaina	20º04'50"S	47º25'17"W	3.5	20.59	78	3
Pedregulho	20º15'25"S	47º28'36"W	15.6	22.26	28	1
Jaboticabal	21º15'17"S	48º19'20"W	71.7	101.39	17	-
Araras	22º21'25"S	47º23'03"W	112.8	175.95	13	-

Proteins were extracted from head of each individual in a 0.2% 2-mercaptoethanol solution and analyzed in 14% starch gels. Samples were evaluated for the polymorphic enzyme systems malate dehydrogenase (*MDH*), acid phosphatase (*ACP*) and esterase (*EST-1*) in a tris-citric acid buffer pH 7.5. These markers were chosen because previous analysis on this species showed polymorphism for these loci (López-Uribe & Del Lama *submitted*).

DNA was extracted from thorax of the specimens following a standard phenol-chloroform protocol (Sheppard and McPheron 1991). Mitochondrial fragments of the 16S regions were amplified using primers 16SWb (Dowton and Austin 1994) and 874-16SlR (Cameron *et al.* 1992). PCR conditions were done as described in López-Uribe & Del Lama (submitted). Both fragments were digested with the restriction enzyme *Vsp*I in a 10µL reaction mixture containing 1µL of PCR product, 1µL of One-for-All (Amershan) buffer and 1U of *Vsp*I. Restriction patterns were visualized in 12% silver stained polyacrylamide gels.

Allele and genotype frequencies, deviations from Hardy-Weinberg equilibrium were calculated for allozyme data. F-statistics were calculated for mitochondrial and allozymic data using the software GENEPOP (Raymond and Rousset 1995). Because of the presence of haploid males and diploid females in the sample, allelic frequencies for each locus were calculated as the number of occurrences of an allele divided by the total number of alleles at that locus. Genetic diversity was estimated defined as:

$$h = 1 - \sum_{i=1}^{q} x_i^2 \qquad (5.1)$$

where x_i is the population frequency of the i-th allele and q is the number of alleles. To measure genetic variation in each population, the average heterozygosity, also known as average gene diversity was estimated by:

$$\hat{H} = \sum_{j=1}^{L} h_j \Big/ L \qquad (5.2)$$

where h_j is the value of *h* at the *j*-th locus. Isolation-by-distance was calculated by the reduced major axis (RMA) regression of the pairwise F_{ST} values to the logarithm of pairwise distance (D) measured in km (Rousset 1997). The significance of this regression was tested after 10000 randomizations of the Mantel test using the IBD 1.52 software (Bohonak 2002).

5.4 Results

Allelic frequencies for all loci are shown in Table 5.2. Locus *MDH* presented two alleles whereas *ACP* and *EST-1* presented four and three alleles respectively. However, the latter two loci showed low polymorphism levels with the frequency of the more common allele being over 95%. Individuals from Araras were monomorphic for all the loci analyzed probably due to the small sample size from this location (n=10). Because Araras was monomorphic for the analyzed loci, this population was not included in population parameters estimates. Distribution of haplotypes for the 16S locus was very different among localities. Haplotype A was more common in Rifaina and Pedregulho, whereas haplotype B was more common in São Carlos, and fixed in Jaboticabal and Araras.

Table 5.2. Allele and haplotype frequencies for the five populations for the three allozymic and the mitochondrial loci analyzed.

		São Carlos	Rifaina	Pedregulho	Jaboticabal	Araras
MDH	100	0.87	0.837	0.759	0.735	1
	92	0.13	0.163	0.241	0.265	-
ACP	108	0.012	0.005	0.069	0.029	-
	105	0.016	0.021	-	0.029	-
	100	0.968	0.953	0.914	0.941	1
	92	0.004	0.021	0.017	-	-
EST-1	106	0.016	0.011	0.035	-	-
	100	0.961	0.963	0.948	1	1
	92	0.024	0.026	0.017	-	-
16S	A	0.284	0.758	0.828	-	-
	B	0.716	0.242	0.172	1	1

Pedregulho was the only population at Hardy-Weinberg (HW) equilibrium for all the nuclear loci analyzed (Table 5.3). *ACP* and *EST-1* were not at equilibrium for populations in which these loci showed polymorphism. Gene diversity ($\hat{H}$) for the 16S locus showed values comparable to *MDH*. Pedregulho showed the highest gene diversity for the nuclear markers, although allele 105 of the *ACP* locus was not found in this population. Overall, São Carlos showed the lowest level of genetic variability.

Table 5.3. Observed heterozygosity (H_o) for the allozyme data and gene diversity ($\hat{H}$) for all the analyzed loci. The average value is calculated for all the nuclear loci. Values in bold indicate loci that are not at Hardy-Weinberg equilibrium for the nuclear markers.

	São Carlos		Rifaina		Pedregulho		Jaboticabal	
	H_o	$\hat{H}$	H_o	$\hat{H}$	H_o	$\hat{H}$	H_o	$\hat{H}$
MDH	0.197	0.226	0.242	0.273	0.414	0.366	0.412	0.390
ACP	**0.048**	0.063	**0.011**	0.091	0.103	0.160	**0.056**	**0.113**
EST-1	**0.063**	0.076	**0.053**	0.072	0.103	0.100	0	0
Average	0.103	0.121	0.102	0.145	0.207	0.208	0.166	0.167
16S	-	0.407	-	0.367	-	0.285	-	0

The pairwise F_{ST} values were non-significant for the nuclear loci (Table 5.4). However, for the mitochondrial locus 16S, the F_{ST} values indicated strong population structure. Rifaina and Predregulho form one group and São Carlos, Jaboticabal and Araras form another group. The pairwise F_{ST} values approached an IBD (r^2=0.452, P=0.033) pattern where pairwise F_{ST} values increased with distance (Fig. 5.1).

Table 5.4. Pairwise F_{ST} values for the five populations of *E. cordata* analyzed. Below diagonal, F_{ST} values for the 16S locus. Above the diagonal, F_{ST} values for the five nuclear loci.

	São Carlos	Rifaina	Pedregulho	Jaboticabal	Araras
São Carlos	*	-0.021	0.0257	0.0316	-0.0495
Rifaina	0.3597	*	0.006	0.0046	-0.00463
Pedregulho	0.4254	-0.0002	*	-0.0174	0.0177
Jaboticabal	0.1165	0.4073	0.4384	*	0.0491
Araras	0.1165	0.4049	0.4483	0.0491	*

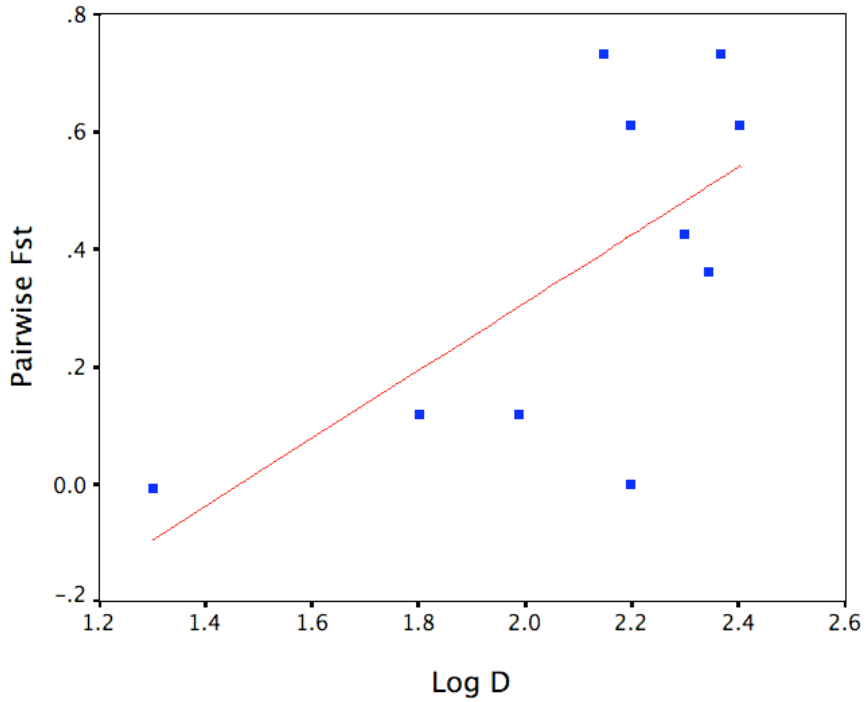

Figure 5.1. Regression between the pairwise F_{ST} values for the 16S locus and the pairwise log distances (D) measured in km.

5.5 Discussion

Our results showed that Pedregulho was the population with the highest level of genetic variability. These findings may be the result of a less severe urbanization process at this locality. However, Rifaina, which has a similar level of urbanization, showed a lower genetic

diversity value suggesting that other environmental variables may be associated to these findings. López-Uribe and Del Lama (submitted) found greater species richness of euglossine bees at Rifaina and Pedregulho when compared to the other cities surveyed. Overall, these results suggest Pedregulho hold a greater euglossine biodiversity and genetic variability due to ecological conditions that should be investigated. We hypothesize that the matrix surrounding São Carlos, Jaboticabal and Araras, which is mainly composed of sugarcane monocultures, is in some way a "barrier" to gene flow. Though it was shown that *E. cordata* has the ability to fly over sugarcane fields (Milet-Pinheiro and Schlindwein 2005), dispersal between populations may be greater through other type of vegetation.

E. cordata is an abundant species in urban areas of the state of São Paulo in Brazil. However, there are no studies comparing populations from urban and forestry areas. These sort of studies are essential to evaluate if urban populations of *E. cordata* are actually less abundant and variable than the population from their original environment, or vice versa. Evaluation of genetic diversity may be a powerful tool for comparing the quality of populations from these two environments.

Departures from HW equilibrium were due to the deficiency of heterozygotes indicating a tendency to inbreeding. However, F_{ST} values showed no structure for nuclear loci of the five populations analyzed. In contrast, the mitochondrial locus 16S showed strong population structure suggesting that males and females have different dispersal ranges for mating. Nonetheless, information from more mitochondrial and nuclear markers is necessary to confirm this hypothesis; our results suggest that females have philopatric behavior. The significance to the IBD model for the mitochondrial data suggests that this genetic differentiation is explained by the geographical distance between populations. Our results

disagree with Dick et al. (2004) who found absence of structure at a broad spatial area for several euglossine bees, suggesting presence of long-distance gene flow even between population across the Andean cordillera.

Our data show heterogeneous genetic variability among five *E. cordata* populations from urban areas of the state of São Paulo (Brazil). We found contrasting results of population structure from nuclear and mitochondrial markers, suggesting that females and males have different dispersal patterns for mating. Even though, urbanization has been considered a process that decreases species richness, it is necessary to study populations in urban areas to evaluate the adaptability species have to this kind of environments. Our results show that urban areas are potential reservoirs of the genetic diversity of the native pollinator *E. cordata*, which appear to be well adapted to the cities of the São Paulo region.

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6. Conclusões e perspectivas

As euglossíneas são um grupo de abelhas que tem merecido especial atenção dos pesquisadores devido ao conjunto de características particulares e interessantes que apresentam. Entre elas, o fato dos machos serem polinizadores específicos de muitas espécies de orquídeas foi o que primeiro chamou a atenção e levou a intensos estudos por parte dos orquidófilos (DRESSLER, 1968). Do ponto de vista ecológico, fêmeas e machos da tribo Euglossini constituem espécies-chaves dos ecossistemas neotropicais, já que muitas espécies de plantas dependem exclusivamente destas abelhas para o processo de polinização. Daí a relevância de estudos sobre a sua biologia e ecologia que possam contribuir para a conservação destes importantes polinizadores.

A metodologia de iscas tem contribuído enormemente para o conhecimento da tribo. Atualmente, mais de 200 espécies são conhecidas, embora muitas ainda estão por serem descritas. Registros sobre a diversidade e riqueza destas espécies são amplamente conhecidos para alguns países, como Panamá e Costa Rica, onde amostragens têm sido realizadas sistematicamente por mais de duas décadas (ROUBIK, 2001; ROUBIK & HANSON, 2004). No entanto, outros países do neotrópico como Brasil, Colômbia, Venezuela, México e Peru possuem registros sobre algumas regiões, porém não têm sido realizadas amostragens por longos períodos de tempo, o que pode subestimar a riqueza de espécies.

À diferença dos machos que são atraídos pelas iscas, a biologia das fêmeas é praticamente desconhecida. Somente para 20% das espécies se conhece a biologia de nidificação devido à dificuldade de se encontrar estes ninhos na natureza. Da mesma forma, a

associação das fêmeas com plantas como fonte de recursos tróficos e para a nidificação é pouco conhecida, o que é de vital importância para a subsistência de uma espécie de abelhas em um lugar.

A interação de *T. peruviana* com as abelhas euglossíneas foi registrada pela primeira vez por Dodson (DODSON, 1966) no Equador, onde esta planta é nativa. No estado de São Paulo, esta árvore é exótica e muito comum em áreas urbanas, onde é utilizada com fins paisagísticos. Este trabalho é o primeiro relato da interação entre abelhas Euglossini e *T. peruviana* em áreas urbanas e evidencia a importância desta planta como fonte de néctar para as abelhas da tribo Euglossini nestes ambientes.

O presente trabalho produziu as seguintes contribuições para o conhecimento das abelhas Euglossini :

1. Descreve as espécies presentes em áreas urbanas de cinco cidades do estado de São Paulo mediante a captura de fêmeas em árvores de *T. peruviana*;
2. Apresenta uma ferramenta molecular para a identificação de fêmeas do gênero *Euglossa,* para as quais não se conhecem caracteres morfológicos que permitam a sua identificação;
3. Estima a abundância das populações de *Eg. cordata* e *El. nigrita* que visitam as flores de *T. peruviana* mediante experimentos de marcação e recaptura;
4. Descreve padrões de forrageio e sazonalidade destas espécies nas áreas urbanas amostradas;
5. Apresenta o primeiro estudo populacional de abelhas Euglossini que sugere diferenças na dispersão entre fêmeas e machos.

Desta forma, foram testadas as duas hipóteses previamente propostas sobre a abundância e padrões de forrageio das abelhas Euglossini. Concluímos que, primeiro, *Eg. cordata* e *El. nigrita* são espécies abundantes nas áreas urbanas onde estão presentes em grande número durante quase todo o ano. Segundo, com respeito aos padrões de forrageio, os experimentos de marcação e recaptura evidenciaram que as fêmeas das duas espécies não se dispersam muito na busca de alimento, ao menos nas áreas urbanas. Da mesma forma, as análises genéticas sugerem que, provavelmente, as fêmeas apresentam algum grau de filopatria, enquanto os machos parecem ser o sexo dispersor. No entanto, é necessário analisar marcadores com maior grau de polimorfismo para corroborar estes resultados preliminares.

Diante dos achados referentes às questões acima, este trabalho abre perspectivas para futuras pesquisas:

- A metodologia de PCR-RFLP pode ser ampliada para outras espécies de *Euglossa* para as quais a identificação de fêmeas não é possível a partir de caracteres morfológicos.
- As coletas em flores de *T. peruviana* permitem capturar um grande número de fêmeas, que são dificilmente encontradas na natureza. Isto possibilita a realização de experimentos de marcação e recaptura que, a longo prazo, podem prover dados sobre longevidade e censos de populações em outras áreas urbanas.
- Trabalhos que permitam comparar a diversidade e abundância da comunidade de abelhas Euglossini em áreas urbanas e fragmentos de mata são de vital importância para estabelecer a qualidade das populações urbanas.

O presente trabalho contribui ao conhecimento da comunidade de abelhas Euglossini em áreas urbanas. No entanto, muitas perguntas ainda precisam ser respondidas para que

conclusões mais consistentes posam ser estabelecidas. Ainda, os resultados aqui apresentados demonstram que as abelhas euglossíneas são um importante componente da comunidade de polinizadores nas cidades e que estes lugares são potenciais reservatórios para algumas espécies da tribo. A partir deste trabalho, é de se esperar que os estudos relativos a este relevante grupo de abelhas em ambientes urbanos sejam intensificados e que o paisagismo nas cidades seja planejado a favor da manutenção destas populações.

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